Disruption of Glut1 in Hematopoietic Stem Cells Prevents Myelopoiesis and Enhanced Glucose Flux in Atheromatous Plaques of ApoE−/− Mice

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Rationale: Inflamed atherosclerotic plaques can be visualized by noninvasive positron emission and computed tomographic imaging with 18F-fluorodeoxyglucose, a glucose analog, but the underlying mechanisms are poorly understood.

Objective: Here, we directly investigated the role of Glut1-mediated glucose uptake in apolipoprotein E-deficient (ApoE−/) mouse model of atherosclerosis.

Methods and Results: We first showed that the enhanced glycolytic flux in atheromatous plaques of ApoE−/− mice was associated with the enhanced metabolic activity of hematopoietic stem and multipotent progenitor cells and higher Glut1 expression in these cells. Mechanistically, the regulation of Glut1 in ApoE−/− hematopoietic stem and multipotential progenitor cells was not because of alterations in hypoxia-inducible factor 1α signaling or the oxygenation status of the bone marrow but was the consequence of the activation of the common β subunit of the granulocyte-macrophage colony-stimulating factor/interleukin-3 receptor driving glycolytic substrate utilization by mitochondria. By transplanting bone marrow from WT, Glut1+/−, ApoE−/−, and ApoE−/−Glut1−/− mice into hypercholesterolemic ApoE-deficient mice, we found that Glut1 deficiency reversed ApoE−/− hematopoietic stem and multipotential progenitor cell proliferation and expansion, which prevented the myelopoiesis and accelerated atherosclerosis of ApoE−/− mice transplanted with ApoE−/− bone marrow and resulted in reduced glucose uptake in the spleen and aortic arch of these mice.

Conclusions: We identified that Glut1 connects the enhanced glucose uptake in atheromatous plaques of ApoE−/− mice with their myelopoiesis through regulation of hematopoietic stem and multipotent progenitor cell maintenance and myelomonocytic fate and suggests Glut1 as potential drug target for atherosclerosis.

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Key Words: atherosclerosis ■ bone marrow ■ cholesterol ■ glucose transporter type 1 ■ glycolysis ■ myeloid cells

Atherosclerosis is a chronic, hypercholesterolemia-driven inflammatory disease that is initiated by the deposition of cholesterol-rich lipoproteins in the artery wall, leading to monocyte–macrophage recruitment. Hypercholesterolemia and defective cholesterol efflux have also been documented to induce myelopoiesis, which contributes to atherosclerotic lesion formation by fueling plaques with monocytes and neutrophils.1,2 The monocyte count, in particular, independently predicts risk for coronary artery disease after adjustment for conventional risk factors.3,4

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Hematopoietic stem cells (HSCs) are quiescent in the bone marrow (BM) niche and are the source of all hematopoietic stem and progenitor cells (HSPCs) and differentiated cells that are critical for the maintenance and replenishment of peripheral leukocytes in adult life, particularly during emergency hematopoiesis. However, we and others have recently shown that chronic cholesterol accumulation in HSPCs because of hypercholesterolemia and defective apolipoprotein (Apo)-mediated

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cholesterol efflux promotes pathogenic HSPC expansion and proliferation leading to uncontrolled myelopoiesis. For instance, in the ApoE−/− mouse model of atherosclerosis, the progressive HSPC expansion that drives myelopoiesis contributed to provide the inflamed atherosclerotic lesions with neutrophils and monocytes. Although recent research has focused on elucidating the roles of cytokines and the microenvironment in the proliferation, mobilization, and commitment of HSPCs in preclinical model of atherosclerosis, the cellular metabolic pathways that regulate these processes remain unknown.

Lessons from various mutant mice displaying a wide range of bioenergetic defects in vivo have pointed to a central role for the mitochondrial energy metabolism in HSC stemness and fate, offering novel therapeutic perspectives. Mounting evidence also suggests that HSC quiescence requires a hypoxic environment to maintain glycolysis-biased metabolic activity instead of mitochondrial oxidative phosphorylation. By limiting mitochondrial respiration and ATP production, this could indeed prevent HSCs from producing reactive oxygen species to avoid their differentiation and exhaustion. In contrast, funneling glucose to the mitochondria for Krebs cycle utilization is required when the HSCs become proliferative or undergo differentiation, most likely because of the high energy demand of these cellular processes.

In an attempt to better understand the relation between the enhanced hematopoietic glycolytic activity, HSPC proliferation, myelopoiesis, and the development of atherosclerotic lesions, we first showed that an enhanced hematopoietic glycolytic activity, HSPC proliferation leading to uncontrolled myelopoiesis, and their myelopoiesis through regulation of HSPC expansion and atherogenesis compared with mice that had received ApoE−/− Glut1+/− BM. Thus, we proposed a causal relation between the enhanced hematopoietic glycolytic activity in ApoE−/− mice and their myelopoiesis through regulation of HSPC expansion and fate, offering novel therapeutic perspectives.

### Methods

**Materials and treatments**

*GluT1−/−* mice (kindly provided by Dr De Vivo, Columbia University) have been crossed to C57BL/6J for >12 generations within our colony. *ApoE−/−* (B6.129P2-*ApoE−/−*) mice were generated. For the neutralizing antibody experiment, WT and *ApoE−/−* mice with single or combined deficiencies of ApoE or the glucose transporter Glut1 into *ApoE−/−* mice on a C57BL/6J background were obtained from the Jackson Laboratory. *ApoE−/−* (B6.129P2-*ApoE−/−*) mice expressing Ly5.1 cyclohexan (CD45.2) were crossed to wild-type (WT) mice expressing Ly5.2 (CD45.1) to generate *ApoE−/−* mice expressing Ly5.2 (CD45.1). For each experiment, littermate controls were generated. For the neutralizing antibody experiment, WT and *ApoE−/−* mice were intravenously injected with IgG control or IL-3R α-1 to maintain glycolysis-biased metabolic activity instead of mitochondrial oxidative phosphorylation. By limiting mitochondrial respiration and ATP production, this could indeed prevent HSCs from producing reactive oxygen species to avoid their differentiation and exhaustion. In contrast, funneling glucose to the mitochondria for Krebs cycle utilization is required when the HSCs become proliferative or undergo differentiation, most likely because of the high energy demand of these cellular processes. More recently, Oburoglu et al. have also reported that glucose utilization can dictate the myeloid lineage commitment in human HSCs. Intriguingly, increased hematopoietic metabolic activity can be visualized by noninvasive positron emission and computed tomographic imaging with 18F-fluorodeoxyglucose, a glucose analog, not only in inflamed atherosclerotic plaques but also in the spleen of patients with cardiovascular diseases, reflecting most likely an extramedullary hematopoiesis. However, the relevance of these observations as well as the underlying mechanisms are not fully understood.

In an attempt to better understand the relation between the enhanced hematopoietic glycolytic activity, HSPC proliferation, myelopoiesis, and the development of atherosclerotic lesions, we first showed that an enhanced hematopoietic glycolytic activity in the aortic arch, the BM, and the spleen of *ApoE−/−* BM-transplanted mice was associated with an enhanced glucose transporter type 1 (Glut1) expression in *ApoE−/−* HSPCs. Mechanistic studies showed that the upregulation of Glut1 in *ApoE−/−* HSPCs was not because of an alteration of the oxygenation status of the BM niche but rather was dependent on Ras signaling downstream of the granulocyte-macrophage colony-stimulating factor (GM-CSF)/interleukin-3 (IL-3) receptor driving glycolytic substrate utilization by mitochondria. Finally, we performed BM transplantation from mice with single or combined deficiencies of ApoE or the glucose transporter Glut1 into *ApoE−/−* mice. Consistent with our hypothesis, *ApoE−/−* mice that had received *ApoE−/−* Glut1+/− BM showed reduced HSPC proliferation and expansion, myelopoiesis, and atherogenesis compared with mice that had received *ApoE−/−* BM. Thus, we proposed a causal relation between the enhanced hematopoietic glycolytic activity in *ApoE−/−* mice and their myelopoiesis through regulation of HSPC expansion and fate, offering novel therapeutic perspectives.

### Flow Cytometry Analysis

**Blood leukocytes**

For identification of peripheral blood leukocytes, 100 μL of blood was collected into EDTA tubes before red blood cell lysis (BD Pharm Lyse, BD Biosciences), filtration, and staining for 30 minutes on ice. Cells were stained with a cocktail of antibodies against CD45, Ly6C/G, CD115, B220, TCR-β, and CD8 as previously described. Briefly, monocytes were identified as CD45+CD115-.
neutrophils as CD45<sup>−</sup>CD115<sup>−</sup>Ly6C/G<sup>−</sup>, B-lymphocytes as CD45<sup>−</sup>B220<sup>−</sup>, T-lymphocytes as CD45<sup>−</sup>TCR<sup>−</sup>β<sup>−</sup> and further subdivided into CD4<sup>+</sup>, CD8<sup>+</sup>, and CD8<sup>+</sup>Ly6C/G<sup>−</sup> subsets.

