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Krüppel-like factor 14 deletion in myeloid cells accelerates atherosclerotic lesion development

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Aims Atherosclerosis is the dominant pathologic basis of many cardiovascular diseases. Large genome-wide association studies have identified that single-nucleotide polymorphisms proximal to Krüppel-like factor 14 (KLF14), a member of the zinc finger family of transcription factors, are associated with higher cardiovascular risks. Macrophage dysfunction contributes to atherosclerosis development and has been recognized as a potential therapeutic target for treating many cardiovascular diseases. Herein, we address the biologic function of KLF14 in macrophages and its role during the development of atherosclerosis.

Methods and results KLF14 expression was markedly decreased in cholesterol loaded foam cells, and overexpression of KLF14 significantly increased cholesterol efflux and inhibited the inflammatory response in macrophages. We generated myeloid cell-selective *Klf14* knockout (*Klf14*^{LysM}) mice in the ApoE$^{-/-}$ background for the atherosclerosis study. *Klf14*^{LysM} *ApoE* $^{-/-}$ and litter-mate control mice (*Klf14*$^{fl/fl}$*ApoE*$^{-/-}$) were placed on the Western Diet for 12 weeks to induce atherosclerosis. Macrophage *Klf14* deficiency resulted in increased atherosclerosis development without affecting the plasma lipid profiles. *Klf14*-deficient peritoneal macrophages showed significantly reduced cholesterol efflux resulting in increased lipid accumulation and exacerbated inflammatory response. Mechanistically, KLF14 upregulates the expression of a key cholesterol efflux transporter, ABCA1 (ATP-binding cassette transporter A1), while it suppresses the expression of several critical components of the inflammatory cascade. In macrophages, activation of KLF14 by its activator, perhexiline, a drug clinically used to treat angina, significantly inhibited the inflammatory response and increased cholesterol efflux in a KLF14-dependent manner in macrophages without triggering hepatic lipogenesis.

Conclusions This study provides insights into the anti-atherosclerotic effects of myeloid KLF14 through promoting cholesterol efflux and suppressing the inflammatory response. Activation of KLF14 may represent a potential new therapeutic approach to prevent or treat atherosclerosis.

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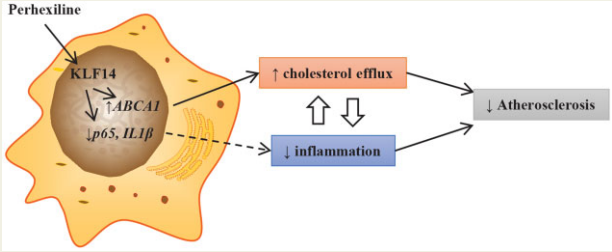
Graphical Abstract



Keyword Krüppel-like factor • Atherosclerosis• Cholesterol efflux • Inflammation

1. Introduction

Atherosclerosis, a chronic and progressive vascular disease, is an important cause of cardiovascular diseases worldwide. It is characterised by an accumulation of excess cholesterol and a chronic inflammatory process in the arterial wall, which triggers acute coronary syndrome when the atherosclerotic plaque ruptures.[1] High low-density lipoprotein cholesterol (LDL-C) level is the strongest and best-studied risk factor for atherosclerosis development.[2] However, despite advances in therapeutics aimed at lowering LDL-C level, residual cardiovascular risks remain in coronary heart disease patients.[3]

Among the protective mechanisms against atherosclerosis, reverse cholesterol transport (RCT) represents an important protective pathway, in which excessive cholesterol from peripheral tissues, including foam cells in the atherosclerotic plaques, is moved by high-density lipoprotein (HDL) particles to the liver and finally excreted into the faeces.[4] As decreased HDL-cholesterol (HDL-C) level is an independent, inverse predictor of cardiovascular risk, raising HDL level was initially proposed to be an operative way to promote atherosclerosis regression. However, many pharmacological approaches to raise HDL levels have not demonstrated an additional beneficial effect on major cardiovascular events in clinical trials. The debate on the HDL hypothesis led to studies showing that low cholesterol efflux capacity of HDL particles is more relevant to cardiovascular disease risks compared with the decreased HLD-C levels.[5] Furthermore, cholesterol efflux is the first and rate-limiting step in RCT, in which free cholesterol inside the arterial foam cells is transported by the ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1) to circulating cholesterol acceptors. ABCA1 controls the cellular cholesterol efflux to lipid poor ApoA-I/small HDL particles, and ABCG1 facilitates cholesterol efflux to mature HDL particles.[4,6–8] In macrophage, liver X receptors (LXRs), LXRα and LXRβ, play pivotal roles in regulating the expression of ABCA1 and ABCG1. Synthetic LXR agonists attenuate atherosclerosis by upregulating ABC transporters and inhibiting inflammation in animal models.[9] However, activation of LXRs triggers liver steatosis and hypertriglyceridaemia, limiting the clinical application of LXR agonists for atherosclerosis treatment. The current challenge is to understand the pathways regulating ABCA1 and ABCG1 expression in macrophages and identify novel therapeutic approaches for atherosclerosis treatment.

Krüppel-like factor 14 (KLF14) was identified by genome-wide association studies (GWASs) as showing direct correlations with both cardiovascular disease and HDL-C level.[10] KLF14 belongs to a large zinc-finger transcription family, the Krüppel-like factor family, which regulates a vast number of genes and is involved in many biological processes.[11,12] Previously, we identified the biological function of KLF14 and its transcriptional activator-perhexiline, a therapeutic agent currently in clinical to treat angina in Australia and New Zealand, in lipid metabolism. We demonstrated that KLF14 has strong anti-inflammatory effect in vascular endothelial cells by directly suppressing the expression of nuclear factor-κB p65[13]. We showed that administration of perhexiline significantly inhibited atherosclerotic plaque development, which could not be fully explained by the effect of KLF14 on raising HDL-C because the HDL-C levels remain intrinsically very low in *ApoE*$^{-/-}$ mice after 12 weeks of western diet challenge.[14] Considering that macrophages are key integrators of the inflammatory response and cholesterol accumulation in the atherosclerotic plaques, in this study, we explore the protective effects of macrophage KLF14 on atherosclerosis. Here, we report that deficiency of KLF14 in macrophages increased the inflammatory response and decreased cholesterol efflux by regulating the expression of IL1β, p65, and ABCA1. Consequently, atherosclerotic plaque lesions were also increased in macrophage-selective *Klf14*-deficient (*Klf14*^{LysM}*ApoE*$^{-/-}$) mice.

2. Methods

2.1 Animals

All animal experiments in this study are approved by the Institutional Animal Care and Use Committee (IACUC) and meet the standards for the animal care and use as established by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. *Klf14* floxed mice were previous generated in our laboratory.[14] *Klf14*$^{fl/fl}$ mice were crossed with *LysM*-Cre transgenic mice (Stock No: 004781 from the Jackson Laboratory) to obtain myeloid cell-selective *Klf14* knockout (KO) mice (*Klf14*^{LysM}). Subsequently, *Klf14*^{LysM} mice were crossed with *ApoE*$^{-/-}$ mice (Stock No: 002052 from the Jackson Laboratory) to generate *Klf14*^{LysM}ApoE$^{-/-}$ mice for the atherosclerosis study. The litter-mate LysM-Cre-negative *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ mice were used as controls. *LXRα*

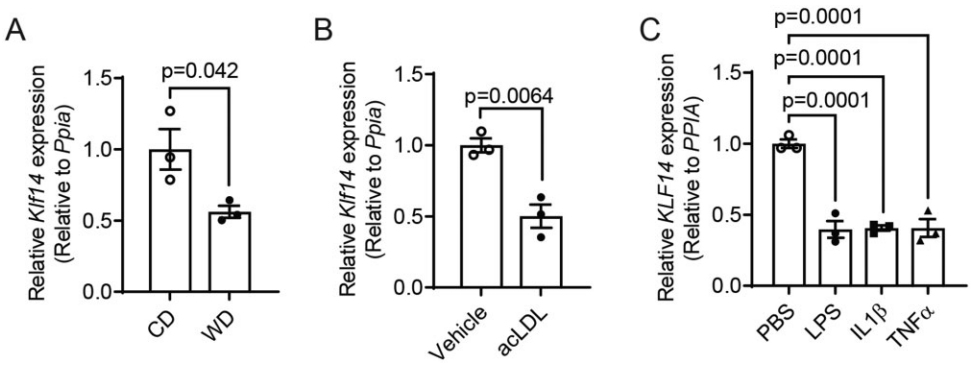


Figure 1 KLF14 expression was significantly decreased under pathological stimuli. (*A*) PMs were isolated from C57BL/6J mice fed a regular chow diet (CD) or western diet (WD), and *Klf14* mRNA levels were measured [three biological replicates for three independent experiments (triplet wells)]. (*B*) PMs were isolated from C57BL/6J mice and treated with acLDL (50 μg/mL) for 24 h, and *Klf14* mRNA levels were measured [three biological replicates for three independent experiments (triplet wells)]. (*C*) THP-1-derived macrophages were treated with PBS, LPS (100 ng/mL), IL-1β (20 ng/mL), or TNFα (20 ng/mL) for 4 h. KLF14 mRNA levels were measured [three independent experiments (triplet wells)]. Data are presented as the mean ± SEM and analysed by two-tailed Student's *t*-test for (*A*,*B*) and one-way ANOVA with Dunnett's correction for (*C*).

knockout mice used for peritoneal macrophages isolation were commercially available (Stock No. 013762 from Jackson Laboratory).

