Defective AMPK regulation of cholesterol metabolism accelerates atherosclerosis by promoting HSPC mobilization and myelopoiesis

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ABSTRACT

Objectives: Dysregulation of cholesterol metabolism in the liver and hematopoietic stem and progenitor cells (HSPCs) promotes atherosclerosis development. Previously, it has been shown that HMG-CoA-Reductase (HMGCR), the rate-limiting enzyme in the mevalonate pathway, can be phosphorylated and inactivated by the metabolic stress sensor AMP-activated protein kinase (AMPK). However, the physiological significance of AMPK regulation of HMGCR to atherogenesis has yet to be elucidated. The aim of this study was to determine the role of AMPK/HMGCR axis in the development of atherosclerosis.

Methods: We have generated a novel atherosclerotic-prone mouse model with defects in the AMPK regulation of HMGCR (Apoe−/−/Hmgcr KI mice). Atherosclerotic lesion size, plaque composition, immune cell and lipid profiles were assessed in Apoe−/− and Apoe−/−/Hmgcr KI mice.

Results: In this study, we showed that both male and female atherosclerotic-prone mice with a disruption of HMGCR regulation by AMPK (Apoe−/−/Hmgcr KI mice) display increased aortic lesion size concomitant with an increase in plaque-associated macrophages and lipid accumulation. Consistent with this, Apoe−/−/Hmgcr KI mice exhibited an increase in total circulating cholesterol and atherogenic monocytes, Ly6-C subset. Mechanistically, increased circulating atherogenic monocytes in Apoe−/−/Hmgcr KI mice was associated with enhanced egress of bone marrow HSPCs and extramedullary myelopoiesis, driven by a combination of elevated circulating 27-hydroxycholesterol and intracellular cholesterol in HSPCs.

Conclusions: Our results uncovered a novel signalling pathway involving AMPK-HMGCR axis in the regulation of cholesterol homeostasis in HSPCs, and that inhibition of this regulatory mechanism accelerates the development and progression of atherosclerosis. These findings provide a molecular basis to support the use of AMPK activators that currently undergoing Phase II clinical trial such as O3—304 and PXL 770 for reducing atherosclerotic cardiovascular disease risks.

Keywords AMPK; Atherosclerosis; HMG-CoA reductase; Cholesterol; HSPCs

1. INTRODUCTION

Atherosclerotic cardiovascular disease, which stems from dyslipidemia and maladaptive inflammatory responses, precedes and predicts the development of cardiovascular complications including stroke and myocardial infarction [1—4]. One of the key contributors to the development and progression of atherosclerosis both in animal models and in humans is the increase in circulating cholesterol levels, which is predominantly produced in the liver [5—7]. Elevated cholesterol can influence the function of hematopoietic stem and progenitor cells (HSPCs) and their propensity to differentiate into monocytes and other immune cells through a process known as myelopoiesis [8—11]. Although it has been suggested that impaired cholesterol regulation in HSPCs play an important role in atherogenesis [8—11], the molecular

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mechanisms that regulate cholesterol homeostasis in HSPCs during the setting of atherosclerotic cardiovascular disease, defective cholesterol efflux in HSPCs results in enhanced proliferation and mobilization of HSPCs that reside in the BM and consequently increased myelopoiesis [9–11]. This in turn increases atherogenic monocyte counts in the circulation, monocyte accumulation and subsequent differentiation of atherogenic monocytes into inflammatory macrophages, accelerating the development of atherosclerotic inflammation and plaque formation [9–12].

