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C/EBP β in bone marrow is essential for diet induced inflammation, cholesterol balance, and atherosclerosis

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Abstract

Background and Objective—Atherosclerosis is both a chronic inflammatory disease and a lipid metabolism disorder. C/EBP β is well documented for its role in the development of hematopoietic cells and integration of lipid metabolism. However, C/EBP β 's role in atherosclerotic progression has not been examined. We assessed the impact of hematopoietic CEBP β deletion in ApoE^{-/-} mice on hyperlipidemia, inflammatory responses and lesion formation in the aorta.

Methods and Results—ApoE^{-/-} mice were reconstituted with bone marrow cells derived from either WT or C/EBP β ^{-/-} mice and placed on low fat or high fat/high cholesterol diet for 11 weeks. Hematopoietic C/EBP β deletion in ApoE^{-/-} mice reduced blood and hepatic lipids and gene expression of hepatic stearoyl CoA desaturase 1 and fatty acid synthase while expression of ATP binding cassette transporter G1, cholesterol 7- α -hydroxylase, and liver X receptor alpha genes were significantly increased. ApoE^{-/-} mice reconstituted with C/EBP β ^{-/-} bone marrow cells also significantly reduced blood cytokine levels and reduced lesion area in aortic sinuses compared with ApoE^{-/-} mice reconstituted with WT bone marrow cells. Silencing of C/EBP β in RAW264.7 macrophage cells prevented oxLDL-mediated foam cell formation and inflammatory cytokine secretion in conditioned medium.

Conclusion—C/EBP β in hematopoietic cells is crucial to regulate diet-induced inflammation, hyperlipidemia and atherosclerosis development.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2016.03.040>.

Keywords

Atherosclerosis; Bile acid; Cytokine; Hematopoietic stem cell; Inflammation; Macrophage foam cells; Cholesterol efflux

1. Introduction

Macrophages play a central role in atherogenesis through the accumulation of cholesterol and the production of inflammatory mediators and cytokines. A critical step in the development of atherosclerosis is the accumulation of cholesterol-laden macrophages, which in the arterial wall is the characteristic of the early atherosclerotic lesion [1,2]. Cellular cholesterol content in macrophages is determined by uptake (mediated by scavenger receptors) and efflux of cholesterol (mediated by cholesterol acceptors) [3], an imbalance of which results in the formation of foam cells, which in turn promote lipid deposition and lesion growth.

The modulation of macrophage accumulation and function represents an appealing therapeutic target for the treatment and prevention of inflammation in a variety of disease pathways. Inflammatory pathways in macrophages are under stringent control by a variety of transcription factors and coregulatory molecules. Among these are NF- κ B, AP1, the PPAR family, liver X receptor alpha (LXR α /Nr1h3), and their associated coactivators and corepressors [4]. However, the signaling pathways that regulate inflammation and trigger foam cell formation and cytokine production in atherosclerosis remain incompletely understood.

C/EBP β is a member of the CCAAT/enhancer binding protein family of basic leucine zipper transcription factors with classical functions in transcriptional and translational regulation of lipid metabolism in the liver and adipose tissue [5,6]. In addition, C/EBP β has been described as a macrophage lineage determination factor, also referred to as a pioneer factor or master regulator because it represents a placeholder that can be replaced by other transcription factors at later stages during development [7]. Beyond its role in early myeloid and adipose cell development [8–10], we demonstrated that adenovirus delivery of C/EBP β to the liver of WT mice re-capitulated many of the nonalcoholic steatohepatitis-like phenotypes including hepatic inflammation, endoplasmic reticulum stress, and lipid accumulation [6]. Conversely, we showed that C/EBP β deletion in *Lepr^{db/db}* mice reduced adiposity, hepatic steatosis, and diabetes [5]. More recently, we showed that hematopoietic deletion of C/EBP β reduced obesity-linked inflammation and insulin resistance in WT mice [11], suggesting that C/EBP β deletion may be important in controlling innate immunity.

Because C/EBP β is known to play an important role in inflammation [9], we hypothesized that hematopoietic deletion of C/EBP β in hyperlipidemic ApoE $^{-/-}$ mice [12] would reduce inflammation and hyperlipidemia, and thereby interrupt processes important for development of atherosclerosis. Surprisingly, our data demonstrate that C/EBP β deletion in hematopoietic cells had pleiotropic effects on lipids and the evolution of atherosclerosis in ApoE $^{-/-}$ mice including reduced total and LDL cholesterol, along with modulation of genes implicated in triglyceride and cholesterol metabolism. Furthermore, adipose tissue

inflammation, circulatory cytokine levels, and atherosclerotic lesion formation in the aortic sinuses of chimeric ApoE^{-/-} mice were suppressed. We documented that shRNA-mediated deletion of C/EBP β in RAW264.7 macrophages attenuated oxidized LDL (oxLDL)-mediated foam cell formation and secretion of inflammatory cytokines in the medium. Altogether, these findings indicate that C/EBP β in hematopoietic-derived cells regulates cholesterol balance in the macrophage and liver and therefore is a crucial transcriptional regulator of diet-induced inflammation, hyperlipidemia, and atherosclerosis development.

2. Materials and methods

2.1. Animals & bone marrow transplants

All animal care and procedures were conducted according to the policies on animal welfare of the National Institute of Health and were approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver and Texas Tech University. Every effort was made to minimize the animal stress and suffering.

Six-to eight-week old B6.SJL (CD45.1; #002104; Jackson Laboratory, Bar Harbor, ME) ApoE^{-/-} female recipient mice were subjected to a total of 1000 rad (2 \times 500 rad 3 h apart) of whole body irradiation to eliminate endogenous bone marrow stem cells. Bone marrow was harvested from donor mice (8–10 week old) by flushing the femur of WT C57BL/6 (CD45.2) and C/EBP β ^{-/-} (CD45.2) with Hank's buffer, as described previously [11]. Recipient mice were then anesthetized with isoflurane and injected with 2.5 \times 10⁶ cells into the retro-orbital sinus cavity. After a 4 week recovery, mice were placed on either low fat (LF; 10 kcal% fat, D12102; Research Diets, New Brunswick, NJ) or high fat/high cholesterol diet (HF/HC; 40 kcal% fat, 1.25% cholesterol, 0% cholic acid; D12108C; Research Diets) for 11 weeks ($n = 6$). Food intake and body weight were measured weekly. To test for engraftment of donor bone marrow, at 10 weeks post-irradiation mice were anesthetized with isoflurane and blood was collected via retro-orbital sinus and analyzed by flow cytometry for CD45.1 or CD45.2 positive cells as described previously [11]. ApoE^{-/-} mice with engraftment of >85% donor cells were continued on the experimental diets and used for final analyses. At the end of the 11 week diet treatment, tissues were collected in mice anesthetized with isoflurane after a 6-h fast. Tissues were immediately placed in tubes with RNAlater (Qiagen, Valencia, CA) or snap-frozen in liquid nitrogen. Blood samples were collected after a 6-h daytime fast from the vena cava.

