Expanded Granulocyte/Monocyte Compartment in Myeloid-Specific Triple FoxO Knockout Increases Oxidative Stress and Accelerates Atherosclerosis in Mice

Kyoichiro Tsuchiya, Marit Westerterp, Andrew J. Murphy, Vidya Subramanian, Anthony W. Ferrante Jr, Alan R. Tall, Domenico Accili

Rationale: Increased neutrophil and monocyte counts are often associated with an increased risk of atherosclerosis, but their relationship to insulin sensitivity is unknown.

Objective: To investigate the contribution of forkhead transcription factors (FoxO) in myeloid cells to neutrophil and monocyte counts, atherosclerosis, and systemic insulin sensitivity.

Methods and Results: Genetic ablation of the 3 genes encoding FoxO isoforms 1, 3a, and 4, in myeloid cells resulted in an expansion of the granulocyte/monocyte progenitor compartment and was associated with increased atherosclerotic lesion formation in low-density lipoprotein receptor knockout mice. In vivo and ex vivo studies indicate that FoxO ablation in myeloid cells increased generation of reactive oxygen species. Accordingly, treatment with the antioxidant N-acetyl-l-cysteine reversed the phenotype, normalizing atherosclerosis.

Conclusions: Our data indicate that myeloid cell proliferation and oxidative stress can be modulated via the FoxO branch of insulin receptor signaling, highlighting a heretofore-unknown link between insulin sensitivity and leukocytosis that can affect the predisposition to atherosclerosis. (Circ Res. 2013;112:992-1003.)

Key Words: atherosclerosis ■ insulin resistance ■ macrophages ■ neutrophils ■ oxidative stress ■ stem cell

Atherosclerotic cardiovascular (CV) disease is the leading cause of death of type 2 diabetic patients, possibly owing to its refractoriness to glucose control. Insulin resistance can also account for the increased vulnerability of diabetic patients to atherosclerosis, but its pathogenetic mechanism is not completely understood, and is likely to involve multiple target organs of insulin action.

In the liver, for example, alterations of insulin receptor (InsR) signaling result in changes of hepatocellular triglyceride content and assembly into or export as apolipoprotein B (ApoB)-containing, very low-density lipoproteins that are typically elevated in the plasma of type 2 diabetic patients. In addition, hepatic InsR signaling also regulates low-density lipoproteins receptor turnover, possibly contributing to the lower than expected low-density lipoproteins-cholesterol levels in these patients.

In the arterial wall, the role of insulin resistance in different cell types and at different stages of disease progression is controversial. In endothelial cells, a burgeoning consensus supports the conclusion that augmenting insulin signaling through Ins2/Akt/FoxO prevents atherosclerosis by pleiotropic mechanisms. In macrophages, another insulin-sensitive cell type with critical functions in disease progression, the data are mixed. At the cellular level, InsR signaling in macrophages modulates inflammation in a context-specific fashion, as well as apoptosis and ER stress. These signals seem to be largely mediated through FoxO. It is unclear how antiatherogenic InsR signals are mediated.

To study the role of the FoxO branch of InsR signaling in macrophages on atherosclerosis, we generated mice lacking the 3 FoxO isoforms (1, 3a, and 4) in this cell type. Our data provide evidence for a dual role of FoxO-dependent signaling in monocyte/macrophages and their progenitors in the pathogenesis of atherosclerosis. First, we show that FoxO ablation increases proliferation of granulocyte-monocyte progenitors, resulting in neutrophilia with monocytosis, a predisposing factor in both human and murine atherosclerosis. Second, myeloid FoxO ablation also increases inducible nitric oxide synthase (iNOS) expression and oxidative stress in...
Macrophages, possibly contributing to endothelial dysfunction. As a result, mice lacking the 3 FoxO proteins in myeloid cells develop larger atherosclerotic lesions than wild-type (WT) controls, with an increased number of intraluminal macrophages, but a decreased percentage of apoptotic macrophages.

Methods
We generated myeloid-specific FoxO knockout mice (MYFKO) by mating FoxO1lox/lox3alox/lox4lox/lox mice with Lysozyme M-Cre mice (Jackson Laboratories). We then crossed MYFKO mice and Ldlr−/− mice to generate Ldlr−/−:MYFKO mice. We fed animals Western diet (WTD, 0.2% cholesterol, 42% from fat-adjusted calorie diet, TD 8137, Harlan Tekland) for the indicated times. We conducted experiments in male Cre(+) and littermate Cre(−) (control) mice. Macrophage isolation and manipulations have been described.20 Blood and bone marrow (BM) analyses using flow cytometry were performed as previously described21,22 (Online Figure IA–IB). The Columbia University Animal Care and Utilization Committee approved all procedures. An expanded Methods section is available in the Online Data Supplement.