2.2 Atherosclerosis model and analysis of atherosclerotic lesions

Klf14$^{fl/fl}$*ApoE*$^{-/-}$ mice and *Klf14*^{LysM}*ApoE*$^{-/-}$ mice (both female and male) were used for the atherosclerosis study. At the end of the experiments, animals were fasted for 4 h and then euthanized by CO_2 overdose. Two quantitative methods were used to analyse the areas of atherosclerotic lesions, as described in our previous study.[14] First, *en face* analysis of the atherosclerotic lesions uses the whole aorta trees: periaortic adipose tissue was carefully removed, and the aortic trees were stained with Oil Red O (ORO) solution and opened longitudinally. The percentage of plaque areas stained by ORO to the total luminal surface area was quantified. Second, three to five locations of the cross-sections (at 80-μm intervals) from the aortic sinus region were examined. The largest plaques of the three valve leaflets were used for morphological analysis. ImageJ analysis software was used to quantify the indicated areas (http://imagej.nih.gov/ij/). All morphometric analyses were performed in a double-blinded manner.

2.3 Statistics

Data are presented as mean ± SEM. Each experiment was performed with at least three experimental replicates and independently repeated at least three times with similar results. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc.). When data passed tests for normality and equal variance, statistical comparisons and analyses between two groups were performed by two-tailed, unpaired Student's *t*-test. Comparisons among three groups or more were analysed with one-way ANOVA with Dunnett's correction or two-way ANOVA with Tukey correction. If the data did not pass those tests, Mann–Whitney was used to compare two groups, and Kruskal–Wallis test followed by a two-stage step-up method of Benjamini, Krieger, and Yekutieli were used for >2 groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 The expression of KLF14 was significantly reduced under pro-atherogenic conditions

The macrophage is one of the most critical cell types that respond to pro-inflammatory stimuli and lipid accumulation during atherosclerosis development.[15] To understand whether KLF14 expression was regulated specifically in macrophages under pathological conditions, we first used a chronic inflammatory animal model by subjecting wild-type (WT) C57BL/6J male mice to challenge with Western diet (WD) for 12 weeks, and isolated primary peritoneal macrophages (PMs) at the end of the treatment. The mRNA level of *Klf14* was significantly reduced in PMs from WD-fed mice compared to a standard chow diet (CD)-fed mice (*Figure 1A*). Next, we induced foam cells[16,17] using PMs isolated from CD-fed WT C57BL/6J mice by acetylated LDL (acLDL) loading, confirmed by an increase of intracellular cholesterol content (Supplementary material online, *Figure S1*), and found that *Klf14* expression was also significantly decreased (*Figure 1B*). Consistent with our previous findings in endothelial cells,[13] *Klf14* expression was also reduced upon pro-inflammatory stimuli (LPS, IL-1β, and TNFα) in THP-1-derived macrophages (*Figure 1C* and Supplementary material online, *Figure S2*). These results indicate that lipid loading and inflammation may lead to *Klf14* downregulation and, therefore, accelerate atherosclerosis progression.

3.2 Klf14 overexpression significantly alleviated lipid accumulation by promoting cholesterol efflux in macrophages

To understand the potential biological functions of KLF14 in macrophages, we first utilised a gain-of-function strategy to determine how KLF14 regulates cholesterol metabolism in these cells. Oxidised LDL (oxLDL) treatment significantly induced lipid accumulation in control THP-1-derived macrophages (AdLacZ-treated cells as control), while KLF14 overexpression significantly decreased lipid accumulation (*Figure 2A*), suggesting that KLF14 may be protective against lipid

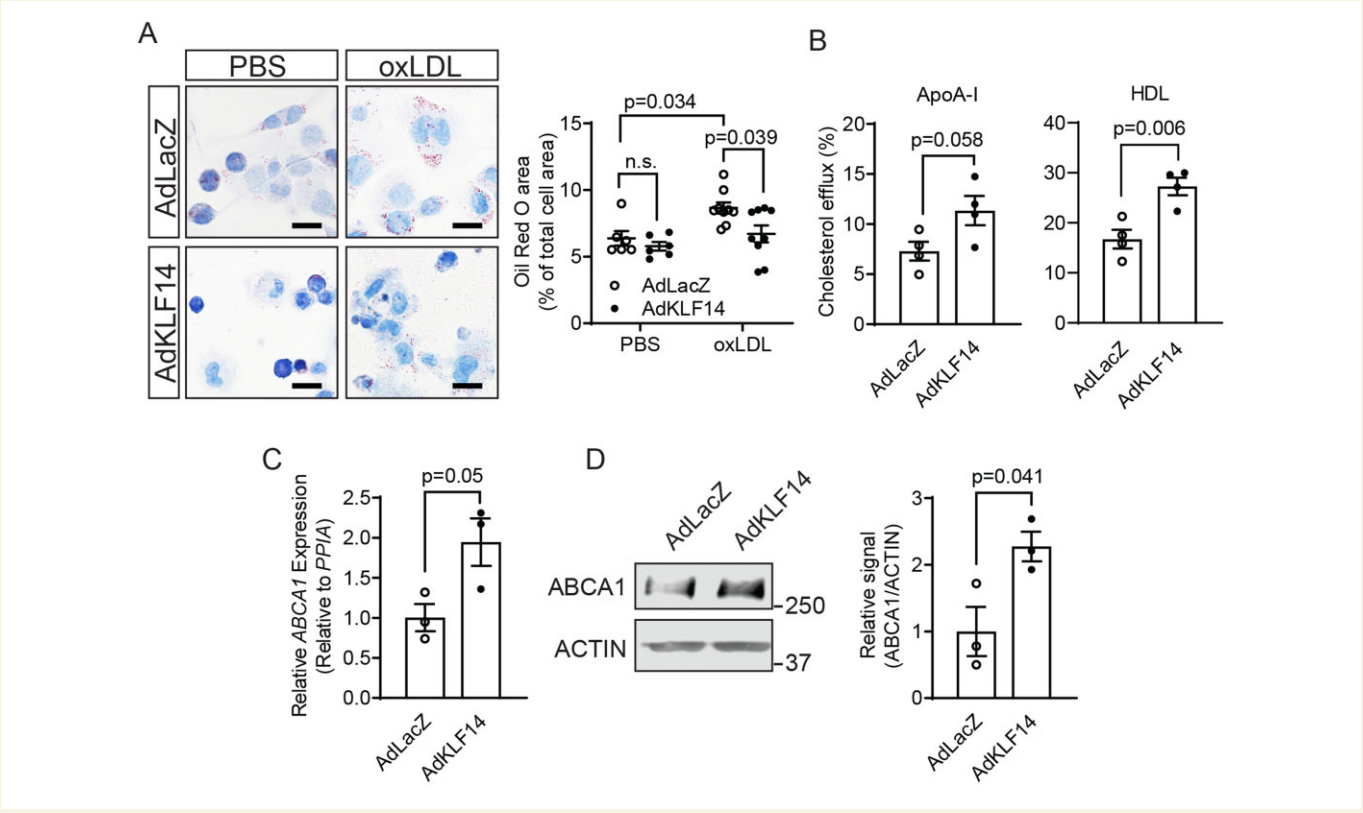


Figure 2 KLF14 overexpression showed decreased lipid accumulation, increased cholesterol efflux, and ABCA1 expression in macrophages. THP-1 cells were differentiated to macrophages by treatment with PMA (100 ng/mL) for 72 h. Adenovirus-mediated overexpression was used as indicated. (*A*) AdLacZ- or AdKLF14-overexpressing macrophages were treated with PBS or oxLDL (25 μg/mL) for 24 h. Neutral lipid in the cells was stained by ORO staining and quantified by ORO area/total cell area [six technical replicates for the PBS group and nine technical replicates for the oxLDL group] Bar = 20μm. (*B*) Cholesterol efflux was measured in AdLacZ- or AdKLF14-overexpressing macrophages with ApoA-I or HDL as acceptors [four independent experiments (triplet wells)]. (*C*) The mRNA level and (*D*) protein levels of ABCA1 were determined by western blotting [three independent experiments (triplet wells)]. Data are presented as the mean ± SEM and analysed by two-tailed Student's *t*-test for (*B–D*) or two-way ANOVA with Tukey correction for (*A*).

accumulation. Next, we determined the effect of KLF14 on cholesterol efflux and found that overexpression of KLF14 significantly increased cholesterol efflux to both ApoA-I and HDL particles as acceptors in THP-1-derived macrophages (approximately 1.5-fold for ApoA-I and 1.6-fold for HDL particles) (*Figure 2B*). In cholesterol loaded foam cells, the cholesterol transporters ABCA1 and ABCG1 play critical roles in the regulation of the net cholesterol efflux to lipid poor ApoA-I and HDL particles.[6–8] Indeed, the expression of ABCA1 was increased by KLF14 overexpression at both the mRNA (*Figure 2C*) and protein levels (*Figure 2D*) compared with AdLacZ-treated macrophages. Our findings suggest that macrophage KLF14 regulates cholesterol efflux by modulation of ABCA1 expression, contributing to the decreased lipid accumulation in macrophages.