2.2. Measurement of serum metabolites

For glucose tolerance tests (GTT), ApoE^{-/-} mice transplanted with WT and C/EBP β ^{-/-} bone marrow cells and fed LF and HF/HC diets were fasted for 4 h and injected intraperitoneally with glucose (2 mg/g body weight), as described previously [11,13]. Blood samples were collected from the tail vein at 0, 30, 60, 90 or 120 min and glucose was measured using a glucometer. Area under the curve (AUC) of glucose was calculated as described previously [14]. To circumvent influences of glucose injection on hepatic gene expression, animals were sacrificed one week after the last GTT, i.e. after 11 weeks on the experimental diets. Serum insulin and adiponectin levels were measured by ELISA kits from ALPCO (Windham, NH). Serum cytokines (IL-1 β , TNF α , MCP1, and IL-6) was measured by

multiplex assay (Bio-Rad, Hercules, CA). Serum total cholesterol, triglycerides, HDL cholesterol, and non-HDL cholesterol were analyzed enzymatically using a Beckman Coulter AU automated chemistry analyzer (Beckman Coulter, Brea, CA).

2.3. Analysis of liver lipids

Lipids were extracted from about 50 mg of frozen liver tissue using a Folch method [15]. Half of the lipid extract was saponified and total cholesterol was extracted as previously described [16]. Cholesterol and stigmasterol (internal standard) were measured using an HPLC system (Waters Corp., Milford, MA) equipped with a C18 reverse-phase column (150 mm × 4.6 mm, 5 μm size). The mobile phase was methanol at 1 mL/min. Detection was monitored at 210 nm by a Waters 2489 UV–visible detector. The tissue pellet was digested by 1 N NaOH and total protein was measured using a BCA kit. Total cholesterol was expressed as mg of cholesterol per mg of protein.

2.4. Evaluation of lesion size

Atherosclerotic lesions at the aortic sinuses were analyzed as described previously after 11 weeks of diet treatment [17]. The upper portion of the heart (aortic sinuses) was obtained, embedded in OCT compound, and stored at –80 °C. Ten μm sections were analyzed for a distance of 800 μm. Sections were stained with Oil Red O. The lipid-staining areas were determined in a blinded fashion by light microscopy. The mean value of lesion area of aortic wall per section was then calculated.

2.5. Quantitative real-time PCR

Total RNA isolation, reverse transcription, and quantitative real-time PCR (qPCR) were performed as described previously [11]. For primers used in qPCR, see Table S1.

2.6. Culture and treatment of RAW264.7 macrophage cells

RAW264.7 macrophage cells were obtained from ATCC (Manassas, VA) and cultured in modified DMEM (4.5 g glucose/L) as described before [11]. The macrophage cells (70–80% confluent) were infected with control (50 pfu/cell) and C/EBPβ-shRNA (50 pfu/cell) for 24 h followed by treatment with nLDL and oxLDL (20 μg/mL; Biomedical Technologies, Inc., Stoughton, MA) for an additional 24 h. Conditioned medium was collected for protein array. Cells were fixed and stained with Oil Red O to detect lipid accumulation. In some experiments, cells were harvested for RNA isolation and Western blot.

2.7. Protein array and immunoblot analysis

Comprehensive analysis of cytokine levels in the conditioned media from RAW264.7 macrophage cell culture was performed using commercially available Mouse Inflammation Antibody Array 1.1 (RayBiotech, Inc., Norcross, GA) according to manufacturer's protocol. Cytosolic and nuclear extracts were prepared from RAW264.7 macrophage cells and subjected to Western blot analysis for P-JNK and C/EBPβ, respectively, as previously described [11]. Primary antibodies used in this study were C/EBPβ and actin (Santa Cruz Biotechnology, Santa Cruz, CA) and P-JNK (Cell Signaling Technology, Danvers, MA).

2.8. Statistical analysis

Statistical comparisons between groups were made either using Student's *t*-test, or two-way ANOVA, followed by Tukey's post hoc test. GTT values were analyzed using two-way repeated measures ANOVA. All values are reported as mean \pm standard error. Differences were considered to be statistically significant at *P* values 0.05 or less.

3. Results

3.1. Hematopoietic deletion of C/EBP β reduced pro-inflammatory cytokines in serum and adipose tissue of ApoE $^{-/-}$ mice

C/EBP β plays an important role in the generation and activation of resident macrophages [10]. Although C/EBP β is induced by inflammatory stimuli and affects cytokine production in several macrophage types [18,19], its role in atherosclerosis is unknown. We therefore evaluated the impact of hematopoietic C/EBP β deletion from bone marrow on mechanisms that provoke atherosclerosis in ApoE $^{-/-}$ mice. ApoE $^{-/-}$ mice transplanted with WT or C/EBP $\beta^{-/-}$ bone marrow cells were placed on high fat/high cholesterol diet (HF/HC) or low fat diet (LF) for 11 weeks. Mice on HF/HC diets gained more weight than animals on LF diet (Fig. 1A) but the difference was not statistically significant. Final body weights were similar between WT \rightarrow ApoE $^{-/-}$ HF/HC and C/EBP $\beta^{-/-}$ \rightarrow ApoE $^{-/-}$ HF/HC mice (Fig. 1A). We also assessed glucose tolerance in the bone marrow transplanted mice. Animals on the HF/HC diet had significantly higher GTT values at baseline than those on a LF diet (*P* = 0.023; Fig. 1B). Area under the curve (AUC) analyses of the GTT curves for the four groups showed differences for LF versus HF/HC diets when using uncorrected AUCs (*P* = 0.006) but not when correcting for the baseline (*P* = 0.57, Fig. 1C). WT \rightarrow ApoE $^{-/-}$ versus C/EBP $\beta^{-/-}$ \rightarrow ApoE $^{-/-}$ was not significantly different in any of the aforementioned analyses (*P* > 0.07 in all cases). There was no evidence in support of an ApoE by-diet interaction in the AUC analyses. Fasting plasma insulin level was similar between the HF/HC groups (data not shown).

