Original Article

Absence of Four-and-a-Half LIM Domain Protein 2 Decreases Atherosclerosis in ApoE−/− Mice

Talin Ebrahimian, David Simon, Catherine A. Lemarié, Stefania Simeone, Maryam Heidari, Koren K. Mann, Sven Wassmann,* Stephanie Lehoux*

Objective—Four-and-a-half LIM domain protein-2 (FHL2) is expressed in endothelial cells, vascular smooth muscle cells, and leukocytes. It regulates cell survival, migration, and inflammatory response, but its role in atherogenesis is unknown.

Approach and Results—To investigate the role of FHL2 in atherosclerosis, FHL2-deficient mice were crossed with ApoE-deficient mice, to generate ApoE/FHL2−/− mice. After high-fat diet, ApoE/FHL2−/− mice had significantly smaller atherosclerotic plaques than ApoE−/− mice in the aortic sinus, the brachiocephalic artery, and the aorta. This was associated with enhanced collagen and smooth muscle cell contents and 2-fold reduction in macrophage content within the plaques of ApoE/FHL2−/− versus ApoE−/− mice. This could be explained, in part, by the reduction in aortic ICAM-1 mRNA and in VCAM-1 protein expression in the plaque. Aortic gene expression of CX3CL1 and CCL5 was increased in ApoE/FHL2−/− versus ApoE−/− mice. Peritoneal thioglycollate injection elicited equivalent numbers of monocytes and macrophages in both groups, but a significantly lower number of proinflammatory Ly6C high monocytes were recruited in ApoE/FHL2−/− versus ApoE−/− mice. Furthermore, mRNA levels of CX3CR1 were 2-fold higher in monocytes from ApoE/FHL2−/− versus ApoE−/− mice. Finally, we investigated the potential importance of myeloid cell FHL2 deficiency in atherosclerosis. After being irradiated, ApoE−/− or ApoE/FHL2−/− mice were transplanted with ApoE−/− or ApoE/FHL2−/− bone marrow. After high-fat diet, both chimeric groups developed smaller plaques than ApoE−/− transplanted with ApoE−/− bone marrow.

Conclusions—These results suggest that FHL2 in both myeloid and vascular cells may play an important role in atherosclerosis by promoting proinflammatory chemokine production, adhesion molecule expression, and proinflammatory monocyte recruitment. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.305071.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ chemokines ■ FHL2 protein, mouse ■ monocytes

Atherosclerosis is a chronic inflammatory disease that is characterized by modifications in the vascular, metabolic, and immune systems. The accumulation of leukocytes within atherosclerotic lesions is a process regulated at various levels, including mobilization, recruitment, and endothelial adhesion of monocytes, followed by their migration into the intima and differentiation into macrophages. Two major monocyte subsets can be distinguished in the blood based on differential expression of the chemokine receptors CCR2 and CX3CR1. The inflammatory monocytes express high levels of CCR2 but low levels of CX3CR1, whereas the patrolling monocytes have low levels of CCR2 and high levels of CX3CR1. Inflammatory monocytes rapidly enter the atherosclerotic plaque where they give rise to macrophages. As macrophages accumulate in the atherosclerotic lesion, they incorporate lipids and become foam cells, eventually forming necrotic cores that fragilize the plaque. The inflammatory milieu attracts diverse immune cells but also promotes vascular smooth muscle cell (VSMC) infiltration from the media. Accumulation of VSMCs and collagen synthesis by these cells is associated with a reduced risk of rupture.

Four-and-a-half LIM domain protein 2 (FHL2, SLIM3) is the best-studied member of the LIM-only subclass of the LIM protein superfamily. LIM proteins are defined by the presence of ≥1 LIM domains composed of a conserved cysteine-rich module that mediates protein–protein interactions. Multiple roles have been ascribed to FHL2. FHL2 exerts its function as a transcriptional cofactor, interacting with a broad range of transcription factors, and modulating the transcription of many proteins such as the androgen receptor, cAMP response element-binding protein, integrins, β-catenin, presenilin-2, ERK-2, AP-1, and sphingosine kinase-1. It modulates cellular processes such as cell survival, proliferation, and signal transduction. FHL2 is strongly expressed in early cardiac

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precursor cells, in the adult heart and in skeletal muscle, as well as at lower levels in most epithelial tissues. FHL2 expression was also recently demonstrated in endothelial cells and VSMCs. Deletion of FHL2 in mice was associated with changes in VSMC contractile protein expression and proliferation, and endothelial function. It was shown to enhance endothelial repair capacity in mice. FHL2 deletion also increased dendritic cell migratory speed, persistence, and directionality, whereas it reduced macrophage activation and expression of inflammatory cytokines interleukin-6 and tumor necrosis factor-α. In summary, FHL2 has been shown to influence many cell types associated with atherosclerosis. Nevertheless, apart from a report that it reduces lesion formation in C57bl/6 mice, nothing is known on how FHL2 actually influences the atherosclerotic process (Table).