AMPK is a highly conserved key regulator of whole-body energy metabolism, including lipid and glucose metabolism, protein synthesis and mitochondrial biogenesis [13–16]. AMPK is present in all tissues as a heterotrimeric complex composed of a catalytic α subunit (α1, α2), a scaffolding β subunit (β1, β2) and a nucleotide-binding γ subunit (γ1, γ2, γ3) and is typically inactive unless phosphorylated on Thr172 in the α subunit activation loop by upstream kinases including liver kinase B1 (LKB1) and calcium/calmodulin dependent protein kinase kinase 2 (CaMKK2) (reviewed in [13–16]). AMPK is activated by metabolic stresses or hormonal changes that signal low energy conditions and acts to inhibit ATP-consuming anabolic processes and promote ATP-generating catabolic pathways [13–16]. The responses induced by AMPK activation are typically short-term, involving phosphorylation of key metabolic enzymes, and long-term, which occurs through modulation of gene transcription [13–16]. Previous studies have demonstrated that the suppression of AMPK activity under conditions of chronic over-nutrition may contribute to the development of metabolic diseases [13–16]. In the context of atherosclerosis, AMPK has been shown to have multiple anti-atherogenic effects through its influence on inflammatory signalling [17], suppressing monocyte to macrophage differentiation [18,19], and increasing cholesterol efflux [20,21], which in combination, decreases foam cell formation and atherogenesis. In line with this, systemic infusion of direct or indirect AMPK activators such as A769662, salutarine or metformin has been shown to decrease lesion size in atherosclerotic prone mice [22–25]. The 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase (HMGR) is the rate-limiting enzyme of the mevalonate pathway that is responsible for the synthesis of important nonsterol isoprenoids and bioactive sterols, such as cholesterol and steroid hormones [5,6]. HMGR is subject to regulation at multiple levels, involving feedback control and cross-regulation by distinct biochemical pathways [5,6]. HMGR enzyme activity is inhibited by phosphorylation at Ser871 (human HMGR Ser872) by AMPK [26,27]. Previous in vitro studies have shown that metabolic stress inhibits cholesterol synthesis but not in cells transfected with HMGR in which Ser871 has been mutated to Ala [28]. We have recently used a mouse model in which the AMPK phosphorylation site Ser871 on HMGR was mutated to Ala (Hmargc KI mice) and demonstrated that this phosphorylation event inhibits cholesterol synthesis in vivo and is important for suppressing the development of hepatic steatosis in response to high carbohydrate feeding [28]. While HMGR regulation by AMPK is important for controlling hepatic cholesterol synthesis, the physiological significance of the AMPK-HMGR axis in the development of atherosclerosis has yet to be elucidated.

2. MATERIALS AND METHODS

2.1. Animals
All animal care and experiments were approved by St. Vincent’s Hospital (Melbourne, Australia) and the Alfred Medical Research Education Precinct Animal Ethics Committee, and conducted in accordance with the National Health and Medical Research Council of Australia’s (NHMRC) guidelines for the Ethical and Humane Use of Animals in research. Hmargc KI mice were generated and genotyped as described previously [23]. Apoe–/– mice were obtained from the Animal Resource Centre (Canning Vale, WA, Australia). Apoe–/–Hmargc KI mice were generated by crossing male Apoe–/– with female Hmargc KI mice in a two-step manner. Mice were genotyped by Transnetyx Inc (Cordova, Tennessee, USA). All mice used for experiments were on a C57BL/6J background. Mice were housed under a controlled temperature of 22 °C and a 12-hour light cycle (lights on from 7 a.m. to 7 p.m.) with ad libitum access to water and a standard chow diet (6% fat, 29% starch, #102108, Barastoc, Ridley Agriproducts) for 12 weeks starting from 8 weeks of age.

2.2. Assessment of atherosclerotic lesions
Aortic atherosclerotic lesions in the aortic root were analysed on 6μm frozen sections.

2.2.1. Lesion size
Sections were fixed (4min, 10% neutral buffered formalin), washed in PBS (4min), stained in Mayer’s Haematoxylin (15min) and washed with running tap water before blueing in Scott’s tap water for 30secs. The slides were then put in 95% ethanol (10 dips), stained in buffered alcoholic eosin (8min), dehydrated in absolute ethanol, cleared with xylene and coverslips were mounted using depex. Sections were imaged on the Olympus FSX100 microscope 4.2x magnification and images were analysed using Adobe Photoshop CC.

2.2.2. Lipid content
Sectioned lesions were fixed in 10% buffered formalin (4mins), washed in PBS (4min), dipped in 60% isopropanol before staining in 60% ORO working solution (2hrs, stock solution: 1% ORO powder in isopropanol). The slides were then washed in 60% isopropanol and distilled water. Sections were stained in Mayer’s Haematoxylin (4mins), washed in tap and distilled water (3min each) and mounted with aquamount. Sections were imaged on the Olympus FSX100 microscope 4.2x magnification and images were analysed using Adobe Photoshop CC.