**BM HSPCs**

BM cells were collected from leg bones, lysed to remove red blood cells, and filtered before use. Freshly isolated BM cells were stained with the appropriate antibodies for 30 minutes on ice. For hematopoietic subsets, the following lineage antibodies were used: a cocktail of antibodies to lineage committed cells (CD45R, CD19, CD11b, CD3e, Ter-119, CD2, CD8e, and Ly6C/G) and the following stem cell markers: c-Kit, Sca-1, FMS-like tyrosine kinase 3 ligand (Flt3), also known as CD135), CD120 (Slamf1), CD34, and FcgRII/III as previously described. Briefly, HSPCs were identified as lin<sup>−</sup>Scal<sup>−</sup>c-Kit<sup>−</sup> (LSks) and HSPC subsets were identified from the most quiescent as long-term (LT)-HSC (CD34<sup>−</sup>CD150<sup>−/hi</sup>Flt3<sup>−</sup>) to the most cycling as short-term–HSC (CD34<sup>−</sup>CD150<sup>−/lo</sup>Flt3<sup>−</sup>) and multipotential progenitors (CD34<sup>−</sup>CD150<sup>−</sup>Flt3<sup>−</sup> → CD34<sup>+</sup>CD150<sup>−</sup>Flt3<sup>−</sup>). Hematopoietic progenitor cells were identified as common myeloid progenitors (CMP, Lin<sup>−</sup>Scal<sup>−</sup>c-Kit<sup>−</sup>CD34<sup>+</sup>FcgRII/III<sup>−</sup>), granulocyte-macrophage progenitors (GMP, Lin<sup>−</sup>Scal<sup>−</sup>c-Kit<sup>−</sup>CD34<sup>+</sup>FcgRII/III<sup>−</sup>), and megakaryocyte–erythocyte progenitors (Lin<sup>−</sup>Scal<sup>−</sup>c-Kit<sup>−</sup>CD34<sup>+</sup>FcgRII/III<sup>−</sup>). For DNA content analysis, HSPC-stained BM cells were fixed in 1% paraformaldehyde in PBS, washed, and stained with 4',6-diamidino-2-phenylindole (5 μg/mL; Molecular Probes). Cell surface expression of Glut1 was quantified using Glut1 FAB1418 antibody from R&amp;D systems or Glut1 receptor binding domain ligand (Metafora Biosystems). To assess the uptake of 2-[(2-NBDG), prestained cells were incubated with 10 μmol/L fluorescent tetramethylrhodamine ethyl ester (AnaSpec) (MMP) and reactive oxygen species production were analyzed with a 4-laser BD FlowJo software (Tree Star Inc). As previously described, short-term–HSC (CD34<sup>−</sup>CD150<sup>−/hi</sup>Flt3<sup>−</sup>) and multipotential progenitors (CD34<sup>−</sup>CD150<sup>−</sup>Flt3<sup>−</sup> → CD34<sup>−</sup>CD150<sup>−</sup>Flt3<sup>−</sup>). Hematopoietic progenitor cells were identified as common myeloid progenitors (CMP, Lin<sup>−</sup>Scal<sup>−</sup>c-Kit<sup>−</sup>CD34<sup>+</sup>FcgRII/III<sup>−</sup>), granulocyte-macrophage progenitors (GMP, Lin<sup>−</sup>Scal<sup>−</sup>c-Kit<sup>−</sup>CD34<sup>+</sup>FcgRII/III<sup>−</sup>), and megakaryocyte–erythocyte progenitors (Lin<sup>−</sup>Scal<sup>−</sup>c-Kit<sup>−</sup>CD34<sup>+</sup>FcgRII/III<sup>−</sup>). For DNA content analysis, HSPC-stained BM cells were fixed in 1% paraformaldehyde in PBS, washed, and stained with 4',6-diamidino-2-phenylindole (5 μg/mL; Molecular Probes). Cell surface expression of Glut1 was quantified using Glut1 FAB1418 antibody from R&amp;D systems or Glut1 receptor binding domain ligand (Metafora Biosystems). To assess the uptake of 2-[(2-NBDG), prestained cells were incubated with 10 μmol/L fluorescent tetramethylrhodamine ethyl ester (AnaSpec) (MMP) and reactive oxygen species production were analyzed with a 4-laser BD FlowJo software (Tree Star Inc).

**Statistical Analysis**

Data are shown as means±SEM. Statistical significance was performed using Prism t test or ANOVA according to the data set. Results were considered as statistically significant when P<0.05.

**Results**

**Enhanced Glucose Uptake in Atheromatous Plaques Under Hypercholesterolemic Conditions Correlates With Higher Metabolic Activity of Hematopoietic Cells and Is Associated With Higher Glut1 Expression in HSPCs**

To monitor the metabolic activity of hematopoietic cells, we first investigated the uptake of the radiolabeled D-glucose analog 2-deoxy[<sup>14</sup>C] glucose in organs isolated from irradiated ApoE<sup>−/−</sup>-recipient mice transplanted with either WT or ApoE<sup>−/−</sup> BM. A >2-fold increase in 2-deoxy[<sup>14</sup>C] glucose uptake was observed not only in the aortic arch of ApoE<sup>−/−</sup> BM-transplanted mice compared with controls but also in their BM and spleen (Figure 1A). A ≈3.5-fold increase in 2-deoxy[<sup>14</sup>C] glucose uptake was also consistently observed in colony forming unit assays with the multipotential progenitors and GMP from the ApoE<sup>−/−</sup> mice (Figure 1B). The oxygen consumption was 1.3-fold higher in ApoE<sup>−/−</sup>-lineage marker-positive (Lin<sup>+</sup>) ApoE<sup>−/−</sup> Lin<sup>+</sup> BM cells, and Lin<sup>+</sup>Scal<sup>−</sup> progenitors; these cell types represent mature leukocytes or a mix of HSPCs, respectively (Figure 1C). The higher oxygen consumption seen in ApoE<sup>−/−</sup> cells was most likely maintained by mitochondrial oxygen consumption because treatment with oligomycin, which inhibits mitochondrial ATP synthase, clearly suppressed their respiration (Figure 1D). Quantification of the citric acid metabolites by liquid chromatography–mass spectrometry showed higher citrate, fumarate, and malate, but not succinate in ApoE<sup>−/−</sup> leukocytes (Figure 1E). This was associated with a 1.7-fold increase in succinate dehydrogenase activity in ApoE<sup>−/−</sup> leukocytes (WT, 13.7±1.9 versus ApoE<sup>−/−</sup>, 24.2±4.1 OD/min per mg protein, respectively). Consistent with these findings, a ≈1.3-fold increase in the MMP was observed in ApoE<sup>−/−</sup> Lineage marker-positive (Lin<sup>+</sup>) and Lin<sup>−</sup> BM cells by flow cytometry using a fluorescent tetramethylrhodamine ethyl ester dye (Figure 1F).