Elevated levels of plasma inflammatory cytokines, and chemokines, have been implicated in the initiation and progression of cardiovascular disease [20,21]. As expected, HF/HC diet significantly increased IL-1 β , MCP1, and IL-6 cytokine levels in WT \rightarrow ApoE $^{-/-}$ mice compared with WT \rightarrow ApoE $^{-/-}$ LF mice (Fig. 1D). By contrast, hematopoietic deletion of C/EBP $\beta^{-/-}$ produced significant reductions (45–81%) in IL-1 β , TNF α , MCP1, and IL-6 cytokine levels in C/EBP $\beta^{-/-}$ \rightarrow ApoE $^{-/-}$ HF/HC mice compared with WT \rightarrow ApoE $^{-/-}$ HF/HC mice (Fig. 1D).

The systemic inflammation that is associated with atherosclerosis is partly derived from adipose tissue [22,23]. In our previous study we found that hematopoietic C/EBP β deletion in wildtype mice prevented high fat diet-mediated induction of adipose tissue inflammation [11]. To investigate whether adipose tissue was the source of reduced circulating cytokines, we determined the effect of hematopoietic C/EBP β deletion on adipose tissue inflammation in C/EBP $\beta^{-/-}$ \rightarrow ApoE $^{-/-}$ mice under LF and HF/HC fed conditions. HF/HC diet resulted in a non-significant increase in *Tnf* and *Mcp1* gene expression in WT \rightarrow ApoE $^{-/-}$ mice compared with LF diet-fed mice (Fig. 2A). Interestingly, despite similar weight gain on the

HF/HC diet, we found reductions in *Tnf* and *Mcp1* gene expression in adipose tissue of C/EBP β ^{-/-} → ApoE^{-/-} HF/HC mice compared with WT → ApoE^{-/-} HF/HC mice although the differences were not significant (Fig. 2A). The increase in adipose tissue inflammation is associated with recruitment of macrophages in adipose tissue and may further increase the magnitude of inflammation [24,25]. Consistent with reduced expression of inflammatory genes in adipose tissues of C/EBP β ^{-/-} → ApoE^{-/-} mice, macrophage marker genes *Cd68* and *Cd11c* showed non-significant reductions in the WT → ApoE^{-/-} compared with C/EBP β ^{-/-} → ApoE^{-/-} mice on HF/HC diet (Fig. 2B).

3.2. A more anti-atherogenic lipoprotein profile in ApoE^{-/-} mice transplanted with C/EBP β ^{-/-} bone marrow cells

An increase in serum and tissue lipids is considered a critical factor in the development of atherosclerosis [26,27]. We assessed the effects of reconstitution with bone marrow lacking C/EBP β on serum lipid levels in ApoE^{-/-} mice. On LF diet, total cholesterol levels were similar in ApoE^{-/-} mice transplanted with C/EBP β ^{-/-} bone marrow cells (Fig. 3A). However, HF/HC diet significantly increased serum cholesterol ($P < 0.0001$), HDL cholesterol ($P = 0.033$) and LDL cholesterol ($P < 0.0001$) in WT → ApoE^{-/-} mice (Fig. 3A). By contrast, hematopoietic deletion of C/EBP β in ApoE^{-/-} mice blunted the HF/HC diet-induced increase in serum total and LDL cholesterol ($P < 0.0001$, $P = 0.0002$, respectively; Fig. 3A). In the liver of ApoE^{-/-} mice with C/EBP β deletion in hematopoietic cells, total cholesterol levels were reduced 60% (Fig. 3B) while liver triglyceride levels appeared to be lower in C/EBP β ^{-/-} → ApoE^{-/-} HF/HC mice compared with WT → ApoE^{-/-} HF/HC mice but was not statistically significant (Fig. 3C). Overall, these data showed that C/EBP β ^{-/-} → ApoE^{-/-} HF/HC mice attenuated serum and liver lipid profiles compared with WT → ApoE^{-/-} HF/HC mice.

3.3. Hematopoietic C/EBP β deletion in ApoE^{-/-} mice reduced *Fasn* and *Scd1* gene expression but increased *Abcg1*, *Lxr/Nr1h3*, and *Gpbar1/Tgr5* gene expression in liver

Atherosclerosis is characterized by a dysfunction of hepatic lipid metabolism [28,29]. In order to investigate the possible mechanisms regarding the observed differences in serum and liver lipid levels, we quantified mRNA expression levels of key liver enzymes in fatty acid and triglyceride synthesis and cholesterol efflux. Expression of fatty acid synthase (*Fasn*) and stearoyl-CoA desaturase (*Scd1*) were reduced (40–50%), while expression of *Lxr/Nr1h3*, a gene that increases cholesterol metabolism, was significantly increased fourfold in liver along with its target gene ATP-binding cassette transporter G1 (*Abcg1*) in C/EBP β ^{-/-} → ApoE^{-/-} HF/HC mice compared with WT → ApoE^{-/-} HF/HC mice (Fig. 3D). HMG-CoA reductase (*Hmgcr*), a gene that controls cholesterol synthesis, was slightly lower in C/EBP β ^{-/-} → ApoE^{-/-} HF/HC mice ($P = 0.14$), and cholesterol 7 alpha-hydroxylase (*Cyp7a1*), which enhances cholesterol to bile acid conversion and plays a crucial role in regulation of serum cholesterol levels, was induced fourfold compared with WT → ApoE^{-/-} HF/HC mice (Fig. 3D). We also analyzed the expression of bile acid sensors farnesoid X receptor (*Fxr/Nr1h4*) and G protein-coupled bile acid receptor 1 (*Gpbar1/Tgr5*) genes, both of which are activated by bile acids and play key roles in hepatic lipid homeostasis and inflammation [30,31]. *Fxr/Nr1h4* gene expression was similar between groups but interestingly, the expression of *Gpbar1/Tgr5* was over fourfold higher (P

= 0.061) in $C/EBP\beta^{-/-} \rightarrow ApoE^{-/-}$ HF/HC mice (Fig. 3D). Overall, these results indicate that $C/EBP\beta$ deletion in bone marrow reduced cholesterol levels in liver and serum, and suggests that despite a substantial increase in $Lxr/Nr1h3$ activation, $C/EBP\beta$ in hematopoietic-derived cells is required to induce liver and serum cholesterol levels in $ApoE^{-/-}$ mice.