2.2.3. Macrophage abundance
Thawed sections were fixed with paraformaldehyde (4%, 20min), washed in PBS (2 x 5min), incubated in pre-chilled 3% H2O2 in methanol (20min) and then washed in PBS (2 x 5min). Each section was blocked with normal goat serum (NGS, 10%, 30min), incubated with AVIDIN blocking solution (15min), rinsed in PBS and then incubated with rat anti-mouse CD68 primary antibody (1:200, 5% NGS, 4 °C) overnight. The slides were then washed in PBS (2 x 5min) before being incubated with the secondary antibody (1:100, 5% NGS, 30min). Next, the sections were washed in PBS (2 x 5min), incubated with ABC avidin/biotin complex (30min) and DAB solution. Staining reaction was terminated with distilled water. The sections were counterstained with Mayer Haematoxylin for 15sec and rinsed in tap water before blueing in scotts tap water and washing in tap water. Finally, slides were dehydrated in ethanol (95% 3min, 100% 3min), cleared in xylene (2 x 5min) and mounted with depex. Sections were imaged on the Olympus FSX100 microscope 4.2x magnification and images were analysed using Adobe Photoshop CC.

2.2.4. Collagen
Sections were thawed and fixed in pre-chilled acetone (15min), washed in PBS (2 x 5min), stained in 0.1% Sirius red F3BA (1hr) and
then washed in 0.01M HCl (2min). Subsequently, the slides were then dehydrated in alcohol (95%, 5mins; 100%, 2 × 5min), cleared in xylene (2 × 5min) and mounted with depeX. Sections were imaged on Olympus BX61 microscope under brightfield and polarised light 4.2x magnification and images were analysed using Adobe Photoshop CC.

2.3. Ex vivo lipogenesis assay

Primary hepatocytes were isolated by collagenase perfusion [30]. Lipogenesis was determined by measuring the incorporation of [3H] acetate or [14C]acetate as described [31]. Briefly, cells were cultured for 2 h in serum-free William’s E medium before pre-treatment for 1 h with 10 μM metformin or 10 μM O-304, followed by incubation with [3H]acetate (5 μCi/mL) and 0.5 mM sodium acetate for 4 h in the continued presence of metformin or O-304. Medium was then removed, and cells washed with PBS before lipid extraction for determination of incorporation into lipid fractions. For cholesterol measurements, the lipid fraction was extracted by homogenizing the hepatocytes in chloroform: methanol extraction as previously described [34]. In brief 10 μl of a 1-butanol and methanol (1:1 v/v) solution containing 5 mM ammonium formate and a series of internal standards were used: gas temperature, 150 °C, gas flow rate 17L/min, nebulizer 20psi, Sheath gas temperature 200 °C, capillary voltage 3500V and sheath gas flow 10L/min. Isolation widths for Q1 and Q3 were set to “unit” resolution (0.7 amu). Free cholesterol was measured as their in-source fragment (Q1 — 369.3 m/z, Q3 — 161.1 m/z), while cholesteryl esters were measured with a common product ion of 369.3 m/z.

2.5.3. Measurement of oxyysterols

To enhance the sensitivity of the low abundant oxyysterols, a derivatization was conducted on an aliquot of the lipid extracts. An aliquot of the lipid extract (40 μl) was dried down using a SpeedVac (Thermo-Fisher) and was subjected to derivatization for oxysterol analysis. To each sample, 200ul of PBS with 96unit/mL of cholesterol oxidase was added and incubated at 37°C with shaking for 1 h and 30 min. Subsequently, 500 μl of methanol containing 10 mM of Girard’s reagent P (TCI America) and 20 μl of acetic acid. This was then incubated in the dark at room temperature on a shaker. Reaction was quenched with 500 μl of acetone, with vortexing over 15 min. The entire mixture was then dried down again using the SpeedVac, reconstituted with 100 μl of butanol and 100 μl of methanol with sonication in a sonicator bath over 10 min. Samples were centrifuged at 13,000 × g for 10 min and supernatant transferred into glass vials with inserts for mass spectrometry analysis. Derivative oxysterols were monitored as a neutral loss of 79.3 specific for the derivative group.

2.6. Competitive BM transplantation study

WT CD45.1 mice were irradiated (2 × 550rads, 4 h apart) and received BM transplantations the next day with equal portions of WT CD45.1 and WT CD45.2 BM or Hmgcr KI CD45.2 BM. Mice were reconstituted for 20 weeks before sacrifice.