Results
MYFKO Mice Display Neutrophilia and Monocytosis
Thioglycollate-elicited peritoneal or BM-derived macrophages from myeloid-specific triple FoxO-knockout mice showed successful ablation of FoxO1, 3a, and 4 mRNA and protein (Online Figure IIA–IID). We determined the cellular composition of peripheral blood and BM in MYFKO mice. Peripheral blood counts showed increased number of white cells in Ldlr−/−:MYFKO mice, with normal numbers of erythrocytes and platelets (Figure 1A). Differential white cell counts showed an increased percentage of neutrophils and monocytes, and decreased lymphocytes (Figure 1B), accompanied by increased total numbers of neutrophils, monocytes, and lymphocytes (Figure 1C). These findings were corroborated by flow cytometry analysis of peripheral blood that showed increased relative and absolute numbers of neutrophils (defined as CD45+ CD115+ Ly6C/G− cells) and monocytes (CD45+ CD115+; Figure 1D through 1F). Ldlr−/−:MYFKO mice showed splenomegaly, secondary to red pulp hypertrophy (Figure 1G–1I), as well as increased Ly6C/G− monocytes, a key contributing population to murine atherosclerosis23 (Figure 1J–1L). Neutrophilia and monocytosis are associated with increased CV disease risk in humans24 and in animal models of atherosclerosis.25

These abnormalities of blood cell composition are likely determined by the combination of triple FoxO knockout and Ldlr nullizygosity, as they were not observed in single LysM-Cre:FoxO1−/− mice (Online Figure IIIA–IIIC), and were considerably less marked in Ldlr-competent MYFKO mice (Online Figure IID–IIIF).

Altered Turnover of FoxO-deficient Granulocyte-Macrophage Progenitors and Macrophages
Granulocytes and monocytes develop from a common myeloid granulocyte-macrophage progenitor (GMP) cell. BM analysis revealed that the abundance of hematopoietic stem and progenitor cells (HSPC), common myeloid progenitor (CMP), and GMP cells were unchanged in Ldlr−/−:MYFKO mice (Figure 2A). However, cell-cycle analysis of BM-derived cells showed an increased proportion of GMP cells from Ldlr−/−:MYFKO mice in G1 phase, and a decreased proportion in G2/M phase (Figure 2B and 2C). In contrast, proportion of cells in G1, S, and G2/M were comparable in HSPC and CMP (Online Figure IVA–IVB). Gene expression analysis revealed the expected reduction of FoxO3a in CMP, and modest reduction of FoxO1, FoxO3a, FoxO4, Cdkn1b, and P53, as reported in another triple FoxO knockout26 (Figure 2D). Consistently, cultured peritoneal macrophages from MYFKO mice showed increased proliferation (Figure 2E through 2G) and bromodeoxyuridine incorporation impairs cell-cycle arrest, resulting in increased proliferation.

We have shown that FoxO ablation protects macrophages from free-cholesterol–induced apoptosis.15 Consistently, peritoneal macrophages from MYFKO mice were refractory to free-cholesterol–induced and 7-ketocholesterol–induced apoptosis compared with WT (Figure 2J and 2K). These data suggest that neutrophilia and monocytosis in Ldlr−/−:MYFKO mice result from increased proliferation and decreased apoptosis in FoxO-deficient GMP cells.

Increased Atherosclerosis and Macrophage Accumulation in Ldlr−/−:MYFKO Mice
We analyzed atherosclerotic lesions in Ldlr−/−:MYFKO mice after 14 weeks of WTD. Analyses of en face aorta preparations revealed a 68% increase of lesion area in Ldlr−/−:MYFKO mice compared with Ldlr−/− mice (Figure 3A and 3B). RNA extracted from whole aortae showed increased expression of macrophage marker Emr1, Il1b, and NADPH oxidase components p47phox and p60phox in Ldlr−/−:MYFKO mice (Online Figure VA). Histological analysis of aortic roots also showed increased lesion area, as well as Mac-3 and α-smooth muscle actin immunoreactivities in Ldlr−/−:MYFKO mice (Figure 3C and 3D). Collagen content and necrotic lesion area were similar (data not shown). In contrast, we observed decreased active caspase-3 immunoreactivity in aortic root plaques of Ldlr−/−:MYFKO mice (Online Figure VB–VC). Peritoneal macrophages from MYFKO mice showed increased expression of chemokine receptors Ccr5 and Cx3cr1, and adhesion molecule Cd44, as reported in regulatory T cells lacking FoxO1 and 3a (Figure 3E), as well as integrins Itgal, Itgb1 and 2, and Psgl1. In contrast, Pecam1, which suppresses atherosclerosis in BM,26 was decreased in macrophages from MYFKO mice. Consistently, adhesion assays using peritoneal macrophages and lipopolysaccharide (LPS)-stimulated MS-1 endothelial cells showed increased adhesion of MYFKO macrophages to endothelial cells (Figure 3F and 3G). Expression of markers of classic (M1) and alternative (M2) peritoneal macrophages was unaltered (Online Figure VD). LPS-induced p105

Nonstandard Abbreviations and Acronyms

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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
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<td>CV</td>
<td>cardiovascular</td>
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<td>GMP</td>
<td>granulocyte-macrophage progenitor</td>
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<td>InsR</td>
<td>insulin receptor</td>
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degradation and p65 phosphorylation were comparable between WT and MYFKO macrophages (Online Figure VE).