3.4. Atherosclerosis is dramatically decreased in $ApoE^{-/-}$ mice transplanted with $C/EBP\beta^{-/-}$ bone marrow cells

Given the reduction in serum cytokines and lipids, we investigated the impact of $C/EBP\beta$ deletion in hematopoietic cells on atherosclerotic lesion development and lesion sizes in the aortic sinuses of $ApoE^{-/-}$ mice. Lesion formation was evaluated by neutral lipid stain Oil Red O and sections of Oil Red O-stained aortic sinuses were quantified after 11 weeks of HF/HC feeding. Quantification of the lesion sizes in Oil Red O-stained sections of the aortic sinuses revealed that deletion of $C/EBP\beta$ in macrophages led to a 50% decrease in the mean atherosclerotic lesion size in $C/EBP\beta^{-/-} \rightarrow ApoE^{-/-}$ HF/HC mice compared with lesions in $WT \rightarrow ApoE^{-/-}$ HF/HC mice (Fig. 4).

3.5. $C/EBP\beta$ regulates oxLDL-mediated macrophage foam cell formation

Macrophages play a critical role in the development of atherosclerosis [32]. Accumulation of macrophage foam cells which results from impaired cholesterol metabolism is a prominent early event in the development of atherosclerosis [33,34]. Given the reduction in lipids, inflammation, and aortic lesions in $ApoE^{-/-}$ mice lacking $C/EBP\beta$ in bone marrow, we hypothesized that $C/EBP\beta$ could have direct effects on macrophage cholesterol balance. Using RAW264.7 cells, we found that macrophage cells treated with oxLDL significantly increased the expression of $C/EBP\beta$ protein (Fig. 5A), along with the cell stress marker phosphorylated JNK (P-JNK). To further investigate whether $C/EBP\beta$ controls cholesterol balance in macrophages, we examined the effects of oxLDL on cholesterol balance in RAW264.7 cells using $C/EBP\beta$ -shRNA knockdown. We found using shRNA-mediated knockdown of $C/EBP\beta$ that oxLDL-mediated induction of lipid accumulation was dramatically decreased as evidenced by Oil Red O staining (Fig. 5B). Consistent with this reduction of lipid accumulation, silencing of $C/EBP\beta$ moderately increased the expression of $Abcg1$ gene (Fig. 5C), which is implicated in cholesterol efflux [35,36] but this change was not statistically significant ($P = 0.11$).

3.6. Attenuated expression of pro-inflammatory proteins in conditioned medium of $C/EBP\beta$ -depleted RAW264.7 macrophages

During atherogenesis, macrophages take up lipid moieties to become foam cells that secrete pro-inflammatory cytokines, which, in turn, propagate lesion formation and further perpetuate vessel wall inflammation [2,37,38]. To gain further insight into $C/EBP\beta$'s role in cytokine production in macrophages, we measured relative levels of key inflammatory cytokines in conditioned medium from RAW264.7 macrophages cultured in the absence or presence of $C/EBP\beta$ -shRNA and oxLDL. We used a protein array method that quantifies the relative levels of cytokines by immunoblot. oxLDL treatment significantly increased IL-6, VEGF, Pro-MMP-9, IL-1 β , and MCP1 protein expression in Cont-shRNA cells compared to nLDL treatment ($P < 0.0001$, $P < 0.0001$, $P = 0.002$, $P = 0.021$, $P < 0.0001$, respectively;

Fig. 5D). Silencing C/EBP β in cells treated with oxLDL significantly reduced the expression of IL-6 and VEGF compared with the Cont-shRNA + oxLDL ($P=0.0115$ and $P<0.0001$, respectively; Fig. 5D). Expression of MCP1 was significantly decreased ($P=0.0008$) while IL-1 β was slightly decreased ($P=0.091$) in C/EBP β -shRNA + oxLDL compared with the ContshRNA + oxLDL group (Fig. 5D). By contrast, the level of IL-10, an anti-inflammatory cytokine, was moderately higher ($P=0.18$) in C/EBP β -shRNA macrophage cells compared with cells treated with control shRNA and oxLDL (Fig. 5D).

4. Discussion

Given the pro-inflammatory role of C/EBP β in tissue macrophages, along with the profound reduction in obesity in global C/EBP $\beta^{-/-}$ mice [11], we aimed to investigate: 1) if bone marrow deficiency of C/EBP β can attenuate the local and systemic inflammation pattern seen in the global ApoE $^{-/-}$ mice, and 2) whether the reduction in inflammation resulting from hematopoietic C/EBP β deletion could attenuate the development of atherosclerosis, a chronic inflammatory disease associated with hyperlipidemia. The present study demonstrated for the first time that C/EBP β deletion in hematopoietic cells significantly reduced atherosclerotic lesion formation in aortic sinuses of ApoE $^{-/-}$ mice on a high fat/high cholesterol diet. This reduction of lesion formation in ApoE $^{-/-}$ mice was associated with marked reduction in circulating cytokine levels while expression of pro-inflammatory and macrophage marker genes in visceral adipose tissue were moderately decreased. In addition, C/EBP β deletion in hematopoietic cells also reduced serum total and LDL cholesterol level without affecting the HDL cholesterol levels. These findings suggest that the underlying mechanisms whereby C/EBP β deletion in hematopoietic-derived cells prevents atherosclerosis may be multifactorial and includes: a) improved mouse serum lipid profiles, specifically with lowered total and LDL cholesterol levels; b) reduced serum pro-inflammatory cytokine levels; and c) decreased macrophage-associated cholesterol accumulation and pro-inflammatory gene expression.