**HIF-1α Is Neither Involved in the Upregulation of Glut1 in ApoE<sup>−/−</sup> HSPCs nor in the Enhanced Myelopoiesis of ApoE<sup>−/−</sup> Mice**

We next set out to better understand the mechanism leading to Glut1 regulation in the ApoE<sup>−/−</sup> HSPCs. Studies have proposed that the HIF-1α upregulates Glut1,17 and HIF-1α contributes to HSPC homeostasis.18,20 We first assessed the hypoxic state of the BM cells isolated from irradiated ApoE<sup>−/−</sup>-recipient mice transplanted with either WT or ApoE<sup>−/−</sup> BM by flow cytometry with a fluorescein-conjugated anti-pimonidazole antibody at 90 minutes after intravenous pimonidazole administration. We did not observe significant changes in pimonidazole staining between WT and ApoE<sup>−/−</sup> BM-transplanted mice could reflect the metabolic state not only of leukocytes but also of HSPCs.
Figure 1. Enhanced glucose utilization in the aortic arch, splenocytes, bone marrow (BM), and hematopoietic stem and multipotential progenitor cells (HSPCs) of ApoE−/− BM chimeras. A, 2-deoxy-[14C]-glucose uptake in aortic arch, BM, and spleen of ApoE−/− recipients transplanted with wild-type (WT) or ApoE−/− BM at 12 weeks after the transplantation procedure. B, 2-deoxy-[14C]-glucose uptake was also determined in colony forming unit assays of multipotential progenitors (CFU-GEMM) and granulocyte-macrophage progenitors (CFU-GM) from the BM of WT and ApoE−/− mice. C, Oxygen consumption of whole BM cells, lineage marker (Lin)+, Lin− BM cells, and Lin− Sca1+ progenitors isolated from the BM of WT and ApoE−/− mice in absence or (D) in presence of oligomycin treatment. E, The citric acid metabolites were determined by liquid chromatography–mass spectometry in BM cells isolated from ApoE−/− recipients transplanted with WT or ApoE−/− BM at 12 weeks after the transplantation procedure. F, The mitochondrial membrane potential was measured by flow cytometry using a fluorescent tetramethylrhodamine ethyl ester dye in Lin−, Lin+, and CD34− or CD34+ HSPCs isolated from the BM of these mice. G, NBD-glucose binding and uptake and (H) cell surface expression of Glut1 were also quantified in these cells. All results are the means±SEM and are representative of at least 1 experiment performed with 6 to 10 animals per group; *P<0.05 vs WT. §P<0.05 vs the untreated condition.
Glut1 gene expression was higher under both normoxic and hypoxic culture conditions in ApoE−/− BM cultures (Online Figure IC). Thus, to directly test the contribution of HIF-1α in the regulation of Glut1-dependent glucose metabolism in ApoE−/− HSPC in vivo, we next generated an inducible, hematopoietic-specific HIF-1α knockout (Mx1-cre HIF1αfl/fl) on a WT or ApoE−/− background. The BM from these mice was transplanted into irradiated ApoE−/− mice and after a recovery period, the recipients were fed with a high-fat diet for 12 weeks to exacerbate their HSPC expansion (Figure 2A). HIF-1α was deleted from hematopoietic cells before the start of the diet by sequential polyI:polyIC injections, which efficiently excised the HIF-1α gene from the BM cells (Figure 2B and 2C). Lactate dehydrogenase mRNA expression was also significantly reduced in the BM of these mice, but Glut1 was only marginally regulated (Figure 2C). Also, HIF-1α deficiency did not alter the cell surface expression of Glut1 in CD34+ HSPCs and CD34− LT-HSCs (Figure 2D) or the frequency of these cells (Figure 2E). Furthermore, quantification of the blood myeloid cells in these mice revealed that HIF-1α deficiency further increased the neutrophil, monocyte, and eosinophil counts in these mice (Figure 2F). Together, these findings suggest that HIF-1α does not mediate the up-regulation of Glut1 in ApoE−/− HSPC or their expansion and minimally contributed to myelopoiesis under hypercholesterolemic conditions.

Figure 2. Hypoxia-inducible factor-1α (HIF-1α)-independent regulation of Glut1 expression and ApoE−/− hematopoietic stem and multipotential progenitor cell (HSPC) expansion and myeloid lineage fate. A, Experimental overview. Bone marrow from Mx1-Cre (controls), Mx1-cre HIF1αfl/fl, ApoE−/− Mx1-Cre, ApoE−/− Mx1-cre HIF1αfl/fl mice were transplanted into ApoE−/− recipient mice and after a 5-week recovery period, the mice were injected with polyinosinic-polycytidylic acid (Poly:IC) and fed with a high-fat diet (HFD) for 12 weeks to induce the expansion of HSPCs. BMT indicates BM transplant. B, Representative Western blots showing HIF-1α levels in bone marrow (BM) cells freshly isolated from these mice at the end of the study period. Quantification (normalized to β-actin) is expressed as arbitrary unit and indicated by numbers below. C, mRNA expression of HIF-1α and HIF-1α target genes Ldha and Glut1 in BM cells freshly isolated from these mice at the end of the study period. D, Histograms showing Glut1 cell surface expression (expressed as the mean fluorescence intensity [MFI]) in CD34− and CD34+ HSPCs. E, Quantification of the CD34+ or CD34− HSPCs by flow cytometry was expressed as the percentage of total BM. F, Peripheral blood neutrophils, monocytes, and eosinophils were also quantified in these mice at the end of the study period. The results are the means±SEM of 6 to 10 animals per group; *P<0.05 vs Mx1-Cre; §P<0.05 vs ApoE−/− Mx1-Cre. ND indicates not detectable.
IL-3Rβ Signaling Pathway Concomitantly Controls the Cycling and the Upregulation of Glut1 in ApoE<sup>−/−</sup> HSPCs

Glut1 can be upregulated by growth hormone-dependent activation of oncogenes, such as Ras or Src. Therefore, we investigated the expression of Glut1 in WT and ApoE<sup>−/−</sup> BM cultures in response to various growth hormones. The Glut1 mRNA levels in WT BM cells were increased on stimulation with GM-CSF and IL-3, but not Flt3L or thrombopoietin, and this response was further increased in the ApoE<sup>−/−</sup> BM cells and blunted by a farnesyl transferase inhibitor that blocks Ras activation (Online Figure IIA). These responses were not observed for the HIF-1α or lactate dehydrogenase mRNAs (Online Figure IIA). Flow cytometry analysis confirmed an increase in Glut1 cell surface expression in ApoE<sup>−/−</sup> HSPCs on IL-3 and GM-CSF stimulation compared with WT HSPCs (Online Figure IIB). These effects were abrogated by blocking the IL-3Rβ signaling pathway and downstream Ras activation with a farnesyl transferase inhibitor, but not by the Jak2 inhibitor, AG490, or the AMP-activated protein kinase activator, metformin (Online Figure IIB). Removing plasma membrane cholesterol with cyclodextrin also prevented the enhanced Glut1 expression in ApoE<sup>−/−</sup> HSPCs confirming the central role of cholesterol in this regulation (Online Figure IIB).

To directly test the relevance of these observations in vivo, an IL-3Rβ blocking antibody was next injected into the WT and ApoE<sup>−/−</sup> mice. Consistent with earlier work, we first showed that this antibody efficiently reduced myelopoiesis in the ApoE<sup>−/−</sup> mice and had no effect in WT mice during the 24-hour period (Figure 3A). An analysis of the genes in the glycolytic pathway in the BM cells at the end of the study period revealed no significant changes in the HIF-1α or lactate dehydrogenase mRNAs, but the Glut1 mRNA was downregulated after treatment with the IL-3Rβ blocking antibody in the ApoE<sup>−/−</sup> BM (Online Figure IIC). Quantification of the HSPCs in the BM of these mice by flow cytometry also revealed reduced numbers of CD34<sup>+</sup>, but not CD34<sup>−</sup> HSPCs, in the ApoE<sup>−/−</sup> mice, but not in the WT mice (Figure 3B), which correlated with reduced cycling of these cells (Figure 3C). This was associated with the reduced cell surface expression of Glut1 in the ApoE<sup>−/−</sup> CD34<sup>+</sup> HSPCs (Figure 3D). These results revealed that the metabolic requirements for proliferation and expansion of the ApoE<sup>−/−</sup> HSPCs are associated with the IL-3/Glut1 axis and not the HIF-1α/Glut1 axis in vivo.

Inhibition of Mitochondrial Glycolytic Substrate Utilization Prevents ApoE<sup>−/−</sup> HSPC Proliferation and Myelomonocytic Fate In Vitro

To determine the contribution of mitochondrial oxidative phosphorylation on ApoE<sup>−/−</sup> HSPC proliferation and lineage specification on IL-3 and GM-CSF treatment, we next artificially suppressed various enzymes that are intricately involved in the regulation of the tricarboxylic acid cycle using pharmacological inhibitors (Figure 4A). We first validated our in vitro BM culture assay by showing that inhibition of the IL-3Rβ signaling pathway (IL-3Rβ blocking antibody), inhibition of Ras signaling (farnesyl transferase inhibitor FTI-277), and plasma membrane cholesterol depletion with cyclodextrin prevented ApoE<sup>−/−</sup> HSPC expansion (Figure 4B) and the generation of CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid cells on IL-3 and GM-CSF stimulation.