These data indicate that ablation of the 3 FoxO in myeloid cells exacerbates WTD-induced atherosclerosis in Ldlr−/− mice. This seems to be due to macrophage accumulation owing to decreased macrophage apoptosis and proliferation.21,27

FoxO Inactivation in Macrophages Reduces Akt Signaling

Next, we examined the consequence of FoxO ablation on macrophage insulin signaling. We and others have described an auto-regulatory FoxO/Akt loop, whereby increased FoxO activity begets a compensatory increase in Akt phosphorylation,28 and vice versa.12 Similar to prior observations in other conditional knockouts,12 we detected decreased InsR and Irs1 levels, as well as decreased phospho-Akt (S473 and T308) generation in response to insulin in FoxO-deficient macrophages (Online Figure VIA). These data phenocopy the decrease in insulin signaling observed after chronic exposure of primary peritoneal macrophages to pharmacological concentrations of insulin, with impaired insulin-stimulated Akt phosphorylation (S473 and T308) associated with marked reduction in InsR and Irs1 levels (Online Figure VIB). It should be pointed out that, despite impaired Akt activation, basal phosphorylation of FoxO1 and 3a was increased in this model, resulting in decreased FoxO activity. These data
Figure 2. Proliferation and apoptosis of bone marrow (BM) and macrophages. A. Percentage of hematopoietic stem and progenitor cells (HSPC), common myeloid progenitors (CMP), and granulocyte-myeloid progenitors (GMP) in BM (n=5). B. Representative scatter plots and (C) quantification of cell-cycle progression in HSPC, CMP, and GMP using 4',6-diamidino-2-phenylindole (DAPI) staining (n=5). D. Expression of apoptosis- and cell-cycle–related genes in cultured peritoneal macrophages (n=5–6). E. Representative pictures and quantification of cell densities assessed by (F) counting and (G) (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay of peritoneal macrophages after 32 h of culture (n=4). H. Representative pictures of bromodeoxyuridine (BrdU) and propidium iodide (PI) staining and (I) percentage of BrdU-positive cells of peritoneal macrophages after 32 h of culture (n=4). J. Representative pictures and (K) quantification of Annexin V (green) and PI staining (red) in macrophages loaded with free-cholesterol (58 035 10 μg/mL+acLDL 100 μg/mL) or 7-ketocholesterol (10 μmol/L) for 20 h. *P<0.05, **P<0.01, and ***P<0.001 vs WT or Ldlr−−.
are consistent with the observation that FoxO phosphorylation is extremely sensitive to basal levels of Akt activity, and allow us to infer that the triple FoxO knockout mimics the biochemical effects of in vivo insulin resistance, thus validating the genetic model as a surrogate of the effects of hyperinsulinemia in vivo.

**Increased Oxidative Stress and NO Production in MYFKO Macrophages**

Next, we analyzed the consequences of the triple FoxO knockout on macrophage function. Expression of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase was lower in peritoneal macrophages from MYFKO mice compared with WT (Figure 4A). Genes encoding catalytic subunits of NADPH oxidase (p22phox and p47phox) were expressed at higher levels in MYFKO macrophages. Consistently, LPS-induced reactive oxygen species (ROS) and superoxide production were enhanced in macrophages from MYFKO mice, as assessed by CM-H2DCFDA (Figure 4B and 4C) and dihydroethidium (Figure 4D and 4E), respectively. Elevated iNOS expression is a marker of Ly6Chi monocytes, and is induced by oxidative stress in macrophages. Accordingly, LPS-dependent iNOS protein and mRNA induction were enhanced in MYFKO macrophages (Figure 4F and 4G), as were palmitate- or H$_2$O$_2$-dependent Nos2 inductions (data not shown). Pretreatment with N-acetyl-l-cysteine (NAC), an antioxidant that promotes GSH synthesis, partly inhibited LPS-induced Nos2 expression, reversing the difference between WT and MYFKO mice (Figure 4G), and normalized NO production, measured by nitrate and nitrite concentration in conditioned media (Figure 4H). Pretreatment with the iNOS inhibitor, L-N6-(1-Iminoethyl)lysine, also blunted LPS-induced NO production (Figure 4I and 4J). The combined reductions of superoxide dismutase and glutathione peroxidase, along with the increase of NADPH oxidase subunits, can increase oxidative stress in MYFKO mice. These data suggest that increased oxidative stress mediates iNOS-derived NO overproduction in macrophages from MYFKO mice.

**Reduced Hepatic Insulin Signaling in WTD-fed Ldlr$^{-/-}$:MYFKO Mice**

Altered insulin signaling in macrophages can affect systemic insulin sensitivity. This, in turn, could affect atherosclerosis development in MYFKO mice. Therefore, we investigated the effect of the triple myeloid FoxO knockout on insulin action. Standard- or high-fat diet–fed MYFKO mice showed no differences in body weight, fasted or refed glucose and insulin levels, or glucose tolerance (Online Figure VIIA–VIIE). In contrast, WTD-fed Ldlr$^{-/-}$:MYFKO mice displayed normal body weight (Figure 5A) and composition (data not shown),
but borderline glucose tolerance without significant alterations of insulin tolerance (Figure 5B and 5C), and modest but significant elevations of fasting and fed glucose, as well as fasting insulin levels (Figure 5D and 5E). Total serum cholesterol, triglyceride, and nonesterified fatty-acid levels were comparable with \( Ldlr^{-/-} \) mice (Online Figure VIIIA–VIIIC).

When we analyzed hepatic insulin signaling after administration of insulin in the portal vein, we detected an attenuation of insulin-induced InsR, Akt (S473), and FoxO1 (S256) phosphorylation (Figure 5F; Online Figure VIIID–VIIIF). Changes in hepatic gene expression were limited to a modest increase of \( G6pc \) (Figure 5G). Liver triglyceride and cholesterol contents were comparable between \( Ldlr^{-/-} \) and \( Ldlr^{-/-}:MYFKO \) mice (data not shown). WTD-fed \( Ldlr^{-/-}:MYFKO \) mice did not show liver, pancreas, and kidney dysfunction by serum chemistry (Online Table I). These data are consistent with a mild impairment of hepatic insulin signaling in WTD-fed \( Ldlr^{-/-}:MYFKO \) mice.