In the present study, we examined the role of FHL2 in atherosclerosis by generating ApoE/FHL2−/− double-knockout mice and by inducing lesions with a high-fat diet (HFD). We demonstrate that absence of FHL2 alters the aortic and leukocyte chemokine/chemokine receptor balance in favor of anti-inflammatory monocyte recruitment. Aortic adhesion molecule expression is lower in ApoE/FHL2−/− mice than their ApoE−/− counterparts, leading to smaller plaques with less macrophage content. Furthermore, we demonstrate that the absence of FHL2 in both the myeloid and the vascular compartment plays a role in the observed atheroprotective effect.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Table. Oligonucleotide Sequences

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Absence of FHL2 Reduces Atherosclerotic Plaque Formation in ApoE−/− Mice

First of all we verified that FHL2 is expressed in mouse atherosclerotic plaques by immunostaining. Starting at ages of 12 weeks, male ApoE−/− and ApoE/FHL2−/− mice were fed with HFD for 7 weeks before euthanization and analysis. Body weight, lipids (total cholesterol, high-density lipoprotein) and triglycerides were equivalent between the 2 mouse groups (Figure I in the online-only Data Supplement). Using cryosections from the brachiocephalic artery, we confirmed that FHL2 is highly expressed throughout the plaques of ApoE−/− mice (Figure 1A) but absent from ApoE/FHL2−/− lesions (not shown).

Atherosclerotic lesions size was analyzed in the aortic sinus, the brachiocephalic artery, and the aorta by Oil Red O staining. We observed a significant reduction in plaque area in all vessel segments of ApoE/FHL2−/− compared with ApoE−/− mice at 7 weeks of HFD (sinus, 0.14±0.02 versus 0.29±0.04 mm²; brachiocephalic artery, 0.03±0.01 versus 0.07±0.01 mm²; aorta, 7.0±0.9% versus 10.3±1.1%; Figure 1B–1D). To characterize plaque composition, we performed immunohistochemical stainings. We found that lesions of ApoE/FHL2−/− mice contained significantly greater proportions of collagen and smooth muscle cells compared with lesions of ApoE−/− mice (Figure 2). These results suggest that ApoE/FHL2−/− mice develop more stable plaques than ApoE−/−.

ApoE/FHL2−/− Mice Develop Plaques With Low Macrophage Content

Next, we characterized macrophage content within the atherosclerotic plaques at the aortic sinus, by MOMA-2 immunostaining. We did not observe any difference in the percent macrophage area between plaques of ApoE−/− and ApoE/FHL2−/− mice (Figure 3A). Because ApoE/FHL2−/− mice develop smaller lesions, this means that total MOMA-2 positive
Absence of FHL2 Decreases Atherosclerosis

To explain this difference in cell infiltration between groups, we investigated adhesion molecule expression in plaques. We observed a significant, 34% reduction in ICAM-1 mRNA levels in the aortas of ApoE/FHL2−/− mice compared with ApoE−/− aortas (Figure 3B). Furthermore, although VCAM-1 mRNA levels were not significantly decreased in ApoE/FHL2−/− aortas (Figure 3B), we found that protein expression of VCAM-1 was significantly reduced in atherosclerotic plaques of these mice (Figure 3C). Lower adhesion molecule expression in the lesions could not be ascribed to differences in reactive oxygen species between ApoE−/− and ApoE/FHL2−/− (Figure II in the online-only Data Supplement). These results suggest that reduced adhesion molecule expression could account, at least in part, for the decrease in macrophage recruitment and reduced plaque size in ApoE/FHL2−/− mice.

Decreased Inflammatory Monocyte Recruitment in ApoE/FHL2−/− Mice

To further explain the differences in lesion formation between ApoE−/− and ApoE/FHL2−/− mice, we evaluated leukocyte content in the spleen, which is a main pool of monocytes and macrophages. We did not observe any differences in the number of CD11b+Gr1− monocytes or CD11b+Gr1−/F4/80+ macrophages between both groups of mice (Figure III in the online-only Data Supplement). Hence, reduced monocyte/macrophage area in ApoE/FHL2−/− plaques could not be explained by reduced availability of these cells.