3.3 *Klf14*-deficient PMs showed increased lipid accumulation and decreased cholesterol efflux and ABCA1 expression

Next, we asked whether *Klf14* is necessary for protecting against lipid accumulation in macrophages. We confirmed the genomic knockout of *Klf14* in PMs by using primers flanking the single exon of the *Klf14* gene in *Klf14*^{LysM}*ApoE*$^{-/-}$ mice (Supplementary material online, *Figure S3A*). In PMs from *Klf14*^{LysM}*ApoE*$^{-/-}$ mice, both mRNA and protein levels of KLF14 were significantly decreased (Supplementary material online, *Figure S3B* and *C*). In PMs isolated from *Klf14*^{LysM}*ApoE*$^{-/-}$ mice, *Klf14* deficiency significantly increased intracellular lipid content upon oxLDL treatment compared to PMs from *Klf14*$^{fl/fl}$ *ApoE*$^{-/-}$ (*Figure 3A*), indicating a loss of the protective effect against foam cell formation in *Klf14* deficient macrophages. Then, we determined if *Klf14* deficiency also leads to a decreased cholesterol efflux in macrophages. Under basal conditions (without LXR agonist-induced expression of ABCA1 and ABCG1), *Klf14*-deficient PMs showed significantly lower cholesterol efflux to ApoA-I as acceptor (approximately reduced by 39.1%), but not to HDL particles (*Figure 3B*), indicating an important regulatory effect of KLF14 on the expression of ABCA1 in macrophages. Upon treatment with TO901317 (TO), an LXR agonist, to induce the expression of ABCA1 and ABCG1, cholesterol efflux was significantly increased in PMs from litter-mate control mice. However, this effect was significantly blocked in PMs isolated from *Klf14* deficient mice (*Figure 3B*), indicating an important role of KLF14 in modulating cholesterol efflux.

In PMs isolated from control mice, the mRNA level of *Abca1* was upregulated by cAMP-mediated transcriptional regulation, but the expression of *Abca1* was significantly blocked in *Klf14*-deficient PMs (*Figure 3C*). However, the expression of *Abcg1* increased in

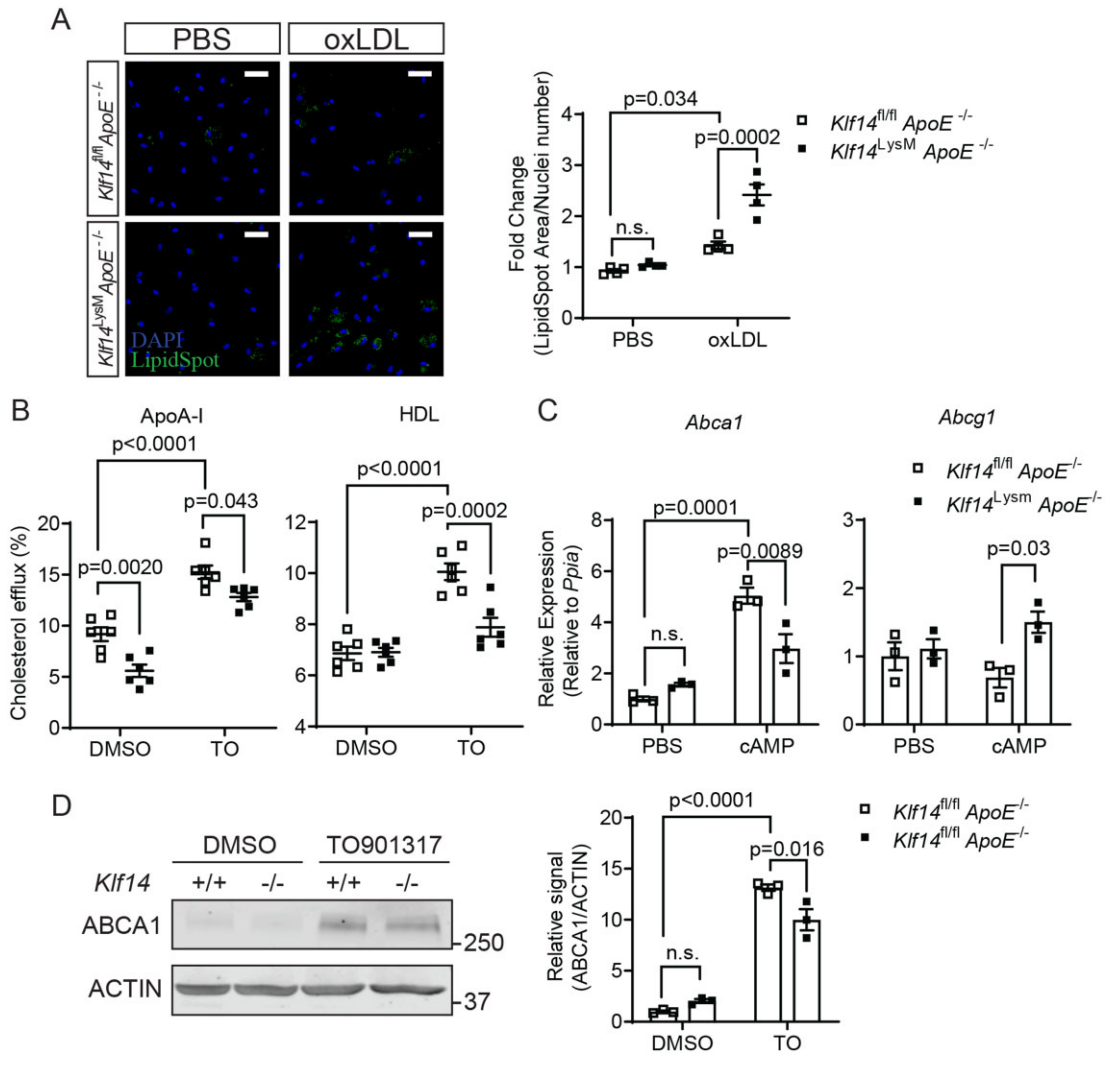


Figure 3 *Klf14*-deficient PMs showed increased lipid accumulation, decreased cholesterol efflux, and ABCA1 expression. PMs were isolated from *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ and *Klf14*^{LysM}*ApoE*$^{-/-}$ mice and given indicated stimuli. (*A*) PMs were treated with PBS or oxLDL (25 μg/mL) for 24 h. Neutral lipid in the cell was stained by LipidSpot™ and quantified by LipidSpot signal/cell counts [PMs differentiated from 3–5 mice, four technical replicates]. Blue: DAPI. Green: LipidSpot. Bar = 20 μm. (*B*) The cholesterol efflux mediated by ApoA-I and HDL was determined in control and *Klf14*-deficient PMs under treatment with DMSO or TO901317 (TO, 0.1 μM) [PMs differentiated from 3–5 mice, six independent experiments (triplet wells)]. (*C*) *Abca1* and *Abcg1* expressions were determined in PMs treated with PBS or cAMP (3 mM) for 24 h [PMs differentiated from 3–5 mice, three independent experiments (triplet wells)]. (*D*) *Klf14*-deficient and control PMs were treated with DMSO or TO901317 (TO, 0.1 μM) for 24 h. Protein levels of ABCA1 were determined and quantified [PMs differentiated from 3–5 mice, three independent experiments (triplet wells)]. Data are presented as the mean ± SEM and analysed by two-way ANOVA with Tukey correction. n.s., not significant.

Klf14-deficient PMs after cAMP induction, which may be caused by a compensatory effect[18] (*Figure 3C*). *Klf14* deficiency also significantly impaired the up-regulation of ABCA1 expression by activating the LXR pathway with TO901317 (*Figure 3D*), which is consistent with the impaired cholesterol efflux to ApoA-I in *Figure 3C*. Considering that increased lipid uptake also contributes to lipid accumulation, the expression of receptors involved in LDL or oxLDL uptake was determined. The expression of *Ldlr* and *Cd36* was higher in *Klf14*-deficient PMs, while *Sra1, Srb1, Lox1* were not changed (Supplementary material online, *Figure S4A*). However, Dil-labelled oxLDL uptake showed no difference between *Klf14*-deficient and control PMs (Supplementary material online, *Figure S4B*), indicating that KLF14 may not regulate lipid uptake in macrophages. Taken together, these data indicate that KLF14 protects against lipid accumulation and maintains cholesterol efflux by regulating *Abca1* expression in macrophages.