Analysis of gene expression in epididymal adipose tissue showed no differences in \( Nos2 \), macrophage markers \( Emr1 \) and \( Cd68 \), and other inflammatory genes (data not shown).
Immunostaining with Mac-3 in epididymal adipose tissue did not show any difference either (data not shown). Thus, it seems unlikely that activated tissue macrophages contribute to the mild metabolic defect of these mice.

**Increased Cysteine Nitrosylation and Tyrosine Nitration of InsR in WTD-fed Ldlr−/−:MYFKO Mice**

To understand the causes of the mild impairment of hepatic insulin signaling, we explored the hypothesis that increased iNOS-dependent NO production in liver caused protein nitrosylation as well as cysteine and tyrosine nitration. Indeed, immunoblotting analysis demonstrated a generalized increase of protein cysteine nitrosylation (Cys-SNO) and tyrosine nitration (3-NT) in livers of WTD-fed Ldlr−/−:MYFKO mice (Figure 6A and 6B). Immunoblotting of InsR immunoprecipitates showed increased Cys-SNO and 3-NT content of InsR in liver from WTD-fed Ldlr−/−:MYFKO mice (Figure 6C). Cys-SNO and 3-NT in aortae of WTD-fed Ldlr−/−:MYFKO mice were also increased (Figure 6D). In contrast, standard- or high-fat diet–fed MYFKO mice showed normal levels of hepatic Cys-SNO and 3-NT (data not shown). These data suggest that increased Cys-SNO and 3-NT of InsR, possibly caused by iNOS-derived NO from macrophages, contribute to the impairment of hepatic insulin signaling in WTD-fed Ldlr−/−:MYFKO mice.

**Antioxidant Treatment Reduces Atherosclerosis in Ldlr−/−:MYFKO Mice**

To test the causative role of oxidative stress and NO-mediated post-translational protein modifications in glucose metabolism and atherosclerosis, we treated WTD-fed mice with the antioxidant, NAC. NAC relieves oxidative stress in hematopoietic cells of mice with Mx1-Cre–mediated triple FoxO ablation. NAC can also lower Cys-SNO levels by displacing NO from cysteine-NO bonds. Seven weeks of oral NAC treatment prevented the increase of white blood cell and monocytes in Ldlr−/−:MYFKO mice, which was seen in vehicle-treated mice (Figure 7A through 7E). Fourteen weeks of NAC treatment reduced WTD-induced atherosclerosis and macrophage accumulation (Figure 7F through 7I), restored glucose tolerance, and decreased fasting glucose and insulin levels in WTD-fed Ldlr−/−:MYFKO mice (Figure 8A through 8D). These changes were reflected in reduced hepatic Cys-SNO and 3-NT levels (Figure 8E through 8G). The data suggest that oxidative stress and NO-mediated protein nitrosylation/nitration play a role in the development of atherosclerosis and hepatic insulin signaling abnormalities in Ldlr−/−:MYFKO mice.

**Discussion**

In the present work, we studied the role of myeloid FoxO in the pathogenesis of atherosclerosis. As FoxO proteins are negative regulators of insulin action, and given our findings...
in other conditional FoxO knockouts, as well as in the converse model of InsR ablation, we expected to find a protective role of this targeted mutation against atherosclerosis. Instead, and consistent with another study on InsR myeloid-specific knockout, we find that MYFKO mice develop more severe atherosclerosis in the Ldlr knockout background. We have identified 2 mechanisms to account for this outcome: (1) a marked expansion of neutrophils and monocytes, likely caused by increased proliferation of GMP and (2) increased oxidative stress and iNOS-derived NO production in macrophages. We also detected a mild impairment of hepatic insulin sensitivity that might contribute to the phenotype.

The induction of GMP proliferation and neutrophil/monocyte number by FoxO ablation is probably owing to inhibition of cell-cycle arrest and decreased apoptosis. In addition, increased oxidative stress, as seen in mice with FoxO ablation, enhances short-term hematopoietic stem cell proliferation and could thus explain the reversal of monocytosis in NAC-treated Ldlr−/−:MYFKO mice.

Correlation studies suggest that neutrophilia and monocytosis are linked with atherosclerosis in humans and in experimental animals. We have reported that deletion of the transporters Abca1 and Abcg1, which promote cholesterol efflux to apoA-1 or high-density lipoprotein, worsens atherosclerosis in mice by increasing neutrophil and monocyte number. Unlike the triple Lys-M-cre FoxO knockout, the pan-BM Abca1/g1 knockout affects proliferation of hematopoietic stem cells. Thus, although the mechanisms of myeloid cell expansion in these 2 models are different, both point to a pathophysiologic link between common correlates of CV disease (high-density lipoprotein-cholesterol and insulin, respectively), white cell counts, and macrophage content of atherosclerotic lesions, suggesting a new therapeutic approach to CV disease aimed at reversing these abnormalities of stem/progenitor cell proliferation. Interestingly, in humans, metabolic syndrome is associated with increased monocyte and neutrophil levels, which, in turn, are linked to increased CHD. Our data indicate that hyperinsulinemia, a common correlate of insulin resistance, increases FoxO phosphorylation and nuclear exclusion in macrophages, establishing a potential mechanism whereby insulin resistance increases CMP/GMP proliferation and myelogenesis.