A surprising finding in the present study was a reduction in serum total and LDL cholesterol levels in C/EBP $\beta^{-/-}$ \rightarrow ApoE $^{-/-}$ HF/HC mice despite no change in HDL cholesterol. Our studies showed that C/EBP β in the hematopoietic compartment modifies liver gene expression towards lowering cholesterol, including a dramatic increase in *Lxr/Nr1h3* expression. Resident macrophages (i.e., Kupffer cells) are derived from hematopoietic stem cells and are primarily responsible for the removal of oxidized forms of LDL from plasma. Thus C/EBP β deletion from bone marrow-derived cells, if infiltrated into the liver immune cell population, could play an important role in improved cholesterol balance in the liver [39]. Since the hematopoietic compartment serves as a reservoir for circulating monocytes and invading macrophages, the deletion of C/EBP β in bone marrow may also play a protective role by transcriptional activation of *Lxr/Nr1h3* and the downstream gene *Cyp7a1*, that contribute significantly to reducing lipids by increasing bile acid synthesis [40]. *Lxr/Nr1h3* induction generally reduces hepatic cholesterol content by inducing reverse cholesterol transport, increasing bile acid production, and inhibiting intestinal cholesterol absorption, but does so at the expense of increases in lipogenesis resulting in hypertriglyceridemia and liver steatosis [41]. Notably, our results show that C/EBP β deletion, specifically in the hematopoietic compartment, induces liver *Lxr/Nr1h3* and

reduces cholesterol without the added complication of liver steatosis. These data suggest that C/EBP β deletion may hold promise for the treatment of lipid disorders, as well as preventing excess hepatic glucose production as shown previously in *Lepr^{db/db}* mouse models [5].

The present study also demonstrated higher expression of *Gpbar1/Tgr5* with bone marrow C/EBP β deletion in ApoE^{-/-} mice on HF/HC diet. The *Gpbar1/Tgr5* receptor is highly expressed in hepatocytes and in macrophage cells as well as in other immune cells [42]. A well-defined function of *Gpbar1/Tgr5* is its potent anti-inflammatory effect in the liver [43]. The activation of *Gpbar1/Tgr5* in LDL^{-/-} mice prevented atheroma development by reducing inflammation and lipid accumulation [44]. Thus the increased *Gpbar1/Tgr5* found in the present study suggests that it may partly be responsible for the reduction of cholesterol and atherosclerosis development in C/EBP β bone marrow-deficient ApoE^{-/-} mice.

Because alterations in the lipoprotein profiles might contribute to the beneficial effect of C/EBP β deletion on the aortic lesions in ApoE^{-/-} mouse, we examined the direct effect of C/EBP β knock-down in RAW264.7 macrophage cells in response to oxLDL-induced foam cell formation. Our study showed that deletion of C/EBP β in RAW264.7 macrophage cells not only prevented macrophage foam cells but also attenuated inflammatory cytokine secretion in the medium. Therefore, it is likely that C/EBP β deletion in macrophages (regardless of the source) may induce anti-atherogenic effects in atherosclerotic lesion by preventing lipid accumulation and pro-inflammatory cytokine production. Foam cell formation by cholesterol accumulation in arterial wall macrophages is a crucial event in the progression of atherogenesis that results from the deregulation of the balance between cholesterol influx and cholesterol efflux [37]. This balance depends on limiting cholesterol inflow through scavenger receptors, as well as maintaining outflow through reverse cholesterol transport, the transport of excess cholesterol from peripheral tissues, including cholesterol laden macrophages in vessel walls, to the liver for excretion [45,46]. Our results showing moderate increase in *Abcg1* gene expression in C/EBP β -depleted RAW macrophage cells suggests an increase in cholesterol efflux which may in part be responsible for the reduction in lipid accumulation in macrophages. However, the molecular signaling pathway(s) involved in C/EBP β -mediated regulation of foam cell formation, macrophage recruitment, or tissue proliferation is not clear and studies are underway in our lab to clarify this point.

In addition to the novelty of our findings, our study is not without limitations. One of these is the small sample size ($n = 4-5$ /group). While more mice would increase the power of some of the findings, we have clearly demonstrated a pronounced change of parameters associated with the progression of atherosclerosis in C/EBP β ^{-/-} \rightarrow ApoE^{-/-} HF/HC mice compared with WT \rightarrow ApoE^{-/-} HF/HC mice and we have been conservative in our interpretation. The other limitation is that reconstitution of the ApoE^{-/-} mice with WT bone marrow cells may compensate for the absence of ApoE and therefore can attenuate the complications and pathogenesis of atherosclerosis in ApoE^{-/-} mice [47]. Future studies should include a C/EBP β ^{-/-}/ApoE^{-/-} double knockout mouse to further clarify C/EBP β 's mechanism in the regulation of atherosclerosis. Further, because RAW246.7 cells may not completely mimic tissue macrophages, these results bear repeating with isolated tissue macrophages from these mice.