3.4 Klf14 overexpression inhibits the pro-inflammatory response in macrophages

Cholesterol crystals (CCs) can be found in early atherosclerotic lesions, and CCs-induced inflammation is critical in all stages of the atherosclerotic process. Consistent with previous studies,[19–21] CCs treatment triggered an inflammatory response in macrophages. In

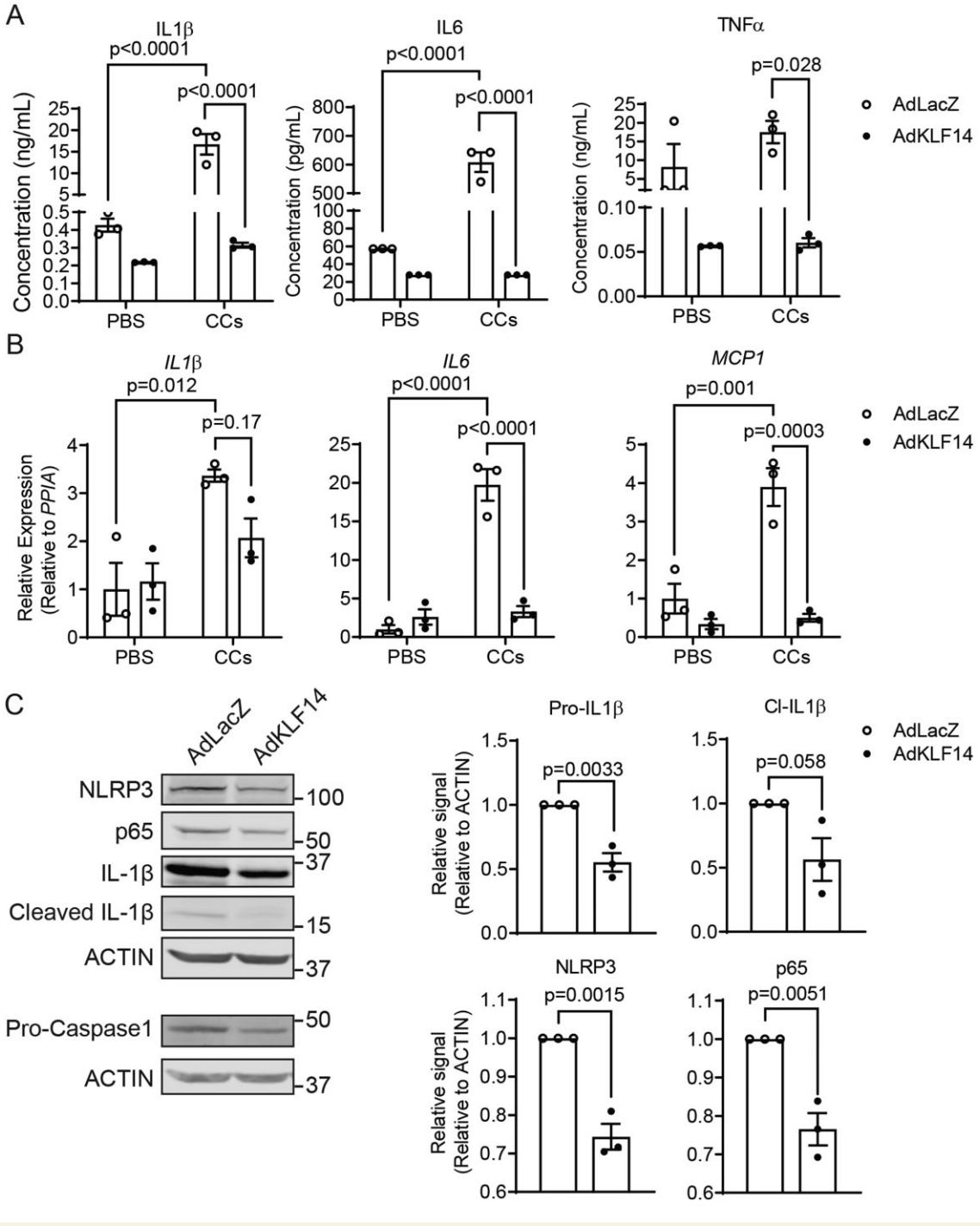


Figure 4 KLF14 overexpression inhibits inflammatory responses in macrophages. THP-1-derived macrophages were transfected with AdLacZ or AdKLF14 for 24 h. After 72 h of differentiation in the presence of 100 nM PMA, THP-1-derived macrophages were treated with cholesterol crystals (CCs, 200 μg/mL) for 24 h, and cell lysates or supernatants were collected at the endpoint. (*A*,*B*) The cytokines release into the supernatant (*A*) and the mRNA level of the indicated cytokines (*B*) were determined and quantified [three independent experiments (triplet wells)]. (*C*) Protein levels of NLRP3, p65, IL-1β, Pro-IL1β, and Pro-Caspase1 were determined and quantified [three independent experiments (triplet wells)]. Data are presented as the mean ± SEM and analysed by two-tailed Student's *t*-test for (*C*) or two-way ANOVA with Tukey correction for (*A*,*B*).

THP1-derived macrophages, KLF14 overexpression significantly reduced the levels of IL-1β, IL-6, TNFα released into supernatant (*Figure 4A*) and downregulated the mRNA expression of pro-inflammatory chemokines, such as *IL1β, IL6,* and *MCP1* (*Figure 4B*). Intriguingly, we found that the expression of inflammasome components (NLRP3 and Pro-Caspase1) and a key effector in the NF-κB pathway (p65) were significantly suppressed by KLF14 (*Figure 4C*). These findings demonstrate that KLF14 suppressed the inflammatory signalling cascade in response to CCs stimulation, suggesting a protective role of KLF14 in atherosclerosis development.