Interestingly, FoxO4−/− mice in the Apoe−/− background display deterioration of atherosclerosis with increased macrophage content of lesions that can be transferred by FoxO4−/− BM and this is associated with increased interleukin-6 and ROS production in cultured FoxO4−/− macrophages. These findings are consistent with the decreased atherosclerosis in Apoe−/− mice lacking InsR in myeloid cells, in which FoxO is expected to be constitutively active in macrophages. The 3 FoxO isoforms function in a redundant manner to suppress proliferation or promote apoptosis, including in BM. We have shown additive effects of FoxO1 and FoxO3a knockdowns on free-cholesterol–induced apoptosis in macrophages. Consistently, we did not find evidence of increased predisposition to atherosclerosis in single FoxO1
knockouts in the present study, even though FoxO1 is the most abundant isoform in macrophages. This finding allays fears that FoxO1-specific inhibitors, which are being developed as insulin sensitizers, might increase CV risk. In addition, the extent of leukocytosis in MYFKO mice on an Ldlr-competent background is modest compared with Ldlr−/−:MYFKO mice. These observations suggest that homozygous loss of all 3 FoxO alleles, as well as Ldlr or Apoe ablation, is required to affect granulocytes, monocytes and their progenitors, and contribute to the development of atherosclerosis.

The decline of superoxide dismutase, catalase, and Gadd45 levels in FoxO-deficient GMP and macrophages is consistent with the role of FoxO in the antioxidant response and is further corroborated by the beneficial effect of antioxidant treatment on metabolism and atherosclerosis in Ldlr−/−:MYFKO mice. These data suggest that increased oxidative stress is pathogenic in atherosclerosis development in Ldlr−/−:MYFKO mice.

Epidemiological studies consistently show a positive correlation between leukocytosis and coronary artery disease. Neutrophils and monocytes play important roles in atherosclerosis. Neutrophils are the first cell type to home into vascular endothelial cells during atherogenesis, triggering inflammatory signals that promote intimal recruitment of monocytes, namely the inflammatory Ly-6C+ subset, which increases steeply in atherosclerotic mice.27,46 These cells, once they enter the lesion, differentiate into macrophages, leading to foam cell formation and progress the disease.

The GMP lineage–restricted proliferation of myeloid cells in Ldlr−/−:MYFKO mice is probably owing to the fact that LysM-cre is hardly expressed in hematopoietic stem cells or CMP.47 The fact that the increased GMP proliferation observed did not result in increased numbers of GMP is likely owing to their accelerated turnover, consistent with the observation that FoxO-deficient BM has enhanced short-term and deficient long-term repopulating ability.24

The increase in lesion macrophages seen in MYFKO mice is likely secondary to combined effects of FoxO ablation to decrease apoptosis and cell-cycle arrest. The former could depend on decreased activation of FasL, the ligand for the Fas-dependent cell death pathway, or proapoptotic Bim. In addition, FoxO can promote cell-cycle arrest and FoxO ablation can increase macrophage survival.15
Interestingly, we find a reduction of cell-cycle genes (Cdkn1b, 2a, 2b, and p53) that is consistent with increased atherosclerosis seen when Cdkn1b or p53 is ablated in BM cells, as well as with the proposed role of Cdkn2a as a modifier of atherosclerosis susceptibility.

The role of macrophage apoptosis in atherosclerosis is context-dependent, as apoptosis is thought to suppress plaque progression in early stages and promote plaque necrosis in advanced stages. However, animal studies suggest that macrophage apoptosis is a negative regulator of plaque growth even after long-term (10–15 weeks) cholesterol-rich diet, which is equivalent in duration to the present study. Therefore, decrease of apoptosis could also partly contribute to the progression of atherosclerosis in Ldlr−/−:MYFKO mice.

We also find increased iNOS-dependent NO production. However, we do not know if this is secondary to iNOS induction or to altered macrophage composition, with increased numbers of Ly6C monocytes. iNOS-derived NO and ROS worsen atherogenesis, and genetic ablation of iNOS in WTD-fed Apoe−/− mice decreases atherosclerosis. In addition, focal ROS and peroxynitrite at the vascular wall can trigger endothelial dysfunction and smooth muscle cell migration, leading to atherosclerosis. Therefore, increase of iNOS-derived NO and ROS in MYFKO macrophages can promote atherosclerosis.

Cys-SNO and 3-NT are biomarkers of nitrosative and nitratative stress and are associated with insulin resistance. In the present study, we observed an increase of Cys-SNO and 3-NT in WTD-fed Ldlr−/−:MYFKO mice, but not in standard- or high-fat diet–fed MYFKO mice. It is possible that it is secondary to increased NO generated by liver macrophages through iNOS.

In conclusion, our study demonstrates that FoxO in myeloid cells plays a significant role in contributing to increased risks of atherosclerosis.