Figure 3. The ApoE<sup>−/−</sup> hematopoietic stem and multipotential progenitor cell (HSPC) expansion and myeloid lineage fate and Glut1 upregulation are driven by the IL3Rβ signaling pathway. A, Twenty-week–old wild-type (WT) and ApoE<sup>−/−</sup> mice were injected with IgG control or 100 μg of the IL-3Rβ blocking antibody for 24 hours and analyzed for peripheral blood myeloid cells by flow cytometry. B, The CD34<sup>+</sup> or CD34<sup>−</sup> HSPCs were quantified in the bone marrow (BM) of these mice and was expressed as the percentage of total BM. C, The percentage of these cells in S/G2M phase was determined by Hoechst staining, and D, Glut1 cell surface expression was expressed as the mean fluorescence intensity (MFI). The results are the means±SEM of 5 to 6 animals per group; *P<0.05 vs WT IgG control; §P<0.05 vs ApoE<sup>−/−</sup> IgG control.
Figure 4. Mitochondrial glycolytic substrate utilization is required for ApoE<sup>−/−</sup> hematopoietic stem and multipotential progenitor cell (HSPC) proliferation and myelomonocytic fate in vitro. 

A, Schematic representation of the metabolic pathways analyzed using pharmacological inhibitors with key enzymes indicated in blue, inhibitors in red, metabolites in black, and cholesterol in green. Red boxes also indicated key signaling molecules. Bone marrow (BM) cells from fluorouracil-treated wild-type (WT) and ApoE<sup>−/−</sup> mice were grown for 72 hours in liquid culture containing 10% FBS IMDM (fetal bovine serum, Iscove’s modified Dulbecco’s medium) in the presence of the indicated chemical compounds and 6 ng/mL IL-3 or 2 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). 

B, Quantification of HSPCs and (C) CD11b<sup>+</sup>Gr<sup>−</sup> myeloid cells after in vitro culture. Arrows on the y axis indicate the starting percentage of cells per well before culture. The results are the means±SEM of an experiment performed with 4 animals per group; ACC indicates acetyl-CoA carboxylase; CD, cyclodextrin; CPI-613 or CPI, 6,8-bis[(phenylmethyl)thio]octanoic acid; GOTs, glutamate oxaloacetate transaminases; LDH, lactate dehydrogenase; NPA, nitropropionic acid; NS, nonsignificant; OAA, oxaloacetate; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase; and TOFA, 5-(tetradecyloxy)-2-furoic acid. P<0.05, genotype effect.
treatment (Figure 4C). In contrast, inhibition of lactate dehydrogenase and acetyl-CoA carboxylase using oxamate and Tofa, respectively, or activation of AMP-activated protein kinase with metformin did not alter ApoE−/− HSPC expansion (Figure 4B) or their myeloid fate (Figure 4C). Surprisingly, inhibition of mitochondrial respiratory chain complex I with rotenone was also not sufficient to dampen ApoE−/− HSPC expansion and myeloid commitment (Figure 4B and 4C). Given the enhanced succinate dehydrogenase activity observed in ApoE−/− BM cells, we next evaluated the contribution of the mitochondrial complex II. Inhibition of succinate dehydrogenase with 3-nitropropionic acid specifically prevented the myeloid fate of ApoE−/− HSPCs, but not their expansion (Figure 4B and 4C). We next assessed whether inhibition of the conversion of pyruvate for entry to the tricarboxylic acid cycle with a pyruvate dehydrogenase inhibitor (CPI-613) and a pyruvate carboxylase inhibitor (chlorothricin) could alter the expansion and myeloid fate of ApoE−/− HSPCs. Although ApoE−/− HSPC expansion required both inhibition of pyruvate dehydrogenase and pyruvate carboxylase (Figure 4B), their myeloid fate was actually suppressed by inhibiting either pyruvate dehydrogenase or pyruvate carboxylase (Figure 4C). This revealed that the conversion of both succinate and pyruvate into the tricarboxylic acid cycle are central metabolic checkpoints for ApoE−/− HSPC lineage specification and to some extent ApoE−/− HSPC expansion. Interestingly, the conversion of succinate to fumarate and pyruvate to oxaloacetate converge and 5D). As a consequence, Glut1 deficiency prevented not only the expansion of the number of cells per well (Online Figure I1D) but also the generation of CD11bGr-1+ myeloid cells both in response to IL-3 and GM-CSF or in ApoE−/− Lin− cultures (Figure 5E and 5F). This mirrored the reactive oxygen species production and MMP assessed by flow cytometry in HSPCs at the end of the culture period (Figure 5G and 5H). Mechanistically, we next tested whether Glut1 may mediate the effect of IL-3 on autophagy because autophagy has recently emerged to regulate HSPC maintenance and a bias toward myelopoiesis.54,45 Western blot analysis of microtubule-associated protein light chain 3-II protein levels, an hallmark of autophagy, revealed that Glut1 deficiency prevented the decrease of microtubule-associated protein light chain 3-II expression in WT and ApoE−/− BM cells under basal and IL-3-stimulated conditions (Online Figure IIIA). To analyze the autophagic flux of HSPCs, we next used the Cyto-ID probe allowing analysis by multicolor flow cytometry. Remarkably, Glut1 deficiency prevented the reduced Cyto-ID staining induced by IL-3 in HSPCs isolated from WT Lin− cultures and restored the autophagic flux of ApoE−/− HSPCs to the level of control cells (Online Figure I1IB and I1IC). These data identify that Glut1 is a key metabolic sensor mediating the growth-regulatory effects of IL-3 through autophagy-dependent modulation of HSPC expansion and myeloid commitment in vitro.

Reduced Glycolytic Activity in Mice With Hematopoietic Glut1 Deficiency Prevents ApoE−/− HSPC Expansion and Proliferation

To directly test the in vivo physiological relevance of Glut1 on ApoE−/− HSPCs, we transplanted the BM of single or combined knockout of ApoE (ApoE−/−) and Glut1 (Glut1−/−) mice. The increased oxygen consumption observed in ApoE−/− BM cells in response to IL-3 and GM-CSF stimulation was severely reduced by Glut1 deficiency (Figure 5A). Remarkably, Glut1 deficiency also prevented the enhanced mitochondrial respiration of ApoE−/− Lin− Sca1+ BM progenitors cultured for 2 hours after isolation (Figure 5B). Thus, we next isolated Lin− BM cells (containing predominantly HSPCs) that were placed in vitro in medium either alone or supplemented with IL-3 or GM-CSF. We found that Glut1 deficiency led to significantly decreased HSPC expansion either in WT Lin− cultures after IL-3 and GM-CSF stimulation or in ApoE−/− Lin− cultures under both unstimulated and stimulated conditions (Figure 5C and 5D). As a consequence, Glut1 deficiency prevented not only the expansion of the number of cells per well (Online Figure I1D) but also the generation of CD11bGr-1+ myeloid cells both in response to IL-3 and GM-CSF or in ApoE−/− Lin− cultures (Figure 5E and 5F). This mirrored the reactive oxygen species production and MMP assessed by flow cytometry in HSPCs at the end of the culture period (Figure 5G and 5H). Mechanistically, we next tested whether Glut1 may mediate the effect of IL-3 on autophagy because autophagy has recently emerged to regulate HSPC maintenance and a bias toward myelopoiesis.54,45 Western blot analysis of microtubule-associated protein light chain 3-II protein levels, an hallmark of autophagy, revealed that Glut1 deficiency prevented the decrease of microtubule-associated protein light chain 3-II expression in WT and ApoE−/− BM cells under basal and IL-3-stimulated conditions (Online Figure IIIA). To analyze the autophagic flux of HSPCs, we next used the Cyto-ID probe allowing analysis by multicolor flow cytometry. Remarkably, Glut1 deficiency prevented the reduced Cyto-ID staining induced by IL-3 in HSPCs isolated from WT Lin− cultures and restored the autophagic flux of ApoE−/− HSPCs to the level of control cells (Online Figure I1IB and I1IC). These data identify that Glut1 is a key metabolic sensor mediating the growth-regulatory effects of IL-3 through autophagy-dependent modulation of HSPC expansion and myeloid commitment in vitro.
capacity of the MMP2 and the Glut1-dependent glucose utilization. Although there was no significant decrease in the S/G2M fraction in the CD34+CD150−Flt3− MMP2 and other populations within the HSPCs of mice receiving Glut1+/− BM, a significant 1.3-fold decrease in the S/G2M fraction was observed in the CD34+CD150−Flt3− MMP2 and downstream CD34+CD150−Flt3− MMP3 of mice receiving ApoE−/−Glut1+/− BM compared with mice receiving ApoE−/− BM (Figure 6E; Online Figure IVF). Quantification of the BM HSPCs confirmed a ~1.4-fold decrease in the frequency and absolute number of the CD34+CD150−Flt3− MMP2 and downstream MMPs in mice receiving ApoE−/−Glut1+/− BM compared with the controls (Figure 6F and 6G). Similar findings were also observed in chow-fed ApoE−/−Glut1+/− BM-transplanted mice compared with the ApoE−/− BM-transplanted mice (data not shown). Together, these findings reveal that Glut1-dependent glucose utilization was required for ApoE−/− MMP2 proliferation and downstream MMP expansion.