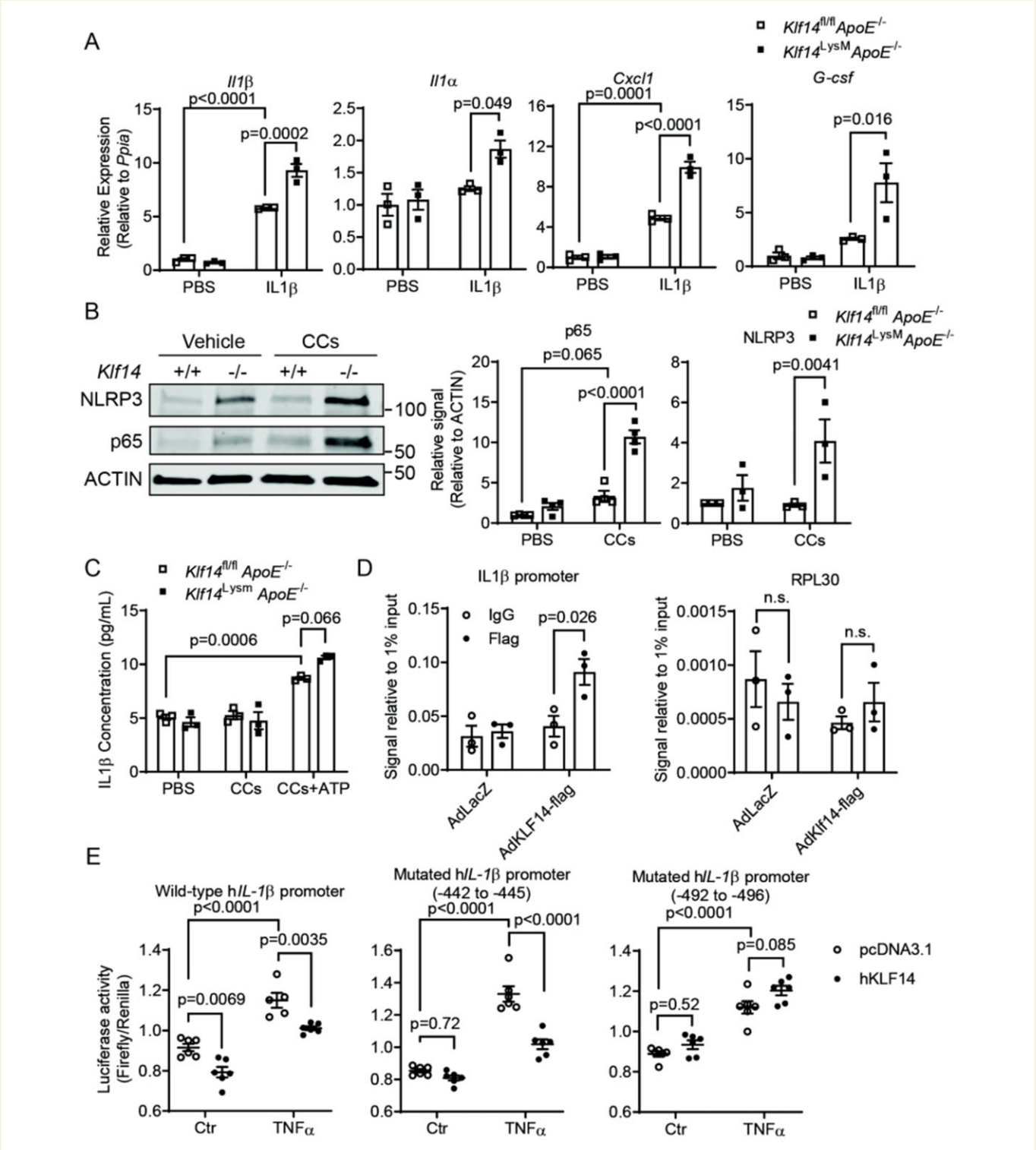


Figure 5 *Klf14* deficiency increases the inflammatory response in macrophages. (*A*,*B*) PMs were isolated from *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ and *Klf14*^{LysM}*ApoE*$^{-/-}$ mice. (*A*) PMs were treated with IL-1β (20 ng/mL) for 4 h. The mRNA level of pro-inflammatory cytokines was determined [PMs differentiated from 3–5 mice, 3 independent experiments (triplet wells)] (*B*) PMs were treated with PBS or cholesterol crystals (CCs, 200 μg/mL) for 24 h. Protein levels of NLRP3 and p65 were determined and quantified. [PMs differentiated from 3–5 mice, 3 independent experiments (triplet wells)] (*C*) PMs were treated with PBS, CCs only (200 μg/mL, 24 h) or CCs+ATP (ATP at 5 mM, CCs 24 h + ATP 1 hr). IL-1β released into the supernatant was measured by ELISA [PMs differentiated from 3–5 mice, three independent experiments (triplet wells)]. (*D*) ChIP assay was conducted in THP-1 cells overexpressing KLF14-flag. Amplification of potential binding site in *IL1β* promoter and the control region of *RPL30* were done by qPCR [three independent experiments]. (*E*) -946/+45bp of the wild-type and mutated human *IL1β* promoter-driven luciferase was transfected in AD293 cells. LPS (100 ng/mL) was used for the induction of inflammation [5–6 technical replicates, repeated by three independent experiments]. Data are presented as the mean ± SEM and analysed by two-way ANOVA with Tukey correction.

3.5 *Klf14* deficiency increases the inflammatory response, but not apoptosis in macrophages

Conversely, and in agreement with the findings above, *Klf14*-deficient PMs from *Klf14*^{LysM}*ApoE*$^{-/-}$ mice showed significantly higher response to inflammatory stimuli, evidenced by higher mRNA levels of *Il1β, Il1α, Cxcl1, G-csf* upon stimulus with IL-1β (*Figure 5A*). Considering that bone marrow-derived macrophages (BMDMs) and PMs may have different responses to inflammatory stimuli,[22] we also repeated this experiment using BMDMs isolated and cultured from *Klf14*^{LysM}*ApoE*$^{-/-}$ and control mice and found that BMDMs showed similar results to the PMs (Supplementary material online, *Figure S6*). CCs activated the NLRP3 inflammasome and the NF-κB pathways, represented by increased expression of NLRP3 and p65 (*Figure 5B*). Under basal conditions (in the absence of CCs), *Klf14* deficiency led to a significant increase of NLRP3 and p65 expression, while, in the presence of CCs, *Klf14* deficiency aggravated the pro-inflammatory response by further increasing the p65 protein level (*Figure 5B*, lane 3 and 4). In accordance, IL-1β released from *Klf14*-deficient PMs was also higher after treatment with CCs+ATP (*Figure 5C*). Although *Klf14* deficiency significantly induced M1 macrophage polarisation when challenged with LPS+INFγ (M1 induction) (Supplementary material online, *Figure S7A*), *Klf14* deficiency did not affect the expression of M2 markers (*Fizz1, Ym1*) when cells were treated with IL-4 (M2 induction) (Supplementary material online, *Figure S7B*) and anti-inflammatory cytokine (IL-10) release under co-treatment with CCs or oxLDL (Supplementary material online, *Figure S7C*). The results show that macrophage *Klf14* deficiency increases the inflammatory response.

Additionally, previous reports showed that free cholesterol accumulation in the macrophages could induce macrophage apoptosis,[23] leading to higher plaque vulnerability in advanced atherosclerosis.[24] Therefore, we determined whether *Klf14* deficiency affects macrophage apoptosis. The full-length caspase-3, an effector caspase in apoptosis, was not significantly changed in *Klf14*-deficient PMs than the control under basal condition or cholesterol crystals (CCs) treatment (Supplementary material online, *Figure S5*). Cleaved caspase-3 was not detectable. To determine the activity of caspase-3, we also probed for a downstream target of active caspase-3, PARP1. PARP1 cleavage was increased by CCs stimulation, illustrated by the ratio of cleaved PARP1/full-length PARP1. There was no difference between the two groups (Supplementary material online, *Figure S5*), indicating that *Klf14*-deficiency did not increase apoptosis of macrophages.

Interestingly, we identified two potential KLF binding sites (-442 to -445 and -492 to -496) upstream of the human *IL1β* transcription start site. Chromatin immunoprecipitation (ChIP) assay performed in the THP-1 derived macrophages using anti-Flag antibody or anti-IgG (negative control), the genomic fragment was applied for PCR amplification using a primer set for this region and we found that KLF14 specifically bound to *IL1β* promoter region, but not to the control *RPL30* promoter (*Figure 5D*). Furthermore, we generated a luciferase reporter construct containing the -946 to +45 region of the human *IL1β* promoter. KLF14 significantly inhibited the luciferase activity in both baseline and LPS stimulated conditions (*Figure 5E*). The mutation of the -442 to -445 region did not affect the regulatory effect of KLF14, while the mutation of -492 to -496 abolished the inhibitory effect of KLF14 on *IL1β* transcription, indicating that -492 to -496 (CACCC) is the functional binding site for KLF14 on the human *IL1β* promoter region (*Figure 5E*). Collectively, we demonstrated that KLF14 inhibits the inflammatory response via suppressing p65 expression[13] and direct binding to the *IL1β* promoter to inhibit its transcription.

3.6 Myeloid-selective *Klf14* deficiency accelerates atherosclerosis in the *ApoE*$^{-/-}$ hyperlipidemic mouse model

To investigate the role of myeloid KLF14 in atherosclerosis development, both male and female *Klf14*^{LysM}*ApoE*$^{-/-}$ and litter-mate control *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ mice were placed on an atherogenic WD for 12 weeks. There were no differences in lipid profiles between *Klf14*^{LysM}*ApoE*$^{-/-}$ and *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ control mice at the end of treatment, including triglycerides, total cholesterol, LDL-C, and HDL-C (Supplementary material online, *Figure S8A*). Nonetheless, both male and female *Klf14*^{LysM} *ApoE*$^{-/-}$ mice showed significantly accelerated atherosclerosis development (*Figure 6A*), shown by *en face* Oil Red O (ORO) staining of the aortic tree. Although male and female *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ control mice have similar plaque burden at the endpoint, male *Klf14*^{LysM} *ApoE*$^{-/-}$ mice were more susceptible to atherogenesis, as evidenced by a 79% increase in the atherosclerotic plaque area in the male knockout group, compared to a 25% increase in the female knockout group. We also observed a significantly higher plaque area at the aortic root in male *Klf14*^{LysM}*ApoE*$^{-/-}$ mice than litter-mate control male mice, but no difference between female groups (*Figure 6B*).

Atherosclerotic plaque components are highly heterogeneous. First, we observed a significantly greater Mac2 (a macrophage marker) positive area in *Klf14*^{LysM}*ApoE*$^{-/-}$ male mice, indicating increased macrophage infiltration (*Figure 6C*). Accordingly, we found that macrophage migration was significantly higher in *Klf14*-deficient macrophages under pro-inflammatory stimulation with LPS (Supplementary material online, *Figure S9*). Second, male *Klf14*^{LysM}*ApoE*$^{-/-}$ mice on WD showed significantly higher CCs contents in the plaque determined by polarised light microscopy, suggesting impaired cholesterol efflux in *Klf14*-deficient macrophages caused more free cholesterol accumulation in the atheroma (*Figure 6D*). Unexpectedly, the content of neutral triglycerides and lipids staining with ORO staining showed no significant difference at the aortic root between *Klf14*-deficient and litter-mate control mice (*Figure 6E*). Considering that a 12-week WD challenge induced atheroma may be too advanced to show a difference in cholesterol ester or neutral lipids, we next investigated the lipid accumulation using both *Klf14*^{LysM}*ApoE*$^{-/-}$ and *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ control mice on a CD. *ApoE*$^{-/-}$ mice fed a CD present spontaneous atherosclerosis, which is considered relatively early-stage atherosclerosis compared to the WD-fed *ApoE*$^{-/-}$ mice.[25] Although there was no difference in lipid profiles (Supplementary material online, *Figure S8B*), *Klf14*^{LysM} *ApoE*$^{-/-}$ male mice on CD showed a significantly higher ORO staining area in the atherosclerosis plaques (*Figure 6F*), indicating that myeloid *Klf14* deficiency increases lipid accumulation in the plaque in the early stages of atherosclerosis development. There were no significant differences in lesion area and macrophage infiltration in this spontaneous atherosclerotic model (Supplementary material online, *Figure S10A,B*). Plaque stability and vulnerability were analysed by Masson trichrome staining (plaque fibrosis) and the area of necrotic cores. There was no difference between male myeloid-*Klf14* deficient mice and controls after treatment (Supplementary material online, *Figure S11A,B*). This is inconsistent with the previous finding that apoptosis did not change in CCs-treated macrophages (Supplementary material online, *Figure S5*).