In summary, C/EBP β deletion, specifically in bone marrow-derived cells, reduced atherogenic lipid levels, total and LDL cholesterol in serum, suppressed inflammatory cytokines in the serum and significantly decreased atherosclerotic lesion formation in the aortic sinus of ApoE $^{-/-}$ mice, despite no differences in body weight. In vitro studies reveal that shRNA-mediated deletion of C/EBP β in RAW macrophage cells attenuated oxLDL-mediated induction of foam cell formation, along with a moderate increase in *Abcg1* gene expression and reduction in secretion of inflammatory cytokines in medium. Conservatively, these results indicate that C/EBP β might be a crucial regulator of diet-induced inflammation, hyperlipidemia, and atherosclerosis. Thus inhibition of C/EBP β might be an important therapeutic strategy for preventing atherosclerosis. Further studies in the time course and molecular events triggered by C/EBP β in the macrophage appear warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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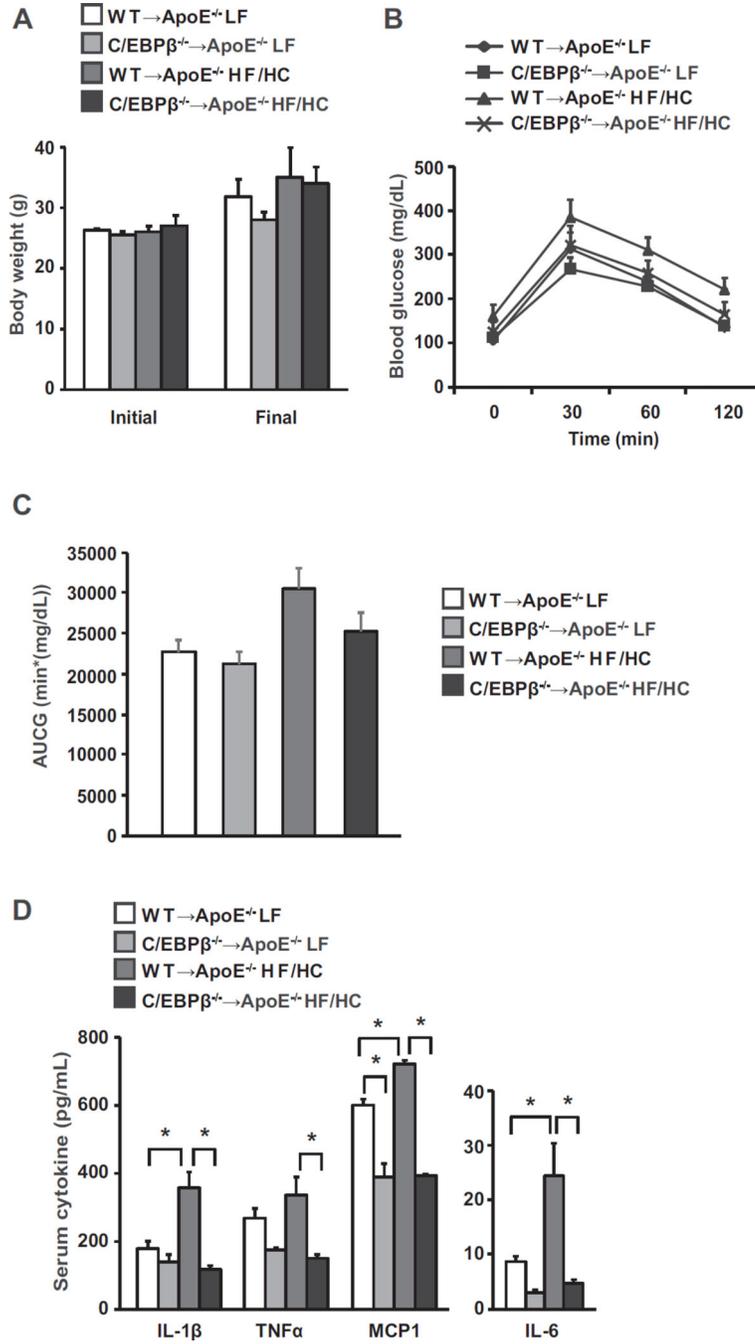


Fig. 1. ApoE^{-/-} mice transplanted with WT or C/EBPβ^{-/-} bone marrow cells were fed either a LF or high fat/high cholesterol (HF/HC) diet for 11 weeks as described in Materials and Methods (*n* = 4–5 per group). Initial and final body weight (A) and GTT results (B). C: AUC of glucose. GTT values were analyzed by two-way repeated measures ANOVA. Data are presented as mean ± SEM. D: Inflammatory cytokines in serum. Data are presented as mean ± SEM. **P* < 0.05 by two-way ANOVA followed by Tukey's post hoc test.