Figure 5. Glut1 is required in vitro for the IL3Rβ-dependent ApoE−/− hematopoietic stem and multipotential progenitor cell (HSPC) expansion and myeloid lineage fate. A, Oxygen consumption of wild-type (WT), Glut1−/−, ApoE−/−, and ApoE−/−Glut1−/− bone marrow (BM) cells cultured for 48 hours in the presence or absence of 6 ng/mL IL-3 or 2 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) or (B) WT, Glut1−/−, ApoE−/−, and ApoE−/−Glut1−/− Lineage marker (Lin)− Sca1+ progenitors cultured for 2 hours after isolation. Bone marrow cells from WT, Glut1−/−, ApoE−/−, and ApoE−/−Glut1−/− mice were sorted for Lin− cells (i.e., enriched in HSPCs) and cultured for 72 hours in liquid culture in the presence or absence of 6 ng/mL IL-3 or 2 ng/mL GM-CSF. C, Representative dot plots and (D) quantification of HSPCs after in vitro culture. E, Representative dot plots and (F) quantification of CD11b+Gr-1+ myeloid cells after in vitro culture. G, Quantification of reactive oxygen species generation and (H) mitochondrial membrane potential by flow cytometry using fluorescent carboxy-H2DCFDA and tetramethylrhodamine ethyl ester dyes, respectively, in HSPCs after in vitro culture. The results are the mean±SEM of n=4 per group. *P<0.05, genotype effect; §P<0.05, Glut1-dependent effect; #P<0.05, growth hormone effect.
Reduced Myeloid Commitment in ApoE−/− BM With Glut1 Deficiency

While working on this article, Pietras et al. elegantly showed that the CD34+CD150−Flt3− MMP2 and downstream CD34+CD150+Flt3+ MMP3 exhibited a myeloid-biased multipotential progenitor phenotype. This prompted us to test whether the decreased MMP2 and MMP3 expansions observed in mice receiving ApoE−/−Glut1+/− BM could be associated with a defective myeloid fate specification. The CMP, GMP, and megakaryocyte–erythrocyte progenitor populations were analyzed by flow cytometry (Online Figure IVG) and were not significantly reduced in the mice receiving Glut1+/− BM, despite a trend toward CMPs (Figure 6H). Nevertheless, the CMP numbers were significantly decreased by >1.2-fold in mice receiving ApoE−/−Glut1+/− BM compared with controls receiving ApoE−/− BM (Figure 6H). We noticed that the splenomegaly in the mice receiving the ApoE−/− BM was rescued by Glut1 deficiency (Online Table I), and the spleen represents an important reservoir of myeloid cells through extramedullary hematopoiesis in ApoE−/− mice. Therefore, the hematopoietic progenitors were next quantified in this organ. Similar to the BM, we observed a 1.6-fold decrease in the frequency of splenic CMPs in mice receiving ApoE−/−Glut1+/− BM and, to some extent, a 1.3-fold decrease in the percentage of GMPs, but no changes in the megakaryocyte–erythrocyte progenitor population (Figure 6I). Consistent with

Figure 6. Glut1-dependence of ApoE−− hematopoietic stem and multipotential progenitor cell (HSPC) expansion and myelopoiesis in vivo. A, Experimental overview. Bone marrow (BM) from wild-type (WT), Glut1+/−, ApoE−−, and ApoE−−Glut1+/− mice were transplanted into ApoE−− recipient mice and, after a 5 week recovery period, the mice were fed a high-fat diet (HFD) for 12 weeks to induce the expansion of HSPCs. B, Glut1 cell surface expression was assessed by flow cytometry in the BM of these mice using Glut1 antibody and Glut1 receptor binding domain (RBD) ligand. Histograms show (C) the Glut1 cell surface expression and (D) NBD-glucose binding and uptake in HSPC subpopulations from the most quiescent (long-term hematopoietic stem cells [LT-HSCs]) to the most cycling (Continued)
these findings, the platelet and red blood cell counts, mean platelet volume, and hematocrit were unchanged in these mice (Online Table I). Peripheral T- and B-cell numbers were also not affected in these mice (Online Figure VA and VB).

In contrast, the blood counts indicated that the leukocytosis, monocytosis, neutrophilia, and eosinophilia of mice receiving ApoE−/− BM in response to feeding a high-fat diet were rescued by Glut1 deficiency (Figure 6J–6M). These data indicate that Glut1-dependent glucose utilization is required at the early stage of ApoE−/− HSPC commitment to the myeloid lineage.

Glut1 Acts in a Cell-Autonomous Fashion to Regulate ApoE−/− HSPC Proliferation and Myelopoiesis

To test whether this phenotype was caused by cell autonomous effects of Glut1 within the myeloid-biased HSPCs or involved in a cell extrinsic effect, we performed a competitive BM transplantation experiment with equally mixed BM cells from CD45.1 ApoE−/− mice and either CD45.2 ApoE−/− or CD45.2 ApoE−/− Glut1+/− BM into irradiated WT recipients. After BM reconstitution, we found that the frequency of CD45.1 ApoE−/− HSPCs, particularly the CD34+ HSPCs, were not affected by the presence of CD45.2 ApoE−/− Glut1+/− BM cells, despite the reduced frequency of the CD45.2 ApoE−/− Glut1+/− HSPCs (Figure 7A). These findings mirrored the reduced S/G2M fraction in the CD45.2 ApoE−/− Glut1+/− HSPCs without altering the S/G2M fraction in the mixed CD45.1 ApoE−/− Glut1+/− HSPCs (Figure 7B). Consistent with these findings on BM HSPCs, there was a preferential accumulation of CD45.1 ApoE−/− versus CD45.2 ApoE−/− Glut1+/− blood monocytes and neutrophils (Figure 7C and 7D), indicative of cell autonomous proliferative disadvantage of Glut1 deficiency.

Glut1 Deficiency Prevents the Progression of Atherosclerosis in ApoE−/− BM-Transplanted Mice

We next explored the in vivo relevance of reducing ApoE−/− HSPC proliferation and myelopoiesis through Glut1
deficiency on the development of atherosclerosis. This was tested in ApoE−/−-recipient mice that received ApoE−/− or ApoE−/− Glut1+/− BM fed with a high-fat diet for 12 weeks (Figure 6A). As shown in Online Table I, the body weight, plasma LDL and HDL cholesterol, or plasma glucose were not significantly different with regard to Glut1 deficiency. However, ApoE−/− mice receiving ApoE−/−Glut1+/− BM showed a ≈1.4-fold decrease in the development of atherosclerosis in their proximal aortas (Figure 8A). Immunohistochemical staining of the aortic root plaques revealed that this phenotype was associated with a massive decrease in the F4/80+ macrophages in the ApoE−/−Glut1+/− BM-transplanted mice (Figure 8B). We also examined the uptake of the radiolabeled D-glucose analog (2-[14C]-DG) after ex vivo incubation of the aortic arch and spleen from the ApoE−/− mice that received ApoE−/− or ApoE−/− Glut1+/− BM. ApoE−/−Glut1+/− BM-transplanted mice showed a significant 1.4-fold and 1.3-fold decrease in total uptake of 2-[14C]-DG in the aortic arch (Figure 8C) and the spleen (Figure 8D), respectively, compared with controls. We next analyzed Ly6C hi monocyte recruitment into atherosclerotic plaques using fluorescent-labeled latex beads, as previously described.10 Figure 8E reveals that 2 days after monocyte labeling, there was a ≈1.8-fold decrease in the number of latex+ monocytes in atherosclerotic lesions of ApoE−/− mice receiving ApoE−/−Glut1+/− BM compared with mice receiving ApoE−/− BM. This reduced recruitment was confirmed by analysis of latex+ monocytes in the aortic arch by flow cytometry (Figure 8F). This paralleled the reduced incorporation of the fluorescent beads in blood monocytes of ApoE−/− mice receiving ApoE−/−Glut1+/− BM compared with controls 2 days after labeling (data not shown) reflecting their reduced monocytopoiesis (Figure 6K). Thus, we showed that Glut1 connects the enhanced glucose uptake in atheromatous plaques of ApoE−/− mice27–30 with their myelopoiesis through regulation of HSPC maintenance and myelomonocytic fate.