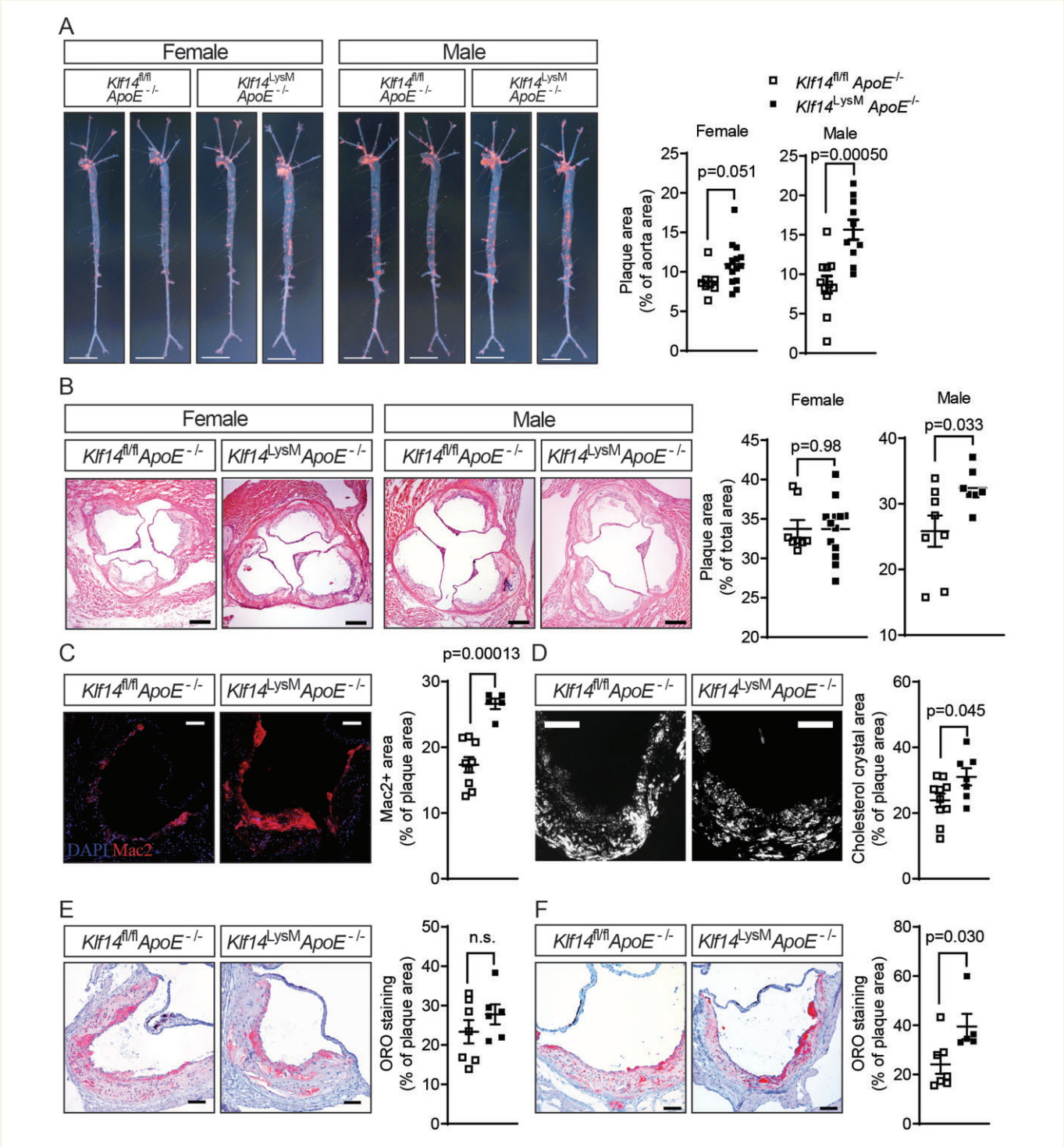


Figure 6 Myeloid *Klf14* deficiency accelerates atherosclerosis development in mice. Both male and female *Klf14*^{LysM}*ApoE*$^{-/-}$ mice and litter-mate control mice were placed on WD for 12 weeks. (*A*) Aortic trees were isolated from those mice, and *En face* Oil Red O (ORO) staining was used to evidence atherosclerotic plaques. $n = 8$ mice for female *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ group, $n = 14$ mice for female *Klf14*^{LysM}*ApoE*$^{-/-}$ group, $n = 11$ mice for male *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ group, $n = 10$ mice for male *Klf14*^{LysM}*ApoE*$^{-/-}$ group. Bar = 5 mm. (*B*) Haematoxylin and eosin (HE) staining was used to evidence the atherosclerotic plaque at the aortic root from male Klf14$^{fl/fl}$ApoE$^{-/-}$ and Klf14^{LysM}ApoE$^{-/-}$ mice. Bar = 100 μm. $n = 8$ mice for female Klf14$^{fl/fl}$ApoE$^{-/-}$ group, $n = 13$ mice for female Klf14^{LysM}ApoE$^{-/-}$ group, $n = 8$ mice for male Klf14$^{fl/fl}$ApoE$^{-/-}$ group, $n = 7$ mice for male Klf14^{LysM}ApoE$^{-/-}$ group. (*C*) Mac2 immunofluorescence staining was used to detect macrophage infiltration at the aortic root plaque from male mice. Bar = 50 μm. *$n = 9$ mice for Klf14$^{fl/fl}$ApoE$^{-/-}$ group, $n = 6$ mice for male Klf14^{LysM}ApoE$^{-/-}$ group.* (*D*) The cholesterol crystals content was determined using a polarised light microscope at the aortic root plaque from male mice after 12 weeks of WD treatment. Bar = 50 μm. *$n = 10$ mice for Klf14$^{fl/fl}$ApoE$^{-/-}$ group, $n = 7$ mice for male Klf14^{LysM}ApoE$^{-/-}$ group.* (*E*,*F*) ORO staining at the aortic root from male *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ and *Klf14*^{LysM}*ApoE*$^{-/-}$ mice after (*E*) 12 weeks of WD treatment or (*F*) on CD. For (*E*), *$n = 7$ mice for Klf14$^{fl/fl}$ApoE$^{-/-}$ group, $n = 6$ mice for male Klf14^{LysM}ApoE$^{-/-}$ group.* For (*F*), $n = 7$ biological replicates for the *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ group, $n = 5$ biological replicates for the *Klf14*^{LysM}*ApoE*$^{-/-}$ group. Bar = 50 μm. Data are presented as the mean ± SEM and analysed by Mann–Whitney test for (*F*) and two-tailed Student's *t*-test for other panels. n.s., not significant.

All similar analyses were also performed in aortic sinus cross-sections from female mice with no significant differences (for CD studies, Supplementary material online, *Figure S12A–C*; and for WD studies, Supplementary material online, *Figure S12D–F*). Contrary to the finding in males, CC area and macrophage infiltration were decreased in female *Klf14*^{LysM} *ApoE*$^{-/-}$ mice compared to control mice (Supplementary material online, *Figure S12G* and *H*). Female *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ control mice have greater plaque area at the aortic root compared to male *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ mice ($P = 0.0064$), while this difference disappeared in *Klf14*^{LysM}*ApoE*$^{-/-}$ mice due to a significantly increased atherosclerotic plaque burden in male mice (*Figure 6B*).

These results indicate that myeloid KLF14 protects against atherosclerosis development without affecting systemic lipid profiles and suggests a potential sex difference in myeloid KLF14 functions between males and females.

3.7 Perhexiline induces KLF14-dependent expression of ABCA1 in macrophages

We previously found that perhexiline is a *bona fide* KLF14 inducer, which directly upregulates the transcription of KLF14 in hepatocytes and endothelial cells.[13,14] Perhexiline, developed initially to treat angina,[26,27] has been identified as a carnitine palmitoyltransferase I (CPT1) inhibitor. In agreement with the previous results,[14] perhexiline increased the expression of KLF14 and ABCA1 in a dose-dependent manner in THP-1-derived macrophages (*Figure 7A and 7B*). Additionally, perhexiline induced *Klf14* and *Abca1*, and inhibited *Il1β* (*Figure 7C*) in mouse PMs. Similar to KLF14 overexpression, perhexiline could also significantly increase cholesterol efflux (*Figure 7D*). Perhexiline significantly increased ABCA1 expression in PMs from control mice, but this effect was largely abolished in the *Klf14*-deficient PMs (*Figure 7E and 7F*), indicating that perhexiline exerted its effect in a KLF14-dependent manner. We also treated J774 macrophages or THP-1 cell-derived macrophages with Etomoxir (Sigma), a well-known CPT1 inhibitor, which failed to upregulate the expression of *Klf14, Abca1*, and *Abcg1* (Supplementary material online, *Figure S13A* and *B*). Considering that perhexiline also inhibits NOX2[27], using siRNA-mediated knockdown of *Nox2* in PMs, we found that NOX2 was not required for the effect of perhexiline on inducing the expression of *Klf14* and *Abca1* (Supplementary material online, *Figure S13C*). These results indicate that perhexiline regulating the expression of *Klf14* and *Abca1* is independent of the inhibitory effects of perhexiline on CPT1 or NOX2.