2.7. Blood leukocytes counts and analysis

Neutrophils, monocytes and monocyte subsets were identified using flow cytometry as previously described [36]. Blood was collected via tail bleeding and collected into EDTA tubes, which were immediately incubated on ice. All subsequent steps were performed on ice. Red blood cells were lysed (BD pharm Lyse; BD Biosciences), and WBCs were centrifuged, washed, and resuspended in HBSS (0.1% BSA w/v, 5 mM EDTA). Cells were stained with a cocktail of antibodies against CD45-PB, Ly6-C/G-PerCP-Cy5.5 (BD Biosciences) and CD115-APC (eBioscience). Monocytes were identified as CD45+CD115+ and further subdivided into Ly6-C and Ly6-Cb, neutrophils were identified as CD45+CD115+Ly6-C/Gb (Gr-1). Samples were run on the Canto II or LSR Fortessa, and analysed using FlowJo.cBMt study: Blood leukocytes were identified as stated above, with the addition of CD45.1 PECy7 (eBioscience) and CD45.2 APCy7 (eBioscience) antibodies.

2.8. Stem and progenitor cells isolation and analysis

2.8.1. Blood stem and progenitor cells

Blood was harvested and WBCs isolated as described above. Cells were then stained as previously described [36]. Briefly, a cocktail of antibodies to lineage committed cells (CD45R, CD19, CD11b, CD3e,
Brief Communication

TER-119, CD2, CD8, CD4, and Ly-6G; all FITC; eBioscience) and stem cell markers Sca1-Pacific Blue and ckit-APC-Cy7. HSPCs were assessed using Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were regarded as statistically significant if \( *P < 0.05, **P < 0.01, ***P < 0.001 \).

3. RESULTS

2.8. BM stem and progenitor cells

BM was harvested from the femurs and tibias. BM was lysed with RBC lysis buffer (BD pharm Lyse; BD Biosciences), and remaining cells were centrifuged, washed, and resuspended in HBSS (0.1% BSA w/v, 5 mM EDTA) buffer. Stem cell proliferation (cell cycle) was measured using DAPI as per the instruction booklet (Sigma). BM cells were then stained, identified and analysed as stated above. In addition, common beta subunit (CBS; CD131)-PE was also added into the BM stem cell markers antibody cocktail.

2.9. Splenic leukocytes, HSPCs and progenitor cells analysis

Spleens were dissected from mice and immediately placed on ice. Spleens were flushed through a 40 μm cell strainer with PBS to obtain a single cell suspension. RBCs were lysed, and cells resuspended in HBSS buffer as previously described [36]. Leukocytes, stem cells and progenitor cells were identified and analysed as previously described.

2.10. Total and membrane cholesterol content (BODIPY-cholesterol and CTxB) analysis

BODIPY-cholesterol was quantified by flow cytometry. Blood and BM cells were isolated and RBCs lysed as previously described [9]. BM and blood cells were incubated with BODIPY-cholesterol (0.03 mM) for 30min (37 °C) or 1hr (4 °C), respectively. Cells were then washed, and stained with a cocktail of antibodies to identify stem cells or leukocytes, and analysed on FlowJo software as previously described.

Cholera toxin subunit B staining: After single cell suspension, BM and splenic cells were incubated with antibody cocktails to stem cells for 30min. Cells were then washed and stained with Cholera toxin subunit B (CT-B) AF647 at 1:2000 for 30min. Cells were then washed and anti-Cholera toxin subunit B antibody was used to crosslink CT-B-labelled lipid rafts at 1:400 for another 30min. Finally, cells were washed and fixed with 4% PFA for 15min and measured using flow cytometry. FlowJo software was used for analysis.

2.11. Statistical analyses

All data are expressed as mean ± SEM. A 2-tailed Student t test was used to test differences between 2 groups of mice. Differences among groups of mice were assessed by two-way analysis of variance (ANOVA) or repeated-measures ANOVA. Bonferroni post hoc was performed to identify differences among means. Statistical analyses were assessed using Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were regarded as statistically significant if \( *P < 0.05, **P < 0.01, ***P < 0.001 \).