**Discussion**

Previous studies have shown that inflamed atherosclerotic plaques can be visualized by noninvasive positron emission and computed tomographic imaging with 18F-fluorodeoxyglucose, a glucose analog, which correlates with macrophage accumulation and inflammation.27–30 However, a recent study has called
into question the relevance of these observations because macrophage-specific overexpression of Glut1 did not aggravate atherosclerosis in mice compared with Glut1 sufficient controls, reflecting the need for a better understanding of the underlying mechanisms. Our study provides direct evidence that Glut1 connects the enhanced glucose uptake in atheromatous plaques of ApoE<sup>−/−</sup> mice with their myelopoiesis through Glut1-dependent regulation of HSPC maintenance and myelomonocytic fate.
Recent studies have suggested that HSPC expansion and the associated myelopoiesis could underlie the hypercholesterolemia-induced atherosclerosis in mice. However, the rate of ATP generation required for cell proliferation and differentiation cannot be directly explained by cholesterol and requires alternative sources of energy. Our observations indicate that the leukocytes and HSPCs from hypercholesterolemic \textit{ApoE}−/− mice exhibited an increased Glut1-dependent glucose uptake that was associated with increased mitochondrial potential, suggesting that the influx of glycolytic metabolites in these cells fuel the mitochondria for oxidative phosphorylation and ATP generation. The low expression of Glut1 in CD34− LT-HSCs compared with other CD34+ HSPCs was first counterintuitive because we initially speculated that the presence of the LT-HSCs in the hypoxic BM niche could favor the expression of Glut1 by HIF-1α. However, increasing glucose metabolism through translocation of Glut1 to the cell surface is thought to be crucial for the active cells and cell cycle entry rather than quiescence. During aerobic respiration, the ATP yield is linked to NAD+-dependent oxidative steps, including oxidative decarboxylation of pyruvate, that requires metabolic shuttle systems to convey reducing equivalents from cytosol to mitochondria. Our findings indicate that both the oxidative decarboxylation of pyruvate and the transamination reactions of the malate–aspartate shuttle were essential for HSPC expansion and commitment to the myeloid lineage. Thus, it is probably not surprising that we did not observe an accumulation of succinate in \textit{ApoE}−/− BM cultures because of higher succinate dehydrogenase activity favoring fumarate and malate production. The absence of succinate accumulation in \textit{ApoE}−/− BM cells could also contribute to the lack of HIF-1α activation in these cells. Consistent with these observations, we did not observe modulation of the oxygenation status of \textit{ApoE}−/− LT-HSCs in chronic hypercholesterolemia, and HIF-1α deficiency in WT or \textit{ApoE}−/− hematopoietic cells did not alter Glut1 expression or the HSPC frequency. In fact, HIF-1α deficiency in hematopoietic cells rather led to increased myeloid expansion, which could contribute to the role of hypoxia in the development of atherosclerosis. In contrast and consistent with the alternative regulation of Glut1 by growth hormone-dependent activation of Ras or Srt3, the enhanced Glut1 expression in proliferating \textit{ApoE}−/− HSPCs was prevented by IL-3Rβ blockade. This metabolic critical regulation was for the expansion of \textit{ApoE}−/− HSPCs and the associated IL-3-dependent down-regulation of autophagy, which is most likely required to limit intracellular lysosomal degradation and fulfill the high-energy demand of these cells for proliferation.

Recently, increased splenic activity in patients with cardiovascular diseases has been demonstrated by noninvasive positron emission and computed tomographic imaging with \textsuperscript{18}F-fluorodeoxyglucose, which could reflect the metabolic activity of extramedullary hematopoiesis required to generate monocytes that infiltrate atherosclerotic plaques. However, these observations do not prove causality. Intriguingly, inhibition of glucose uptake with a 2-deoxyglucose (2-DG) analog has recently been shown to inhibit myelopoiesis in human HSCs, but the relevance to atherosclerosis has not been tested. The present study clearly establishes that the increased Glut1-dependent glucose utilization in the \textit{ApoE}−/− HSPCs could divert these cells to a myelomonocytic fate leading to extramedullary myelopoiesis and subsequent macrophage deposition–dependent atherosclerotic plaque formation. Indeed, we now provide direct in vivo evidence that Glut1 deficiency can significantly reduce the number of CMPs in the \textit{ApoE}−/− BM as well as the number of CMPs and GMPS in the \textit{ApoE}−/− spleen, and this was associated with reduced splenic glucose uptake. This led to inhibition of the monocytosis, neutrophilia, and eosinophilia in the \textit{ApoE}−/− mice transplanted with the \textit{ApoE}−/− BM. Consistent with the lack of effect of Glut1 deficiency on resting T-cells, we also did not observe variations in the number of lymphocytes in our models. While working on this article, Pietras et al elegantly showed that the CD34+CD150+Flt3−MMP2, which is now showed to express the most Glut1, and downstream CD34+CD150+Flt3−MMP3 exhibited a myeloid-biased multipotential progenitor phenotype, offering an alternative explanation to the role of Glut1 in favoring myelopoiesis that is independent of a change in other lineage commitments. Together, these findings reveal that the mechanism by which defective \textit{ApoE}-dependent cholesterol efflux pathways skew HSCs toward myelopoiesis relies on the regulation of Glut1-dependent glucose uptake by the IL-3Rβ signaling pathway.

The metabolic phenotype of \textit{ApoE}−/− HSPCs outlined here could be relevant to the adaptability of HSPCs to cholesterol overload and may indicate that the glycolytic phenotype of HSPCs is not merely a product of their hypoxic environment. Thus, the existence of different molecular mechanisms underlying the different glycolytic phenotypes in HSPCs may suggest strategies for specifically modulating the pool of HSPCs that are committed to the myeloid lineage under stressed conditions, such as in myeloproliferative disorders, sepsis, myocardium infarction, or chronic atherosclerosis, as shown in the present study. Inhibition of glucose uptake by a Glut1 inhibitor that does not cross the blood–brain barrier could ultimately provide a novel therapeutic approach to prevent myelopoiesis-driven diseases, such as atherosclerosis.

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Disclosures
None.

References


These findings suggest the presence of proatherogenic cross-talk between nutritional and growth factor signaling pathways in hematopoietic stem and progenitor cells.
Disruption of Glut1 in Hematopoietic Stem Cells Prevents Myelopoiesis and Enhanced Glucose Flux in Atheromatous Plaques of ApoE−/− Mice

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Supplemental Material

Disruption of Glut1 in hematopoietic stem cells prevents myelopoiesis and enhanced glucose flux in atheromatous plaques of ApoE−/− mice.


Supplemental Experimental procedures

Competitive BM transplantation
Competitive BM transplantation was performed as previously described. In brief, CD45.1+ ApoE−/− BM isolated from congenic CD45.1+ ApoE−/− mice on the C57/BL6 background was mixed with either CD45.2+ ApoE−/− or CD45.2+ ApoE−/−Glut1+/− BM (ratio 1:1) before transplantation into lethally irradiated C57/BL6 WT recipient mice.

White blood cell counts
Leukocytes, differential blood counts, platelets, mean platelet volume (MPV), erythrocytes and hematocrit were quantified from whole blood using a hematology cell counter (Hemavet, Beckman Coulter).

Blood parameters
Plasma LDL-C, HDL-C and glucose levels were determined using commercial kits (all from Wako Diagnostics).

Histological analysis of proximal aortas
Mice were sacrificed and heart was harvested. Heart was washed with PBS then fixed with 4% paraformaldehyde. Heart was embedded in paraffin and 5µm sections of proximal aortas were performed using a Microm HM340E microtome (Microm Microtech, Francheville France) and stained with H&E as previously described. Aortic lesion size of each animal was calculated as the mean of lesion areas in 6 sections from the same mouse using ImageJ software calibrated with parameters of the Leica DM5500 B (Leica Microsystems SAS, Nanterre, France) microscope. Atherosclerotic lesions were expressed in µm2 per section.

Immunohistochemistry
Cells fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X100 (Sigma). Unspecific staining was avoided with a blocking step in PBS 0.1% Triton X100 3% FBS. Antibodies anti F4/80, was from Cell Signaling and used as recommended by the manufacturer. Dilution for antibodies was 1/100. Slides were mounted in fluorescence mounting medium Vectamount (Vector laboratories, Inc, Burlingame, CA, USA). Pictures were taken as previously described, enhanced for publication purposes and the number of positive cells were counted using ImageJ software. Count was performed 3 times per slice of tissue and the average reported.