3.8 LXRs are dispensable for the induction of Abca1 by perhexiline

LXRα and LXRβ are two critical nuclear receptors that control cholesterol efflux through regulating the expression of ABCA1 and ABCG1.[28,29] Synthetic LXR agonists attenuate atherosclerosis via the upregulation of the ABC cholesterol transporters in both mouse and rabbit atherosclerosis models.[30,31] To understand whether LXRs are mediators of the KLF14 function in macrophages, we isolated PMs from LXRα knockout mice and then knocked down *Lxrβ* by using *Lxrβ* siRNAs to generate LXRα/β knockout/knockdown PMs (referred to as LXR KO, with the LXRα/β mRNA levels shown in Supplementary material online, *Figure S14A* and *B*). In PMs isolated from WT control mice, TO901317 significantly induced the expression of *Abca1* (Supplementary material online, *Figure S14C*) but did not affect the expression of *Klf14* (Supplementary material online, *Figure S15*), indicating that *Klf14* is not regulated by LXR activation in macrophages. We found that perhexiline significantly increased *Klf14* expression in PMs of both LXR KO and WT group, indicating that the induction of *Klf14* by perhexiline is independent of LXRs (*Figure 7G*). While LXRs deficiency largely abolished the induction of *Abca1* by TO901317 (Supplementary material online, *Figure S14C*), LXR KO did not affect the induction of *Abca1* by perhexiline (*Figure 7G*), indicating that LXRs are dispensable for the effect of perhexiline on *Abca1* in macrophages.

In addition, LXR agonists induce apparent side effects such as increased triglycerides and fatty liver, partly through upregulation of the expression of LXR target genes in the liver, like sterol regulatory element-binding transcription factor 1 (SREBF1) and fatty acid synthase (FASN), which limited their clinical use.[32] Therefore, we also determined whether perhexiline affects the levels of lipogenic genes. In HepG2 cells, TO901317 treatment significantly increased the expression of *SREBF1* and *FASN*. However, perhexiline treatment did not affect the expression of the two lipogenic genes (*Figure 7H*), indicating that perhexiline might induce macrophage cholesterol efflux with lower liver side effects compared to LXR agonists. Indeed, using adenovirus-mediated overexpression of *KLF14* or *LacZ* in C56BL/6J mice,[14] we did not observe any upregulation of *Lxrα, Lxrβ, Srebf1*, or *Srebf2* in the liver (Supplementary material online, *Figure S16*), indicating KLF14 itself does not induce liver lipogenesis. Collectively, these data indicate that perhexiline, through upregulation of KLF14 in macrophages, may be an effective drug to induce cholesterol efflux for the treatment of atherosclerosis.

4. Discussion

Human genetic studies have identified several risk alleles leading to the decreased expression level of KLF14 in association with increased CAD risks.[10,33] We previously demonstrated that hepatocyte-selective KLF14 deficiency gives rise to lower HDL-C level in plasma as well as a decrease in serum cholesterol efflux capacity due to reduced ApoA-I expression in the liver.[14] Although the KLF14 inducer, perhexiline, inhibits atherosclerosis development in *ApoE*$^{-/-}$ mice, we noticed that HDL-C levels are very low in *ApoE*$^{-/-}$ mice after 12-week WD challenge,[14] suggesting a yet undefined mechanism underlying the protective effects of KLF14 and perhexiline. Here, our findings demonstrate the anti-atherosclerotic effects of macrophage KLF14 through promoting cholesterol efflux and suppressing the inflammatory response.

Uptake and accumulation of cholesterol by macrophages promotes the formation of foam cells and drives the development of atherosclerotic lesions. Epidemiological studies have consistently shown that HDL-C levels are inversely related to atherosclerotic cardiovascular disease. One of the underlying mechanisms by which HDL and ApoA-I reduce lesion formation is reverse cholesterol transport. This process is regulated by the quality and quantity of HDL particles and the efficiency of cholesterol efflux from the peripheral tissues to lipid-poor ApoA-I or lipidated HDL particles.[4,34] In the present study, we found that KLF14 also plays a critical role in macrophage biology. We demonstrate for the first time that mice with macrophage-selective *Klf14*-deficiency had increased atherosclerosis development due to impaired cholesterol efflux and increased inflammatory response, while KLF14 overexpression or induction by perhexiline significantly increased cholesterol efflux in macrophages. These findings agree with genetic studies that the T/T allele carriers in rs4731702, compared to the C/C allele carriers, show higher expression of KLF14 in an eQTL study[24] and have lower cardiovascular risks. The present study builds upon and expands our previous study by demonstrating that KLF14 induction by perhexiline inhibits