Figure 8. Metabolic parameters and protein nitrosylation/nitration after N-acetyl-l-cysteine treatment. A, Intraperitoneal glucose tolerance tests and (B) area under the curve (AUC) of 18-week-old Western diet (WTD)–fed Ldlr−/− and Ldlr−/−:MYFKO mice treated with Vehicle (V) or N-acetyl-l-cysteine (N) for 12 weeks after an 18-h fast (n=6–9). Serum (C) glucose and (D) insulin levels in 20-week-old mice fed WTD and fasted for 16 h (n=6–9). E, Representative immunoblots and quantification of (F) S-nitroso-cysteine (Cys-SNO) and (G) 3-nitro-tyrosine (3-NT) in liver from 20-week-old mice after 14 weeks on WTD and treatment with V or N (n=3–4). *, #P<0.05 vs vehicle-treated Ldlr−/−, or Ldlr−/−:MYFKO mice, respectively. LE indicates long exposure; and SE, short exposure.
of atherosclerosis and glucose metabolism, through neutrophilia/macrophages, oxidative stress, and iNOS-derived NO overproduction. Our study suggests that reversing these abnormalities benefits diabetes mellitus and its macrovascular complications.

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Disclosures
None.

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Expanded granulocyte/monocyte compartment in myeloid-specific triple FoxO knockout increases oxidative stress and accelerates atherosclerosis in mice

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ONLINE METHODS

**Reagents.** We purchased N-Acetyl-L-cysteine from Sigma. Sources of antibodies are: anti-p-InsR-β, phospho-FoxO1, FoxO3a, phospho-FoxO3a, FoxO4, Akt, p-Akt (Ser-473 and Thr-308), β-actin, and iNOS from Cell Signaling; anti-insulin-receptor, and α-tubulin from Santa Cruz Biotechnology; anti-nitrotyrosine, actin, and Irs1 from Millipore; anti-nitrosocysteine from Abcam.

**Animal experiments.** We measured body composition by NMR (Bruker Optics), blood glucose with a glucometer (One Touch Ultra, Lifescan), plasma insulin (Mercodia) by ELISA, triglyceride (Cayman chemical), cholesterol, and nonesterified fatty acids by colorimetric assays (Cholesterol E and NEFA C, Wako Pure Chemicals). We have described the procedure for intraperitoneal glucose (2g/kg) and insulin (0.75U kg⁻¹) tolerance tests. In some experiments WT and MYFKO mice are maintained with standard or high-fat (60% of calories from fat, Research Diets) diet for indicated periods.

**Macrophage culture.** We harvested peritoneal macrophages from mice by peritoneal lavage 3 days after intraperitoneal injection of 4% thioglycolate, and cultured them in DMEM supplemented with 10% fetal bovine serum and 20% L929 cell-conditioned medium. Bone marrow cells were collected from the femurs and tibias of mice, pooled, and differentiated in vitro by incubating for 7 days at 37°C in RPMI 1640 medium containing 10% FBS and 20% L929 cell-conditioned medium.

**Blood analysis.** Mouse peripheral blood was collected by tail vein puncture into heparin-coated tubes. Total RBC, WBC, and platelet counts were quantified using the FORCYTE Veterinary Analyzer (Oxford Science Inc.). The cells were subjected to RBC lysis and were stained using an antibody cocktail including CD45-APC-Cy7, CD115-APC, and Ly6C/G-PerCP-Cy5.5 (BD Pharmingen). Monocytes were identified as CD45⁺CD115⁺ cells and further gated as Ly6C⁺ or Ly6C⁻ (Online Figure IA). For some experiments neutrophils and monocytes were identified as CD45⁺CD11b⁺Ly6G⁺ and CD45⁺CD11b⁺Ly6G⁻, respectively. Multiparameter analyses were
performed using a LSR II flow cytometer (Becton Dickinson) with DiVa software. Data were analyzed using FlowJo software (Tree Star, Inc.).

**Bone marrow analysis.** Bone marrow (BM) analysis using flow cytometry was performed as described (Online Figure IB) \(^2\)\(^-\)\(^4\). We harvested BM from femurs and tibiae and lysed red cells, followed by resuspension in HBSS (0.2% BSA/0.5mM EDTA) and incubation with a cocktail of antibodies to lineage (lin) committed cells (B220, CD2, CD3e, CD4, CD8, CD11b, CD19, Gr-1, and TER-119; all FITC; eBioscience) and stem cell markers Sca1-PE-Cy7 and ckit-APC-Cy7. HSPCs were identified as lin\(^-\)Sca1\(^+\)ckit\(^+\). Where further identification of hematopoietic progenitor cells was required, we used antibodies to CD16/CD32 (FcγRII/III) and CD34 to separate CMP (lin\(^-\)Sca1\(^-\)ckit\(^+\)CD34\(^{int}\)FcγRII/III\(^{int}\)), and GMP (lin\(^-\)Sca1\(^-\)ckit\(^+\)CD34\(^{int}\)FcγRII/III\(^{hi}\)).

**Cell proliferation measurement.** For cell cycle analysis in BM cells, we fixed and stained cells in 1μg/ml DAPI at 4°C for 30 minutes, followed by staining with lineage, stem and progenitor markers. We cultured thioglycollate-elicited peritoneal macrophages at 2.5x10\(^5\) cells/cm\(^2\) and cultured them with DMEM supplemented with 10% fetal bovine serum and 20% L929 cell-conditioned medium. After 32h, we manually counted cell number and assessed their viability by MTT (Life Technologies). Staining of 5-bromo-2'-deoxyuridine (BrdU, Life Technologies) was performed using biotin-conjugated anti-BrdU antibody (Life Technologies) and streptavidin-Alexa Fluor 488 (Jackson Immunoresearch). We quantified percentage of BrdU-positive cells as the ratio of BrdU-positive to PI-positive cells.