Monocyte labeling and tracking in vivo. Classical Ly6C− monocytes were labeled as previously described. Briefly, mononuclear cells were first transiently depleted by i.v. injection of 250µl clodronate-loaded liposomes (FormuMax, Sunnyvale, CA, USA) and 2 days later, Ly6C− monocytes were labeled in vivo by retro-orbital i.v. injection of 1µm Fluoresbrite green fluorescent (YG) plain microspheres (Polysciences Inc.), diluted 1:4 in
sterile PBS. Labeling efficiency (i.e., engulfment of latex beads) was verified in peripheral monocytes by flow cytometry 1 day after labeling and 2 days later, mice were sacrificed and the heart and aorta were perfused and isolated. Histological analysis of proximal aortas was performed as described above in a blinded manner and the mean number of latex+ cells per section was determined. Nuclei were revealed with DAPI staining. Aortic arches were subjected to enzymatic digestion with a mixture of collagenases I and XI (450 and 125 U/mL, respectively) and prepared for flow cytometry analysis of latex+ cells per aortic arch.

**Ex vivo 2-[14C]-DG uptake**

In some experiments, mice were sacrificed and aortic arch and spleen were harvested and weighed. Tissues were perfused with PBS. BM cells were collected from leg bones. Splenocytes were isolated by manual disruption of the spleen through a 40-µm cell strainer with PBS. Whole aortic arch (normalized to similar tissue weight) and isolated BM cells and splenocytes were incubated with 0.1µCi 2-[14C]-deoxyglucose (2-[14C]-DG) in 2% BSA Krebs-Ringer bicarbonate buffer, pH 7.4 for 10min at 37°C, washed 3times with PBS and homogenized with 5% HClO4 solution. The radioactivity incorporated was measured and expressed as total radioactivity per tissue weight.

**Splenic HSPCs and hematopoietic progenitors**

Isolation of splenocytes for flow cytometry analysis was achieved as follows. Spleens were dissected from mice, perfused with PBS, and a cell suspension was obtained by manual disruption through a 40-µm cell strainer with PBS. The cellular mix was then centrifuged and subjected to a brief RBC lysis. Specific HSPC and hematopoietic progenitor subsets were identified by flow cytometry as described for BM cells.

**Flow cytometry analysis of intracellular pimonidazole (Pimo)**

12-week old WT and Apoe−/− mice were i.v injected with 60mg/kg Pimonidazole (Pimo; Hydpoxyprobe) 90 min before sacrifice according to the manufacturer’s instructions. BM cells were stained as decribed in the ‘flow cytometry analysis’ section. Cells were then fixed and permeabilized with BD Perm Wash kit (BD Biosciences). Intracellular Pimo adducts were detected using an anti-Pimo FITC antibody provided in the Hydpoxyprobe Kit.

**Bone marrow harvest and treatment.** Primary BM cells were resuspended in IMDM (Gibco) containing 10% FCS (Stemcell) and cultured for 1h in tissue culture flasks to remove adherent cells, including macrophages. Succinate dehydrogenase (SDH) activity was determined by an ELISA kit according to the manufacturer’s instructions (MitoSciences). In some experiments, mice were i.p injected with 150mg/kg 5-fluorouracil for 3 days to enrich hematopoietic stem cells in BM cultures as indicated in the figure legend. Suspended cells were then cultured for 72h in the presence of 6ng/mL IL-3 or 2ng/mL GM-CSF, Flt3L or TPO (all from R&D Systems). In some experiments, the farnesyl transferase inhibitor (FTI, Calbiochem) was used at the final concentration of 1μM, the lactate dehydrogenase inhibitor (oxamate, Sigma-Aldrich) at 50mM, the pyruvate dehydrogenase inhibitor (CPI-613, Tocris Bioscience) at 200µM, the pyruvate carboxylase inhibitor (chlorothricin, Cayman Chemical) at 100µM, the glutamate oxaloacetate transaminase inhibitor (AOA, Sigma-Aldrich) at 2mM, the mitochondrial complex I inhibitor (rotenone, Cayman Chemical) at 1μM, the succinate dehydrogenase inhibitor (3-NPA, Sigma-Aldrich) at 100μM, the acetyl-coA carboxylase inhibitor (Tofa, Sigma-Aldrich) at 10μM, the AMPK activator (Metformin, Sigma-Aldrich) at 5mM, the IL3Rβ blocking antibody (R&D Systems) at 10μg/mL and Cyclodextrin (CD, Sigma-Aldrich) at 2.5mM. BM cells were also cultured in an hypoxic chamber (1% oxygen) for 6hours as indicated in the figure legend.

**Lin’ bone marrow cell cultures.** Hematopoietic stem and progenitor cells were isolated by FACS sorting of lineage depleted (Lin’) bone marrow from ApoE−/− recipient mice transplanted with WT, Glut1−/−, ApoE−/− and ApoE−/−Glut1−/− BM. Lin’ BM cells (0.5x10⁵ cells) were
resuspended in IMDM (Gibco) containing 10% FCS (Stemcell) and cultured for 72h in the presence or absence of 6ng/mL IL-3 before analysis by flow cytometry.

**Colony-Forming Assay and in vitro 2-[14C]-DG uptake**
Primary bone cells (4x10^5) were plated in methylcellulose-based media containing a cocktail of recombinant cytokines including SCF, IL-3 and IL-6 (Methocult, Stemcell) supplemented with 2% FCS to generate multipotential progenitor cells (CFU-GEMM) and in presence of additional GM-CSF (2ng/mL, R&Dsystems) to generate granulocyte-macrophage progenitors (CFU-GM) as previously described. In some conditions, cultures were performed in 5.6mM glucose (DMEM low glucose, Gibco) rather than 25mM glucose (standard DMEM, Gibco) as described in the figure legends. After 10 days of differentiation, the number of CFUs per dish was scored or colonies were extensively washed with PBS to dissociate them from the methylcellulose-based media and were incubated with 0.1μCi 2-deoxy-[14C]-glucose (2-[14C]-DG) in 2% BSA Krebs-Ringer bicarbonate buffer, pH 7.4 for 10min at 37°C. After 3 washes with PBS, colonies were homogenized with 5% HClO4 solution and the radioactivity incorporated into CFUs was determined by standard procedures using liquid scintillation counter.

**Directed Metabolomic assays**
Metabolomics analyses were performed at the Michigan core facility of the University of Michigan as previously described. Briefly, Metabolites from Krebs cycle (Citrate, Succinate, Fumarate and Malate) were analyzed in WT and Apoe−/− deficient BM cells. Metabolites were extracted by exposing cells to a chilled mixture of 80% methanol, 10% chloroform, and 10% water and analyzed by LC-MS.

**Respiration assays**
Oxygen consumption was measured with the Oxygen Biosensor System in accordance with the manufacturer’s recommendations (PreSens GmbH) and as previously described. A total of 0.5 to 3x10⁵ cells/well were used. In studies where Lineage marker-positive and negative (Lin+ and Lin−) BM cells and Lin−Sca1+ progenitor cells were isolated for respiration analysis, magnetic lineage depletion and Sca1 enrichment (autoMACS separator, Miltenyibiotec) were used. Adequate lineage depletion was confirmed by flow cytometry as previously described. Oxygen consumption was determined after 2h of culture in the biosensor in presence or absence of 2μM oligomycin to inhibit mitochondrial ATP synthase.

**Western blot analysis**
The expression of HIF1α and LC3-II were measured in BM cell extracts by Western blot analysis as previously described. Primary antibodies for HIF1α and LC3-II were purchased from Novus Biologicals and Cell Signaling Technology, respectively

**qPCRs**
qPCRs were performed on StepOne device from Applied Biosystem (France) using sybergreen reaction system. All the results were normalized on m36B4 gene expression. All conditions were performed in at least one experiment performed in triplicates.

**Antibodies.** CD45.1 (clone A20)-PE and CD45.2 (clone 104)-PerCPCy5.5 were purchased from eBioscience. CD2 (RM2-5), CD3e (145-2C11), CD4 (GK1.5), CD8b (53-6.7), CD19 (eBio1D3), CD45R (B220, RA3-6B2), Gr-1 (Ly6G, RB6-8C5), Cd11b (Mac1, M1/70), Ter119 (Ly76) and NK1.1 (Ly53, PK136)-FITC were all from eBioscience and used for lineage determination. c-Kit (CD117, ACK2)-APCeFluor780 from eBioscience, Sca-1-Pacific blue from Biolegend, FcgRlll/III-PE (CD16/32, 2.4G2), CD34 (RAM34)-AlexaFluor 647, CD135 (Flt3, A2F10)-PE, CD150 (Slamf1, TC15-12F12.2)-PECy7 were from Biolegend and used to quantify HSPCs and progenitor subsets. Peripheral leukocytes were stained with CD115 (AFS98)-APC, B220 or CD45R (RA3-6B2)-FITC, CD45 (30-F11)-APCCy7, Ly6C/G or Gr-1
(RB6-8C5)-PercPCy5.5, TCRβ (H57-597)-Pacific Blue, CD3 (17A2)-PE and CD8a (53-6.7)-PECy7 from eBioscience and BD Biosciences, respectively.