3. RESULTS

3.1. Inhibition of AMPK-HMGCR signalling increases atherogenesis

To determine the effect of blocking AMPK-HMGCR signalling on atherosclerosis progression, we crossed Hmgcr KI mice with atherosclerotic-prone Apoe deficient mice to generate Apoe−/−/Hmgcr KI double mutant mice. Apoe−/− mice were used as a control. Mice were aged to 20 weeks old on a standard chow diet to develop atherosclerosis, before the aortic sinus was collected to evaluate atherosclerotic lesion size and plaque composition in male (Figure 1A–E) and female (Figs. S1A–E) mice. While the atherosclerotic lesion size in the control mice corresponded to those expected of Apoe−/− mice at this age and diet, the cross-sectional lesion areas of the aortic sinuses from Apoe−/−/Hmgcr KI were increased by approximately 40% compared to Apoe−/− control mice (Figure 1A,B). We next characterised the atherosclerotic lesion composition and plaque remodelling by determining lipid, macrophage and collagen content. Interestingly, not only were the lesions found to be larger, but we also observed a significant increase in lipid content in Apoe−/−/Hmgcr KI mice as determined by Oil-Red-O staining (Figure 1A,C). Furthermore, in lesions from the aortic sinuses, the areas that positively stained for CD68, a macrophage marker, were also profoundly increased in Apoe−/−/Hmgcr KI mice (Figure 1A,D). Moreover, we found that Apoe−/−/Hmgcr KI mice exhibited a significant reduction in collagen deposition across their aortic sinus (Figure 1A,E). Importantly, these atherosclerotic plaque phenotypes were also observed in female Apoe−/−/Hmgcr KI mice (Figs. S1A–E). Collectively, these data indicate that blocking AMPK signalling to HMGCR not only accelerates the development of atherosclerosis but also renders the atherosclerotic plaque less stable and more susceptible to rupture.

3.2. Blocking AMPK signalling to HMGCR leads to increased circulating cholesterol and its metabolites 27-hydroxysterol levels

Our previous studies have shown that the inhibition of AMPK regulation of HMGCR leads to increased hepatic cholesterol synthesis [29]. We next asked if increased hepatic cholesterol synthesis and thus circulating cholesterol levels could also contribute to enhanced atherogenesis in Apoe−/−/Hmgcr KI mice. We assessed hepatic cholesterol synthesis ex vivo using hepatocytes isolated from control and Apoe−/−/Hmgcr KI mice. While the basal rates of synthesis were not different between the genotypes, Apoe−/−/Hmgcr KI hepatocytes showed reduced sensitivity to the cholesterol-lowering effects of the AMPK activators metformin and O-304 when compared to vehicle treated hepatocytes (Figure 2A,B). Consistent with this, we found that Apoe−/−/Hmgcr KI mice exhibited significant increases in hepatic and serum total and free cholesterol as well as cholesterol esters, the main cholesterol form stored within the cell or exported into the blood stream (Figure 2C,D). Serum concentrations of LDL/VLDL cholesterol were increased in Apoe−/−/Hmgcr KI mice (Figure 2E), whereas serum HDL cholesterol levels were comparable between genotypes (Figure S2A). Similarly, mass spectrometry-based lipidomic analysis revealed a significant increase in serum free cholesterol as well as cholesteryl ester in Apoe−/−/Hmgcr KI mice, with comparable triglyceride and diglyceride levels between genotypes (Figure 2F and S2B–E). More importantly, we identified that the serum levels of the cholesterol metabolite 27-hydroxycholesterol, which is the most abundant oxysterol in circulation and previously found to positively correlate with the development and progression of atherosclerosis both in humans and in rodents [37,38], was also upregulated in Apoe−/−/Hmgcr KI mice (Figure 2G–H and Fig. S2F), while serum bile acids remain comparable (Figure S2G). Consistent with increased atherogenesis, we found that hepatic Mcf3 expression and its downstream target genes such as Srebfl and Abca1 mRNA levels were significantly reduced Apoe−/−/Hmgcr KI mice (Fig. S2H–I). Given that hyperglycaemia and inflammation are also known to be involved in atherogenesis [39,40], we determined if glucose homeostasis and

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circulating inflammatory cytokines were altered in Apoe−/−/Hmgcr KI mice. We observed no differences in fed and fasted blood glucose levels, whole body glucose tolerance or insulin sensitivity between control and Apoe−/−/C0−/−/C0−/−/Hmgcr KI mice (Figs. S3A–C). Similarly, circulating inflammatory cytokines IL-1β, TNF and MCP1 levels were comparable between control and Apoe−/−/C0−/−/C0−/−/Hmgcr KI mice (Figs. S3D–F). These data suggest that elevated circulating cholesterol levels and its metabolite, 27-hydroxysterol, due to loss of HMGCR regulation by AMPK, are associated with enhanced atherogenesis in Apoe−/−/C0−/−/C0−/−/Hmgcr KI mice.