**Apoptosis detection.** We loaded cultured peritoneal macrophages with free-cholesterol (58035, 10 mg/l and acetylated low-density lipoprotein, 100 mg/L) or 7-ketocholesterol (10μM) for 20h. We measured apoptosis by Alexa 488-labeled annexin V and propidium iodide staining (Vybrant Apoptosis Assay kit, Invitrogen). For each condition, we randomly selected four separate fields, counted > 100 positive cells and plotted them as percentage of total cells.

**Atherosclerotic lesion Analysis.** We pinned aortae on silicon dishes and performed Oil Red-O staining, using Image J software to quantify lipid-laden areas as a percentage of total area.
We isolated and fixed hearts in phosphate-buffered formalin then dehydrated and embedded them in paraffin.

**Macrophage adhesion assay.** Peritoneal macrophages were subjected to RBC lysis and were labeled with APC-anti-CD11b antibody (BD) for 30min. Then cells were washed and seeded (5x10^5 cells/cm^2) onto confluent MS1 cells stimulated with 20μg/ml LPS for 6 h. After 20 min, we washed non-adherent macrophages 3 times with PBS. We quantified adherent macrophages per field by fluorescent intensity and analyzed the data using ImageJ.

**NO production.** We loaded peritoneal macrophages with DAF-2 DA (0.5μM, invitrogen) for 30 min at 37 °C, washed 3 times with phenol red-free DMEM and incubated them in the dark. We used fluorescent microscopy to score cells, followed by ImageJ analysis. We assayed nitrate and nitrite levels in conditioned media of cell culture using Greiss assay kit (Enzo). Nitrate and nitrite levels are normalized by protein concentration of cell extracts.

**Reactive oxygen species (ROS)/peroxynitrite, and superoxide production.** We assessed intracellular ROS/peroxynitrite and superoxide using fluorescent dye 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H2DCF-DA, Invitrogen) and dihydroethidium (DHE, Invitrogen), respectively. We treated serum- and growth supplement-starved cultured macrophages with or without LPS for 30 min, washed twice with Krebs-Ringer buffer and loaded them with CM-H2DCFDA (5 μM) or DHE (5 μM) for 20 min at 37°C. We visualized cells and quantified fluorescence using ImageJ software.

**Hepatic lipid content.** We measured liver lipid content by tissue saponification in ethanolic KOH after neutralization with MgCl2 5, and hepatic triglyceride and cholesterol content by colorimetric assay (Wako Pure Chemicals).

**mRNA analysis.** We extracted RNA using TRIzol (Invitrogen), synthesized cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and performed quantitative RT-PCR using GoTaq SYBR Green qPCR Kit (Promega) in a Chromo4 Real-Time PCR Detection System (Bio-Rad). For analysis of GMP, we sorted cells using FACSARia (BD), collected them
into 0.35 ml of buffer-RLT and stored them at ~80°C. RNA extraction and DNase treatment were performed with the RNeasy Micro kit (Qiagen Inc., California) according to manufacturers’ instructions. Eluted RNA samples were reverse transcribed using SuperScript III and random hexamers (Invitrogen) according to protocol supplied by the manufacturer. Primer sequences are available on request.

**Protein analysis.** We lysed tissues or cells in buffer containing 2% SDS, 50mM Tris-HCl, and 5mM EDTA. For immunoprecipitation, we lysed liver or cells in RIPA buffer with protease/phosphatase inhibitors. We immunoprecipitated lysates with anti-InsR-β antibody, carried out immunoblotting and visualized the signal with ECL (GE Lifescience).

**Statistical analysis.** We show data as mean ± SE. We used the customary threshold $P < 0.05$ to declare statistically significant differences using unpaired t-test or ANOVA with Dunn’s post hoc test.
**Online Table I Serum chemistry panel of WTD-fed mice**

<table>
<thead>
<tr>
<th></th>
<th><em>Ldlr</em>^−/−^ (n=11)</th>
<th><em>Ldlr</em>^−/−^: MYFKO (n=13)</th>
</tr>
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<tr>
<td><strong>ALP (U/l)</strong></td>
<td>184 ± 28</td>
<td>218 ± 21</td>
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<tr>
<td><strong>γ-GTP (U/l)</strong></td>
<td>2.4 ± 0.9</td>
<td>4.1 ± 1.7</td>
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<tr>
<td><strong>AST (U/l)</strong></td>
<td>64 ± 18</td>
<td>78 ± 18</td>
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<tr>
<td><strong>ALT (U/l)</strong></td>
<td>59 ± 35</td>
<td>74 ± 26</td>
</tr>
<tr>
<td><strong>Amylase (U/l)</strong></td>
<td>1280 ± 118</td>
<td>1407 ± 105</td>
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<tr>
<td><strong>BUN (mg/dl)</strong></td>
<td>11 ± 0.9</td>
<td>10 ± 0.5</td>
</tr>
<tr>
<td><strong>Albumin (g/dl)</strong></td>
<td>2.4 ± 0.2</td>
<td>2.6 ± 0.2</td>
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<tr>
<td><strong>UA (mg/dl)</strong></td>
<td>2.5 ± 0.3</td>
<td>2.6 ± 0.5</td>
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<tr>
<td><strong>TP (g/dl)</strong></td>
<td>4.1 ± 0.1</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td><strong>Globulin (g/dl)</strong></td>
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<td>2.0 ± 0.2</td>
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Data were obtained after a 16-hr fast in 20-week-old mice fed WTD for 14 weeks. None of the differences is statistically significant.
ONLINE FIGURE LEGENDS