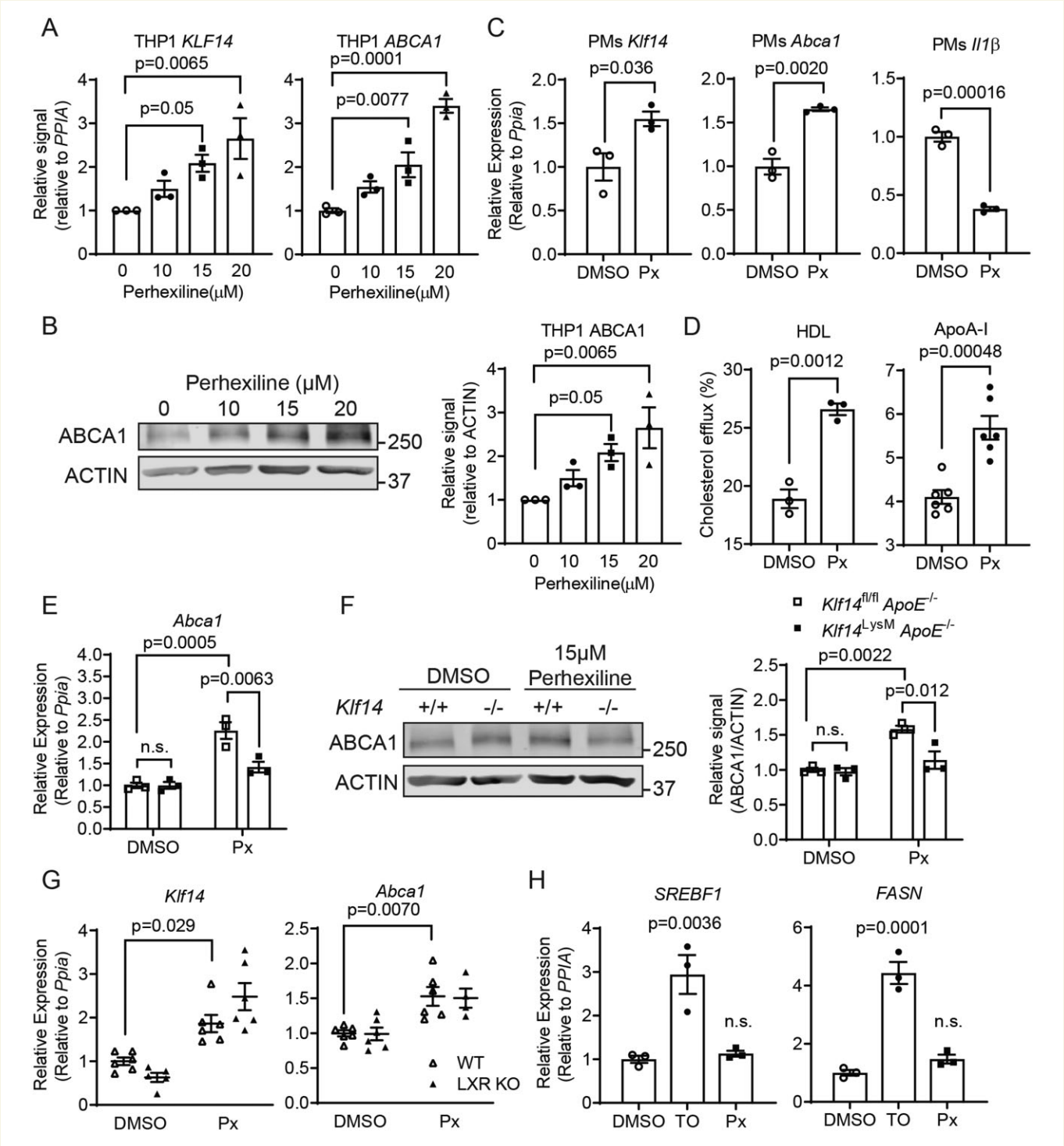


Figure 7 Perhexiline increases *Abca1* expression in a KLF14-dependent manner. (*A,B*) In THP-1 differentiated macrophages, perhexiline treatment increases KLF14 and ABCA1 expression at both the mRNA (*A*) and protein levels (*B*) in a dose-dependent manner [three independent experiments (triplet wells)]. (C) PMs were isolated from WT mice and treated with 15 μM perhexiline for 24 h. The expression of *Klf14, Abca1,* and *IL1β* was determined by qRT–PCR [PMs differentiated from 3–5 mice, three independent experiments (triplet wells)]. (*D*) The cholesterol efflux mediated by HDL or ApoA-I was determined in THP-1-derived macrophages treated with DMSO or Perhexline (Px, 15 μM) for 24 h [three independent experiments (triplet wells) for the left panel, six independent experiments (triplet wells) for the right panel] (*E,F*) PMs from *Klf14*$^{fl/fl}$*ApoE*$^{-/-}$ and *Klf14*^{LysM}*ApoE*$^{-/-}$ mice were treated with DMSO or 15 μM perhexiline for 24 h. The expression of ABCA1 was determined at the (*E*) mRNA or (*F*) protein level and quantified [PMs differentiated from 3–5 mice, three independent experiments (triplet wells)]. (*G*) PMs of WT or *Lxr* KO group were treated with DMSO or perhexiline (15 μM) for 24 h. The *Klf14* and *Abca1* mRNA levels were determined by qRT–qPCR [PMs differentiated from 3–5 mice, 4–6 independent experiments (triplet wells per experiment)]. (*H*) HepG2 cells were treated with DMSO, TO901317 (TO, 0.1 μM), or perhexiline (Px, 15 μM) for 24 h. *SREBF1* and *FASN* mRNA levels were determined by RT–qPCR [three independent experiments (triplet wells)]. Data are presented as the mean ± SEM and analysed by one-way ANOVA with Dunnett's correction for (*A,B*) and (*H*), two-tailed Student's *t*-test for (*C,D*), or two-way ANOVA with Tukey correction for (*E–G*). n.s., not significant.

atherosclerosis by synergistically 1) increasing HDL-C in the circulation by modulating ApoA-I expression in the liver, 2) enhancing cholesterol efflux through modulating ABCA-1 expression in macrophages, and 3) suppressing the inflammatory response via inhibition of the expression of p65 and IL1β in macrophages.

Atherosclerosis-relevant stimuli, including cholesterol crystals, can trigger inflammasome activation and redundant inflammatory signalling pathways.[21] In vitro, this requires a two-step activation: (1) NF-κB activators,[35] adenovirus-mediated penetration,[36] or other stimuli-induced priming processes upregulating NLRP3 expression, and (2) a CC-induced NLRP3 inflammasome activation.[21] Besides, primary macrophages could also be '*in vivo* primed' by hypercholesterolaemia in *ApoE*$^{-/-}$ mice.[37] In our experiments, the priming process was accomplished by adenovirus-mediated penetration in the overexpression experiments and hypercholesterolaemia-induced '*in vivo priming*'. Furthermore, the expression of NLRP3 was regulated by KLF14, which may also affect the priming process during NLRP3 activation.

Despite examining cholesterol efflux and inflammation separately in our study, cholesterol efflux and inflammation homeostasis are two processes that reciprocally affect each other. Lower cholesterol efflux leads to cholesterol accumulation in the macrophages, which triggers the inflammasome activation and the release of IL-1β.[18,20,21,38,39] On the other hand, higher inflammation decreases cholesterol efflux.[40] Additionally, we provide evidence that KLF14 directly binds to the promoter of the IL1β gene to suppress its expression, suggesting that *Klf14* deficiency leads to loss of direct inhibition of IL1β transcription and initiates the pro-inflammatory cascades which disrupt inflammation homeostasis and exacerbate cholesterol accumulation in macrophages. Although IL-1β has manifold effects on the cardiovascular system, the Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) has demonstrated that administration of canakinumab to neutralise IL-1β activity improves outcomes in individuals with post-acute myocardial infarction.[41] Our findings indicate that KLF14 blocks multiple inflammatory pathways and presents protective effects on atherosclerosis.

W. Xie *et al.* also demonstrate that KLF14 plays an anti-atherogenic role via a miR-27a-dependent downregulation of lipoprotein lipase and subsequent inhibition of pro-inflammatory cytokine secretion.[42] Nonetheless, a study from X. Wei *et al.* showed a contradictory result indicating that KLF14 induces inflammation in macrophages and increases atherosclerosis development using adenovirus-mediated gene silencing both *in vivo* and *in vitro*.[43] However, we used Cre-loxP system-mediated myeloid cell-selective gene knockout *in vivo* and primary cell culture *in vitro*. The uncomparable methods maybe generate the seemingly opposite results. A recently published paper showed that endothelial KLF14 may induce macrophage M2 polarization,[44] indicating a potential role of KLF14-mediating cell–cell interaction.

Previous genetic studies showed sex-specific metabolic phenotypes of KLF14 with female risk allele carriers having lower hip-to-waist ratio but higher type 2 diabetes risk.[45] However, the KLF14 risk allele does not show such sex differences on cardiovascular and lipid profile phenotypes.[10] Accordingly, we found that atherosclerosis was increased in both female and male mice, though *Klf14*^{LysM} *ApoE*$^{-/-}$ male mice showed higher susceptibility to WD-induced atherosclerosis compared to their female counterparts. The *ApoE*$^{-/-}$ mice model does not exhibit consistent sex differences during atherosclerosis development,[46–48] which does not reflect the sex differences observed in humans[49] and may not be an appropriate model to study sex differences in the biological functions of KLF14. The effects and underlying mechanisms of KLF14 in sex differences will be further investigated.

Perhexiline was used as a potent anti-anginal[50] drug in the clinic even before its mechanism was fully understood.[27] Later on, perhexiline has been administrated as a CPT1 inhibitor to increase metabolic efficiency in heat failure patients.[51–53] Interestingly, perhexiline improves cardiac energetics and symptom status without significantly altering cardiac substrate utilization,[53] suggesting possible unanticipated mechanisms. Perhexiline was also found to inhibit neutrophil NADPH oxidase.[54] In our study, we found that perhexiline induces ABCA1 expression independent of CPT1 or NOX2, addressing a new mechanism of action of perhexiline.

ABCA1 is a target gene of LXR,[9] and ABCA1 deficiency in patients results in Tangier disease and familial HDL deficiency.[55,56] Although preclinical studies showed strong inhibition of atherosclerosis by LXR agonists,[30,31,57] the clinical use of LXR agonists was halted due to their side effects, which is triggered mainly through upregulation of lipogenic genes in the liver.[32] We did not detect a direct protein–protein interaction between LXR and KLF14 using co-immunoprecipitation (data not shown). Then, we determined that KLF14 induces cholesterol efflux is independent of LXRs using LXR KO PMs. We also found that increased KLF14 expression induced neither LXRα/β expression nor lipogenic gene (*SREBF1* or *FASN*) expression, indicating that KLF14 upregulates the cholesterol efflux in macrophages without triggering lipogenesis in hepatocytes in an LXR-independent manner, which is confirmed in adenovirus-mediated overexpression of KLF14 in the liver. These findings define KLF14 as a promising therapeutic target for treating atherosclerosis.

5. Limitations

Although the *ApoE*$^{-/-}$ mouse model is one of the most commonly used mouse models for atherosclerosis studies, macrophage-specific *ApoE* deficiency itself demonstrated biology on cholesterol efflux and inflammation.[58,59] Therefore, although we carefully designed and controlled our study, the deletion of *ApoE* in macrophages may reduce or enhance the observed phenotypes. LysM-Cre is expressed in all myeloid cells, including macrophages and neutrophils.[60,61] We also determined the neutrophil infiltration in the plaque by using anti-Mouse Gr-1 monoclonal antibody (RB6-8C5), which has been widely used to detect neutrophils.[62–64] In both male and female animals, Gr1^{+} cells were minimal and showed no significant difference (Supplementary material online, *Figure S17*) in this study. Gr-1 antibodies react with both the Ly6G (exclusively expressed on granulocyte) and Ly6C (monocyte/macrophage)[62]. LysM-Cre mice, and perhexiline treatment may regulate the biological functions of KLF14 in other subset of myeloid cells such as neutrophils and dendritic cells, causing another limitation to address the contribution of macrophage KLF14 to atherosclerosis *in vivo*. Other approaches should be necessary to study the contribution of macrophages and neutrophils to vascular inflammation and atherosclerosis in the future.

6. Conclusions

Our results uncovered a pivotal role of myeloid KLF14 in protecting against atherosclerosis development by regulating cholesterol efflux and inflammation. These findings provide new insights into a previously unrecognised effect of KLF14 in *myeloid cells* and extend our

understanding of the underlying mechanism of perhexiline as a KLF14 activator to support its potential use for atherosclerosis treatment.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Author contributions

Y.G. and Y.E.C. conceived, designed, and supervised the study. H.W., W.H., Y.L., and Y.G. performed the experiments. H.L., L.C., M.T.G.-B., A.S., and J.Z. provided the technical support and contributed to the discussion of the project and article. H.W. and Y.G. analysed the data and wrote the article.

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Conflict of interest: none declared.

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Data availability

The expanded Methods section and other supporting data are available in the Supplementary material online. The primary data for this article will be shared on reasonable request to the corresponding authors.

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Translational Perspective

Here, using both gain- and loss-of-function strategies, we show that KLF14 regulates cholesterol efflux by regulating the expression of ABCA1 and inhibits inflammatory response in macrophages. These findings, along with our previous data, put activation of KLF14 forward as a prospective therapeutic target for atherosclerotic cardiovascular disease.