3.3. Ly6-c hi monocytosis is observed in Apoe−/−/C0−/−/Hmgcr KI mice

Besides elevated circulating cholesterol levels, circulating monocytes also play a causal role during the development of atherosclerosis. More importantly, defective cholesterol regulation in HSPCs, which are indispensable for the generation of all circulating immune cells, has been shown to contribute to enhanced atherogenesis [10,12,41]. Therefore, we sought to understand whether cholesterol dysregulation via AMPK-HMGCR inactivation could influence myelopoiesis. By assessing circulating leukocytes, we found that Apoe−/−/C0−/−/Hmgcr KI mice exhibited increased circulating monocytes, driven by the atherogenic Ly6-C hi subset of monocytes, while neutrophil levels remained comparable between control and Apoe−/−/C0−/−/Hmgcr KI mice (Figure 3A–E).

3.4. HSPC mobilisation and extramedullary myelopoiesis contribute to monocytosis in the Apoe−/−/Hmgcr KI mice

To investigate the mechanisms contributing to the increased abundance of circulating monocytes, we explored whether the impaired AMPK-HMGCR pathway may influence myelopoiesis. This was achieved by assessing the number of BM-derived cells from control and Apoe−/−/C0−/−/Hmgcr KI mice. Somewhat unexpectedly, we observed a reduction in BM HSPCs in Apoe−/−/C0−/−/Hmgcr KI mice (Figure 4A) while BM monocyte and neutrophil counts remained comparable in both genotypes (Figs. S4A–B), suggesting that myelopoiesis did not originate from the BM in Apoe−/−/C0−/−/Hmgcr KI mice. Consistent with the reduction in BM HSPCs, flow cytometry analysis revealed a significant increase in the abundance of circulating HSPCs (Figure 4B) and an increase in granulocyte-macrophage (GM)-colony-forming unit (CFU) s (Fig. S4C) in Apoe−/−/C0−/−/Hmgcr KI mice, suggesting that HSPCs from the BM had exited the BM and moved into the blood. During atherogenesis, HSPCs mobilise to extramedullary sites such as the spleen, which provides a more permissive microenvironment for proliferation and myeloid differentiation in a process of extramedullary myelopoiesis [12]. In line with this, Apoe−/−/C0−/−/Hmgcr KI mice exhibited increased splenic HSPCs (Figure 4C), common myeloid progenitors (CMPs) (Figure 4D) and granulocyte macrophage progenitors (GMPs) (Figure 4E), concomitant with a trend (p = 0.07) in increased spleen weight (Fig. S4D). More importantly, we also

Figure 1: Inhibition of AMPK-HMGCR signalling increases atherogenesis. (A-E) Apoe−/− and Apoe−/−/Hmgcr KI mice were aged to 20 weeks old on a standard chow diet and aortas were collected to assess for plaque size and composition. In the aortic sinus, (A, B) lesion size were assessed by hematoxylin and eosin (H&E), (A, C) lipid content was assessed by Oil Red O (ORO) staining, (A, D) macrophages were assessed by staining for CD68, (A, E) collagen content was assessed by staining for picrosirius red. Results shown are a representative image. Scale bars = 50 µM. Data are means ± SEM and analysed using a Student unpaired t test, n = 8–16 per group (**P < 0.05, ***P < 0.001, when comparing Apoe−/− versus Apoe−/−/Hmgcr KI).
found increased splenic Ly6-Chi monocytes by approximately 50% in \( \text{Apoe}^{-/-}/\text{Hmgcr} \) mice, which have been shown previously to track into atherosclerotic lesions [12] (Figure 4F). Consistent with a selective increase in the Ly6-Chi monocytes, splenic levels of Ly6-Clo monocytes and neutrophils remained comparable (Figs. S4E–F).