Online Figure I. Multiparameter flow cytometric analysis

(A) After selection of live blood cells and exclusion of doublets, leukocytes were gated as CD45+ cells. Monocytes (Mo) were gated as CD45+CD115+, and neutrophils (Neu) as CD45+CD115−Ly6C hi. Monocytes consist of distinct CD45+CD115+Ly6C lo and CD45+CD115+Ly6C hi populations that correspond to Ly6C hi (hi) and Ly6C lo (lo) monocyte subsets, respectively. (B) After selection of live BM cells and exclusion of doublets, cells were gated for Lineages− (Lin−). In Lin− cells, HSPC were gated as Sca-1−c-Kit+ (gate 7). Sca-1−c-Kit+ progenitor (Pro) cells were further divided into GMP (lin−Sca1−ckit+CD34 intFcγRIII/IIII hi) and CMP (lin−Sca1−ckit+CD34 intFcγRIII/IIII int).

Online Figure II. FoxO expression in macrophages

FoxO mRNA and/or protein expression in (A and B) peritoneal and (C and D) bone marrow macrophages from WT and MYFKO mice (n=4). ** p < 0.01, *** p < 0.001 vs. WT.

Online Figure III. Peripheral blood analysis of single Foxo1 knockouts and MYFKO mice

(A) WBC number, (B) percentage and (C) absolute number of CD45+CD11b+Ly6G− (neutrophils) and CD45+CD11b+Ly6G+ (monocytes) cells in 6-weeks-old LysM-Cre: FoxO1 floxed mice (n=4-5). (D) RBC, WBC and Plt number, and (E) percentage and (F) absolute number of neutrophils (Neu), lymphocytes (Ly), monocytes (Mo), eosinophils (Eo), and basophils (Ba) in 6-week-old WT and MYFKO mice with Ldlr+/− background (n=6-8). (G) Gene expression in peritoneal macrophages (n=4). (H) Representative pictures and (I) quantification of adherent CD11b-labelled peritoneal macrophages to MS1 cells (n=4). * p < 0.05, * p < 0.05, *** p < 0.001 vs. WT.

Online Figure IV. FoxO or cell cycle-related genes in HSPC, CMP, and GMP

Relative populations of cells in G1, S, and G2/M phase of (A) HSPC and (B) CMP (n=6). Foxo expression in (C) HSPC, (D) CMP, and (E) GMP sorted from BM of 6-week-old Ldlr+/− and Ldlr−/−
Online Figure V. Gene expression in aorta, caspase-3 immunostaining in aortic root, M1/M2 markers, and NF-κB signaling.

(A) Gene expression in whole aortae of mice after 14 weeks on WTD (n=4-6). (B) Representative pictures and (C) quantification of aortic root with active caspase-3 (n=6-7). (D) M1/M2 markers in peritoneal macrophages from WT and MYFKO mice (n=4). (E) LPS-induced p105 and p65 phosphorylation in peritoneal macrophages from WT and MYFKO mice. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. Ldlr⁻/⁻ or WT.

Online Figure VI. Insulin signaling in cultured macrophages

(A) Immunoblot of insulin signaling proteins in cultured peritoneal macrophages from WT and MYFKO mice. (B) Immunoblots of insulin signaling proteins in cultured peritoneal macrophages pretreated with insulin (10nM) or vehicle for 24 h prior to 3-hr incubation in serum-free medium followed by the addition of insulin for the indicated times.

Online Figure VII. Metabolic characterization of SD- or HFD-fed MYFKO mice

(A and B) Body weight (n=10-14) and (C and D) intraperitoneal glucose tolerance tests (n=6-10) after 18-hr fast in 12- or 16-week-old WT or MYFKO mice fed standard (SD) or high-fat diet (HFD), respectively. (E and F) Glucose and (G and H) insulin levels in 16- or 18-week-old mice fed SD or HFD fasted for 16 h (0h, Fasted), or fasted for 16 h and refed for 4 h (Refed) (n=6-8).

Online Figure VIII. Serum lipid and liver insulin signaling in WTD-fed Ldlr⁻/⁻: MYFKO mice

Serum (A) TC, (B) TG, and (C) NEFA levels in 20-week-old mice fed WTD and fasted for 16 hr (n=6-9). Quantification of insulin-stimulated (D) p-InsRβ, (E) p-Akt (S473), and (F) p-FoxO1
(S256) levels in liver of 14-week-old \(Ldlr^{-/-}\) and \(Ldlr^{+/+}\): MYFKO mice fed WTD for 8 weeks. After a 16-hr fast, mice were injected with insulin or PBS and livers were collected 3 min later (n=3). * \(p < 0.05\) vs. \(Ldlr^{+/+}\).
REFERENCES


Online Figure I
Online Figure V

A

$mRNA (AU)\quad |\quad Ldlr^{\text{--}}\quad |\quad Ldlr^{\text{--}}:MYFKO$

B

Active caspase-3

C

Active caspase-3

D

$mRNA (AU)\quad |\quad WT\quad |\quad MYFKO$

E

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p105

p-p65-S536

p65
Online Figure VI

A

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B

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