**Statistical analysis**

Data are shown as mean ± SEM. Statistical significance was performed using Prism t-test or ANOVA were performed according to the dataset. Results were considered as statistically significant when P<0.05.

**References**


Supplemental Table I: Hematologic and metabolic parameters of Apoe\(^{-/-}\) mice transplanted with WT, Glut1\(^{+/+}\), Apoe\(^{-/-}\), Apoe\(^{-/-}\)Glut1\(^{+/+}\) BM after 12 weeks of high-fat diet. Values are mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>WT BM (\rightarrow) Apoe(^{+/+}) (n=6)</th>
<th>Glut1(^{+/+}) BM (\rightarrow) Apoe(^{+/+}) (n=6)</th>
<th>Apoe(^{-/-}) BM (\rightarrow) Apoe(^{-/-}) (n=10)</th>
<th>Apoe(^{-/-})Glut1(^{+/+}) BM (\rightarrow) Apoe(^{-/-}) (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematologic parameters</strong></td>
<td></td>
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</tr>
<tr>
<td>Spleen (mg)</td>
<td>90±14</td>
<td>64±8</td>
<td>124±13*</td>
<td>85±9§</td>
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<tr>
<td>Platelets (\times 10^3/\mu L)</td>
<td>665±17</td>
<td>646±15</td>
<td>728±59</td>
<td>671±67</td>
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<tr>
<td>MPV (\times 10^3/\mu L)</td>
<td>5.3±0.1</td>
<td>5.4±0.1</td>
<td>5.1±0.1</td>
<td>5.3±0.1</td>
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<tr>
<td>Erythrocytes (\times 10^6/\mu L)</td>
<td>8.2±0.7</td>
<td>8.3±0.6</td>
<td>8.9±0.2</td>
<td>8.4±0.3</td>
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<tr>
<td>Hematocrit (%)</td>
<td>37.1±3.1</td>
<td>36.0±2.2</td>
<td>43.7±0.9</td>
<td>37.9±1.3</td>
</tr>
<tr>
<td><strong>Metabolic parameters</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Body weight (g)</td>
<td>21.7±0.5</td>
<td>22.5±0.6</td>
<td>21.9±0.6</td>
<td>21.2±0.7</td>
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<tr>
<td>Plasma LDL-C (mg/dL)</td>
<td>146±36</td>
<td>126±43</td>
<td>1055±124</td>
<td>1002±102</td>
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<tr>
<td>Plasma HDL-C (mg/dL)</td>
<td>68±22</td>
<td>94±12</td>
<td>164±23</td>
<td>142±27</td>
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<tr>
<td>Plasma glucose (g/L)</td>
<td>1.8±0.2</td>
<td>1.8±0.3</td>
<td>3.1±0.2</td>
<td>3.0±0.2</td>
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</table>
Supplemental Figure I. HIF1α-independent regulation of Glut1 in ApoE⁻/⁻ BM cells. (A) Oxygenation status measured by flow cytometry using a fluorescent pimonidazole (Pimo) dye in Lineage marker (Lin), Lin⁻ and CD34⁻ or CD34⁺ HSPCs isolated from the BM of chow fed ApoE⁻/⁻ recipients transplanted with WT or ApoE⁻/⁻ BM, 12weeks after the transplantation procedure. (B) Western blot analysis of HIF1α and (C) mRNA expression of HIF1α and HIF1α target genes (Ldha and Glut1) in BM cells exposed to normoxia (21% O₂) or hypoxia (1% O₂) for 6hours.
Supplemental Figure II. IL3Rβ-dependent regulation of Glut1 and Glut1 dependency on cell expansion in ApoE⁻/⁻ BM cells. (A) mRNA expression of HIF1α and HIF1α target genes Ldha and Glut1 in BM cells treated for 72 hours with the indicated growth hormones and in the presence or absence of 1µM farnesyl transferase inhibitor (FTI). (B) Glut1 cell surface expression (expressed as the mean fluorescence intensity (MFI) in HSPCs after BM cells were cultured for 72 hours with the indicated treatments (10µg/mL IL3Rβ blocking antibody; 1µM farnesyl transferase inhibitor (FTI); 5mM AMPK activator (Metformin); 2.5mM Cyclodextrin (CD)). (C) mRNA expression of HIF1α and HIF1α target genes Ldha and Glut1 in BM cells freshly isolated from 20 weeks old WT and ApoE⁻/⁻ mice that were injected with IgG control or 100µg IL-3Rβ blocking antibody for 24h. Values were normalized to ribosomal m36B4. (D) WT, Glut1⁺, ApoE⁻, and ApoE⁻ Glut1⁺ Lin cells (i.e, enriched in HSPCs) were cultured for 72h in liquid culture in presence or absence of 6ng/mL IL-3 or 2ng/mL GM-CSF and the number of cells per well was quantified. Results are means ± SEM of experiments performed with 6-10 animals per group or cultures performed in triplicates. *P<0.05 vs. WT, §P<0.05 vs. ApoE⁻ and #P<0.05 vs. untreated conditions.
Supplemental Figure III. IL3Rβ-dependent regulation of autophagy in ApoE⁻/⁻ BM cells. (A) Western blot analysis of LC3 in BM cells cultured with or without 6ng/mL IL-3 for 24 hours. Bars represent LC3-II/β-actin relative expression. (B) Autophagic flux was quantified using the Cyto-ID probe by flow cytometry and expressed as the mean fluorescence intensity (MFI) in HSPCs after in vitro culture with or without 6ng/mL IL-3 for 72 hours. Results are means ± SEM of experiments performed with 6-10 animals per group or cultures performed in triplicates. *P<0.05 vs. WT, †P<0.05 vs. ApoE⁻/⁻ and §P<0.05 vs. untreated conditions.
Supplemental Figure IV. Multiparameter flow cytometric analysis. Representative dot plots and gating strategies used to characterize hematopoietic stem and progenitor cells. (A) Single live BM cells were selected by exclusion of low forward and side scatter populations (debris) and doublets. (B) Cells were then gated for Lin− and Sca−c−kit+ (HSPCs) or Sca−c−kit+ populations. (C) HSPC gates were further subdivided into four HSPC populations based on differential expression of CD34, CD150 and Flt3 (CD135): CD34+CD150−Flt3− long-term LT-HSCs and CD34+CD150+Flt3−, CD34+CD150−Flt3+ and CD34+CD150+Flt3+ multipotential progenitors (MPP2, MPP3 and MPP4, respectively). Representative flow cytometry analysis of (D) Glut1, (E) NBD-glucose and (F) DAPI stainings in LT- and MMP2 from the BM of high-fat diet fed ApoE−/− mice transplanted with WT, Glut1+/−, ApoE−/−, ApoE−/− Glut1+/− BM. (G) Lin− Sca−c−kit+ gates were further divided into granulocyte-macrophage progenitor (GMP), common myeloid progenitor (CMP) and megakaryocyte/erythrocyte compartments.
Supplemental Figure V. Glut1 deficiency does not alter lymphocyte homeostasis in ApoE\textsuperscript{−/−} BM transplanted mice. Analysis of peripheral blood lymphocyte subsets by flow cytometry in ApoE\textsuperscript{−/−} recipient mice transplanted with WT, Glut1\textsuperscript{+/−}, ApoE\textsuperscript{−/−}, ApoE\textsuperscript{−/−}Glut1\textsuperscript{+/−} BM and fed a high-fat diet for 12 weeks. (A) Representative dot plots and (B) histograms showing the quantification of B220\textsuperscript{+} B-lymphocytes, CD4\textsuperscript{+} T-lymphocytes, CD8\textsuperscript{+} T-lymphocytes and CD8\textsuperscript{+}Gr1\textsuperscript{+} T-lymphocytes. Results are means ± SEM and are representative of an experiment performed with n=6 (WT and Glut1\textsuperscript{+/−} BM transplanted into ApoE\textsuperscript{−/−} recipients) or n=10-12 (ApoE\textsuperscript{−/−} and ApoE\textsuperscript{−/−}Glut1\textsuperscript{+/−} BM transplanted into ApoE\textsuperscript{−/−} recipients) animals per groups.