Similarly, these differences in BM HSPCs, and circulating Ly6-C hi monocytes and HSPCs were also evidenced in female \( \text{Apoe}^{-/-}/\text{Hmgcr} \) mice (Figs. S5A–D). Collectively, these results suggest that inhibition of AMPK regulation of HMGCR promotes extramedullary myelopoiesis, thereby contributing to the abundance of circulating atherogenic monocytes.

#### 3.5. Increased cholesterol accumulation in \( \text{Apoe}^{-/-}/\text{HMGCR} \) mice

3.5. Increased cholesterol accumulation in \( \text{Apoe}^{-/-}/\text{HMGCR} \) mice contributes to HSPC mobilization and monocytosis

Previously, we have shown that defective HSPC cholesterol regulation is a key determinant of enhanced myelopoiesis, contributing to increased monocyte production and accelerated atherogenesis [10,12,41]. To understand whether dysregulated HSPC cholesterol synthesis could be contributing to enhanced myelopoiesis in \( \text{Apoe}^{-/-}/\text{Hmgcr} \) mice, we performed ex vivo cholesterol synthesis assay using BM-derived HSPCs isolated from \( \text{Apoe}^{-/-}/\text{Hmgcr} \) and \( \text{Apoe}^{-/-}/\text{Hmgcr} \) mice. In support of this hypothesis, we found that \( \text{Apoe}^{-/-}/\text{Hmgcr} \) HSPCs exhibited significant increases in cholesterol synthesis.
More importantly, we also observed an increase in cholesterol content, as shown by the increased staining with BODIPY-cholesterol, in BM- and blood-derived HSPCs of ApoE−/−/Hmgcr KI mice (Figure 5B,C). Moreover, membrane cholesterol content as determined by Cholera toxin subunit B (CTxB) staining was also increased in BM-derived HSPCs of ApoE−/−/Hmgcr KI mice (Fig. S6A). Interestingly, elevated cellular cholesterol content was associated with increased HSPC proliferation (Figure 5D), concomitant with increased cell surface abundance of the common B subunit (CBS) of the Interleukin 3 (IL-3)/Granulocyte-macrophage colony-stimulating factor (GM-CSF) in the BM-derived HSPCs of ApoE−/−/Hmgcr KI mice (Figure 5E). These results suggest that increased cholesterol content in ApoE−/−/Hmgcr KI HSPCs may lead to enhanced HSPC expansion and myelopoiesis. Consistently, we observed increased total and membrane cholesterol (Figure 5F and Fig. S6B) and proliferation in splenic HSPCs (Figure 5G–H) and CMPs (Figure 4G–L and Figs. S6C–D) in ApoE−/−/Hmgcr KI mice, suggesting that increased monocytosis in ApoE−/−/Hmgcr KI mice is driven by extramedullary hematopoiesis in the spleen. Previous studies have shown that increased cholesterol content in mature myeloid cells, including monocytes and macrophages, promotes cell formation and contributes to accelerated atherogenesis [42,43]. We hypothesized that this might also be occurring in the mature myeloid cells of ApoE−/−/Hmgcr KI mice, which transition to lipid-laden macrophages in the atherosclerotic lesion. Consistent with this, atherogenic monocyes, Ly6-Clo subset from ApoE−/−/Hmgcr KI mice exhibited significantly increased cholesterol content (Figure 5I), suggesting that the increased cholesterol content in HSPCs is retained during extramedullary myelopoiesis. To test whether blocking AMPK-HMGCR signalling in HSPCs is responsible for driving extramedullary myelopoiesis independent of circulating cholesterol levels, we performed a competitive BM transplantation (cBMT). This allowed us to assess whether HSPCs with defective AMPK-HMGCR signalling would outcompete wildtype (WT) HSPCs. This was achieved by transplanting equal portions of BM cells from CD45.1 WT mice and either CD45.2 WT or Hmgcr KI CD45.2 into lethally irradiated WT recipient mice (Figure 5J). Consistent with our hypothesis, there was significantly greater proportion of circulating Hmgcr KI Ly6-Clo monocytes (Figure 5K). Analysis of the BM HSPCs revealed greater proportion of Hmgcr KI CD45.2 HSPCs within the BM (Figure 5L), thus suggesting that the Hmgcr KI CD45.2 cells have a competitive advantage. Taken together, these results reveal that increased cholesterol accumulation in Hmgcr KI HSPCs also intrinsically drives extramedullary myelopoiesis, at least in part advancing atherosclerosis progression.
Figure 4: Inhibition of AMPK-HMGCR signalling promotes HSPC mobilization and extramedullary myelopoiesis. (A-F) Apoe\(^{-/-}\) and Apoe\(^{-/-}\)/Hmgcr KI mice were aged to 20 weeks old on a standard chow diet. HSPCs in the (A) BM, (B) blood and (C) spleen were measured using flow cytometry. In the spleen, (D) CMPs, (E) GMPs and (F) Ly6-C\(^{hi}\) monocytes were measured via flow cytometry. Results are means ± SEM and analysed using a Student unpaired t test, n = 7–11 per group (*P < 0.05, **P < 0.01 when comparing Apoe\(^{-/-}\) versus Apoe\(^{-/-}\)/Hmgcr KI).
4. DISCUSSION