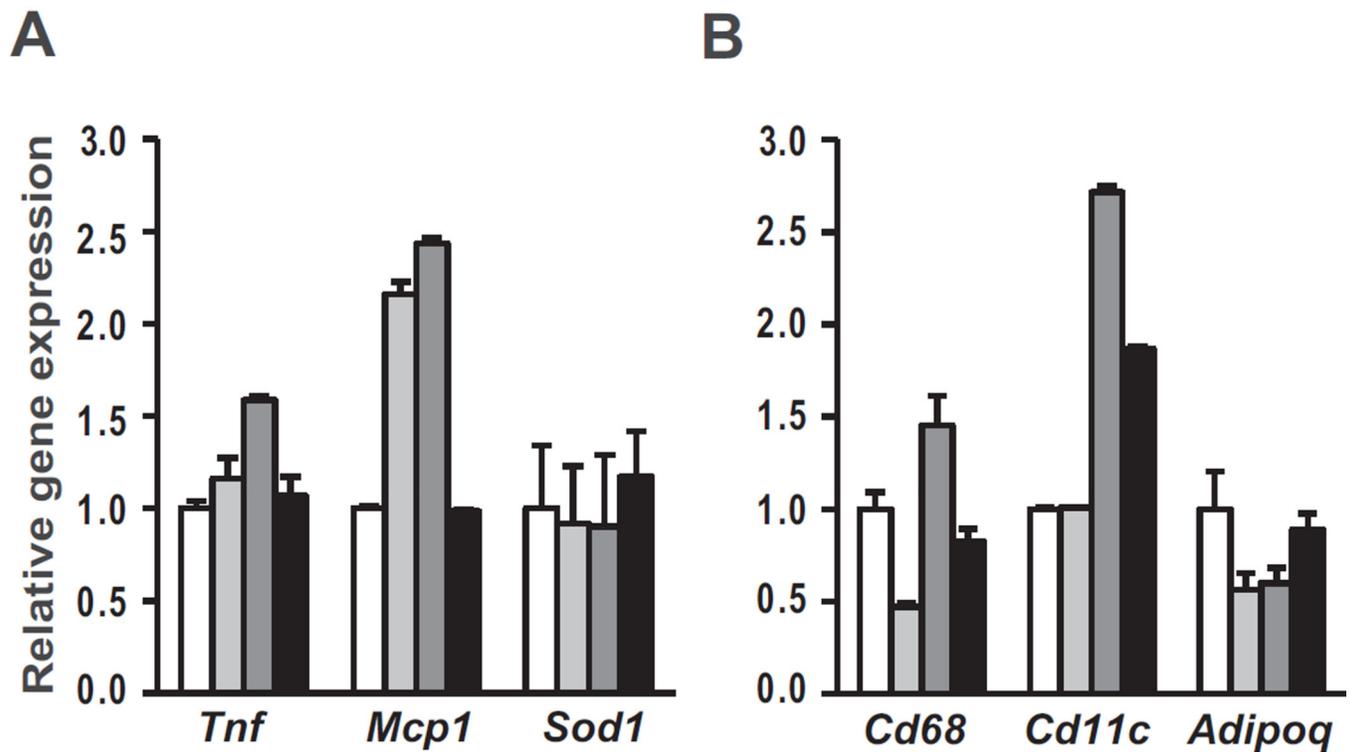
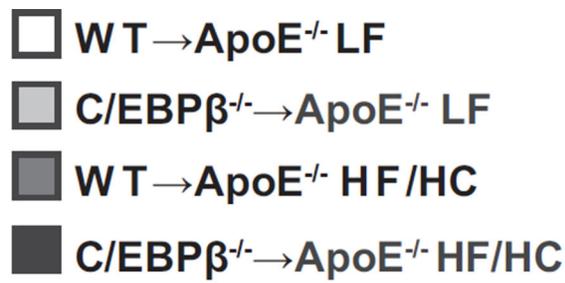
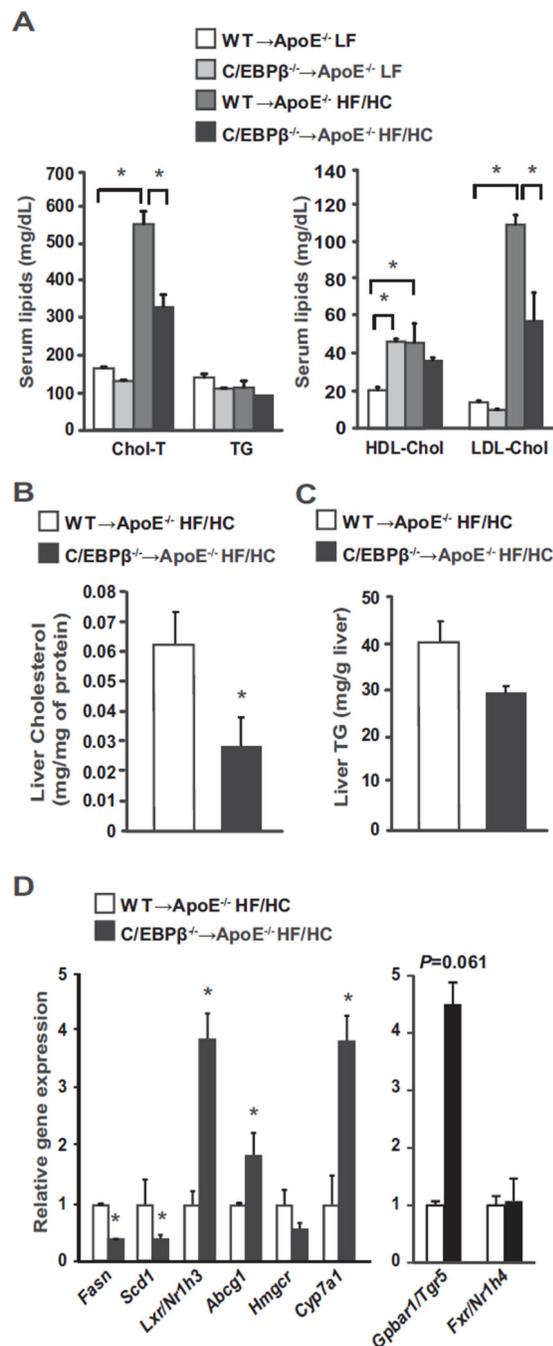


Fig. 2. Gene expression analyzed by qPCR in epididymal adipose tissue from ApoE^{-/-} mice reconstituted with C/EBP β ^{-/-} or WT bone marrow cells (A-B; $n = 4$ per group). Data are presented as mean \pm SEM and analyzed by two-way ANOVA followed by Tukey's post hoc test.

**Fig. 3.**

Lipoprotein profiles and gene expression in liver from ApoE^{-/-} mice transplanted with WT or C/EBPβ^{-/-} bone marrow cells ($n = 4-5$ per group). A: Serum lipid analysis for total cholesterol (Chol-T), triglyceride (TG), HDL cholesterol (HDL-Chol) and LDL cholesterol (LDL-Chol). Data are presented as mean \pm SEM. * $P < 0.05$ by two-way ANOVA followed by Tukey's post hoc test. Cholesterol (B) and triglyceride (TG; C) levels in liver. * $P < 0.05$ by Student's t tests. D: Gene analyses in liver tissue. B–D: Data are presented as mean \pm SEM. * $P < 0.05$ versus WT \rightarrow ApoE^{-/-} HF/HC group as tested by Student's t -test.