In the present study, we demonstrated that AMPK regulation of the rate limiting enzyme, HMGCR, in the mevalonate pathway plays a critical role in controlling cholesterol homeostasis in the liver and in HSPCs. Our findings show that blocking AMPK inhibition of HMGCR (Apoε−/−/Hmgcr KI) exhibited significantly increased atherosclerotic lesion size combined with elevated lipid and macrophage content.

Figure 5: Increased cholesterol accumulation in Apoε−/−/HMGCR KI HSPCs contributes to HSPC mobilization and Ly6-CHi monocytosis. (A-I) Apoε−/− and Apoε−/−/Hmgcr KI mice were aged to 20 weeks old on a standard chow diet. (A) BM HSPCs were sorted using FACS before ex vivo cholesterol synthesis was measured via 14C-acetate labelling and TLC fractionation. n = 4 independent experiments. BODIPY-Cholesterol levels were measured in (B) BM HSPCs and (C) blood HSPCs. BM HSPC (D) proliferation and (E) CBS were measured via flow cytometry. BODIPY-Cholesterol levels were measured in (F) splenic HSPCs, and (G) splenic HSPC proliferation and (H) CBS were measured via flow cytometry. BODIPY-Cholesterol levels were measured in (I) blood Ly6-CHi monocytes. (J) Experimental overview. (K-L) Representative flow plots, and ratio of CD45.2 to CD45.1 cells in (K) blood Ly6-CHi monocytes and (L) BM HSPCs. (M) Graphic abstract. Results are means ± SEM and analysed using a Student unpaired t test, n = 4–9 per group (* P < 0.05, ** P < 0.01 when comparing Apoε−/− versus Apoε−/−/Hmgcr KI or Wt/Wt versus Hmgcr KI/Wt).
These findings are consistent with previous studies showing that genetic ablation of AMPK α2 subunit increases atherosclerosis [44] and that pharmacological activation of AMPK in atherosclerotic-prone Apoe<sup>—/—</sup> mice, with either direct or indirect AMPK activators [22–24], protected against the development and progression of atherosclerosis. More importantly, in line with our previous studies [29], we further showed that in primary hepatocytes isolated from Apoe<sup>—/—</sup>/Hmgcr<sup>KI</sup> mice, the Ser871Ala mutation rendered HMGCR insensitive to the inhibitory effects of the AMPK activators metformin and 304 for reducing atherosclerotic cardiovascular disease risk.

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**AUTHOR CONTRIBUTIONS**

M.K.S.L designed and performed research experiments, contributed discussion, and edited manuscript. O.D.C, X.L, S.N, D.D, K.H and D.A.O contributed to research experiments and edited manuscript. S.G, P.J.M, T.E, M.D.F and B.E.K contributed discussion and edited manuscript. A.J.M and K.L contributed conceptualization, study design, discussion, wrote and edited manuscript. All authors have read and agreed to the published version of the manuscript.

**DATA AND MATERIALS AVAILABILITY**

Further information and requests for reagents may be directed to corresponding authors.

**MATERIALS AVAILABILITY**

This study did not generate new unique reagents.

**DATA AND CODE AVAILABILITY**

This study did not generate any unique datasets or code.

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**CONFLICT OF INTEREST**

Dr Thomas Edlund is the Chief Scientific Officer and a shareholder of Betagenon AB.

**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.molmet.2022.101514](https://doi.org/10.1016/j.molmet.2022.101514).
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