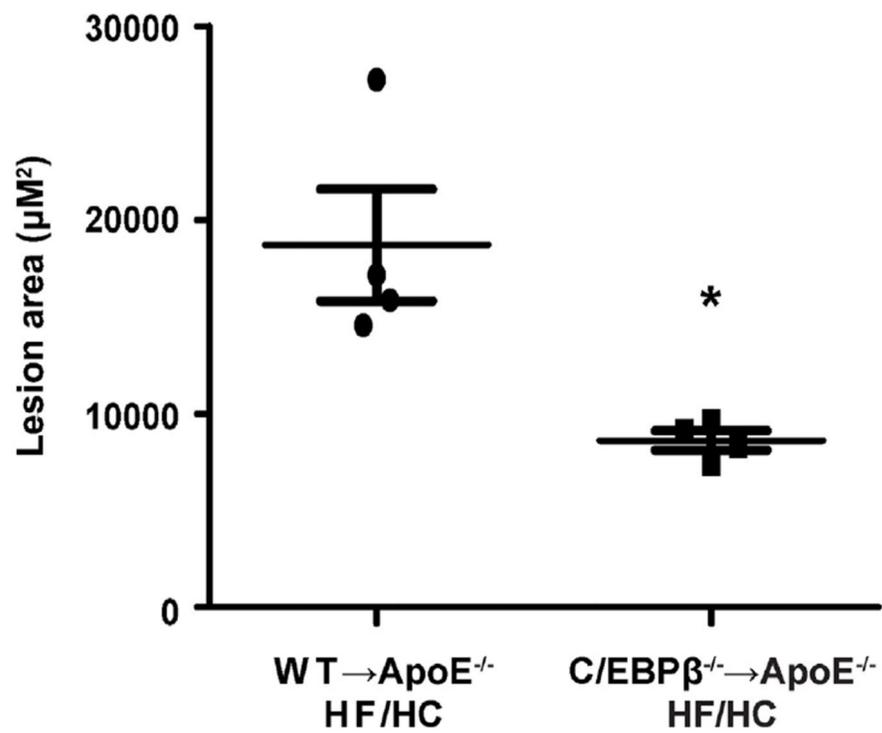
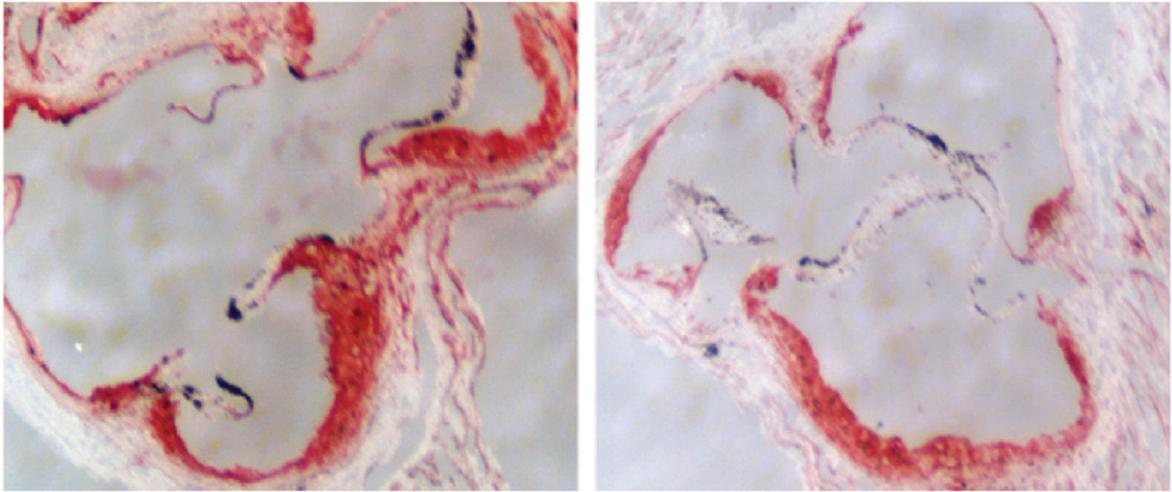
WT → ApoE^{-/-} HF/HCC/EBPβ^{-/-} → ApoE^{-/-} HF/HC

Fig. 4. Oil Red O staining of aortic sinuses and quantitation of lesion area ($n = 4$ per group). * $P < 0.05$ versus WT → ApoE^{-/-} HF/HC group as tested by Student's t -test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

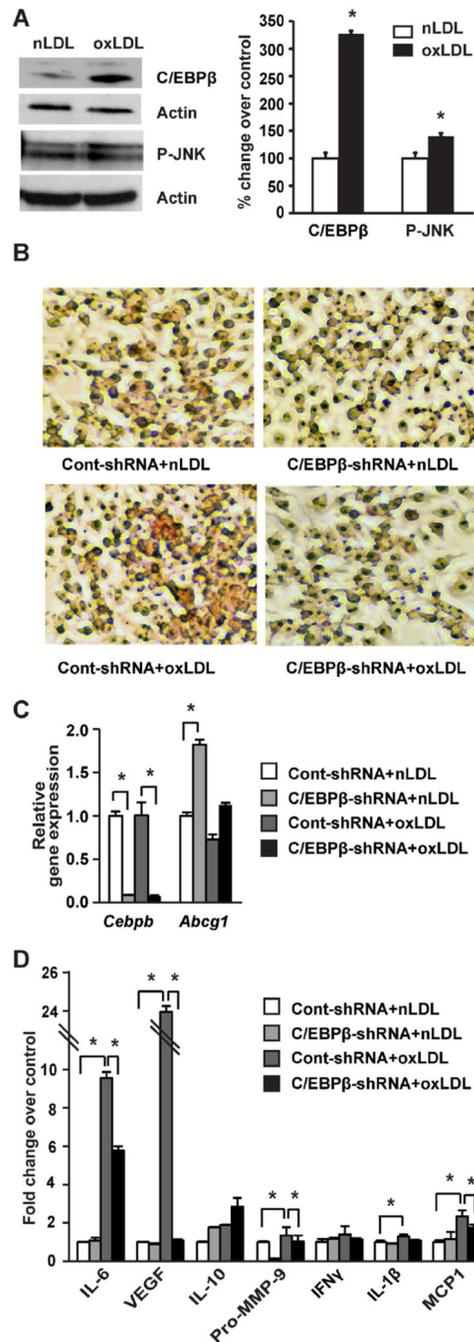


Fig. 5. RAW264.7 macrophage cell treatment with oxLDL and effects of shRNA knockdown of C/EBPβ. **A:** RAW264.7 cells were treated with nLDL or oxLDL (20 μg/mL) for 24 h ($n = 3$ per experiment). Immunoblots and densitometric values for C/EBPβ (in nuclear fraction) and P-JNK (in cytosolic extract), with representative blots shown. Data represent the mean \pm SEM, expressed as percent change over control after normalizing to actin. $*P < 0.05$ versus nLDL group as tested by Student t -test. **B–D:** RAW macrophage cells were transduced with control-shRNA (50 pfu/cell) or C/EBPβ shRNA (50 pfu/cell) for 24 h followed by treatment

with nLDL or oxLDL (20 $\mu\text{g}/\text{mL}$) for an additional 24 h ($n = 3$ per experiment). B: Cells were fixed and stained with Oil Red O to detect lipid accumulation. C: Gene expression analysis by qPCR in RAW264.7 macrophage cells. Data are presented as mean \pm SEM. $*P < 0.05$ as tested by two-way ANOVA followed by Tukey's post hoc test. D: Protein array data using conditioned medium collected from RAW cell experiments. Data are presented as mean \pm SEM. $*P < 0.05$ as tested by two-way ANOVA followed by Tukey's post hoc test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)