Next, we investigated monocytes recruited to the peritoneal cavity in response to 96-hour thioglycollate. The number of CD11b+Gr1− monocytes recruited by thioglycollate was equivalent in both groups of mice, as were numbers of Ly6C+ monocytes (Figure 4A) and CD11b+Gr1−/F4/80+ macrophages (Figure 4B). However, we observed a significantly lower number of Ly6C+ monocytes in ApoE/FHL2−/− mice (8±3%) versus ApoE−/− mice (18±4%; Figure 4A). A series of functionality tests indicated that MCP-1–induced migration, proliferation, low-density lipoprotein (LDL) uptake and efferocytosis were not different between peritoneal monocytes from ApoE−/− and ApoE/FHL2−/− mice (Figure IV in the online-only Data Supplement). The migratory capacity of bone marrow–derived monocytes was equivalent as well between groups (data not shown). These results indicate that the absence of FHL2 limits the recruitment of inflammatory Ly6C+ cells by a proinflammatory stimulus.

ApoE/FHL2−/− Mice Exhibit Higher Levels of Anti-Inflammatory Chemokines and Receptors

Chemokine/chemokine receptor systems are key regulators of monocyte chemotaxis toward sites of inflammation, such as atherosclerotic lesions. We observed a 4-fold elevation in CX3CL1 and CCL5 mRNA levels (P<0.05) in aortas of ApoE/FHL2−/− mice compared with ApoE−/− mice (Figure 5A). In parallel, we also found a significant, 2-fold increase in CX3CR1 mRNA levels in monocytic cells of ApoE/FHL2−/− versus ApoE−/− mice, but no differences in CCR5 mRNA levels (Figure 5B). However, CCL2 and CXCL1 mRNA levels were equivalent in aortas of both mouse groups, as were monocyte mRNA levels of CCR2 and CXCR2. Similarly, mRNA levels of other proinflammatory cytokines, such as interleukin-1β, interleukin-12β, and tumor necrosis factor-α, as well as mRNA and protein levels of the anti-inflammatory cytokine interleukin-10, were equivalent in monocytic cells from ApoE/FHL2−/− and ApoE−/− mice (Figure V in the online-only Data Supplement). These findings suggest that different monocyte subsets could be recruited in ApoE/FHL2−/− mice. More specifically, in the absence of FHL2 there is a preferential elevation...
of CX3CR1/CX3CL1 that potentially favors the recruitment of patrolling Ly6C<sub>lo</sub> rather than inflammatory Ly6C<sub>hi</sub> monocytes.

**Many Functions of ECs and SMCs Are Regulated by FHL2**

To test whether absence of FHL2 could also influence the function of vascular cells, we conducted in vitro studies. The proliferation of ECs from ApoE/FHL2<sup>−/−</sup> mice was significantly increased, and basal as well as tumor necrosis factor-α-induced apoptosis was reduced, compared with ECs from ApoE<sup>−/−</sup> mice. In contrast, uptake of Dil-LDL by ApoE/FHL2<sup>−/−</sup> mice was significantly decreased compared with ECs from ApoE<sup>−/−</sup> mice. As regards migration of ECs between genotypes, and microvessel sprouting in matrigel from the aorta of ApoE/FHL2<sup>−/−</sup> mice, was significantly decreased compared with ApoE<sup>−/−</sup> (Figure VI in the online-only Data Supplement). Hence, ECs and SMCs from ApoE/FHL2<sup>−/−</sup> mice display many characteristics that could contribute to the atheroprotective phenotype.

**Absence of FHL2 in the Bone Marrow and Vascular Cells Is Involved in the Atheroprotective Effect Observed in ApoE/FHL2<sup>−/−</sup> Mice**

We undertook studies to elucidate the origin of FHL2 involved in the regulation of atherosclerotic plaque formation by performing irradiation-transplantation experiments. Bone marrow from ApoE<sup>−/−</sup> or ApoE/FHL2<sup>−/−</sup> mice was transplanted into γ-irradiated ApoE<sup>−/−</sup> (A→A) or to ApoE/FHL2<sup>−/−</sup> (A→F or F→F) recipients. Four weeks after transplantation the mice were fed a HFD for 7 weeks, and atherosclerotic plaques were analyzed thereafter. Mean body weight was not significantly different between groups before and after HFD feeding (Figure VIII.A in the online-only Data Supplement). About the lipid profile, there was a decrease in total cholesterol and triglyceride levels in A→F compared with A→A mice, but no differences were observed in other groups, and high-density lipoprotein cholesterol levels were equivalent in all the mice (Figure VIII.B in the online-only Data Supplement).

In concurrence with the data presented in Figure 1, we observed a significant reduction in plaque size in the sinus and aorta of F→F mice compared with A→A mice (sinus, 0.19±0.03 versus 0.26±0.04 mm<sup>2</sup>; aorta, 9±1.5 versus 16±1% plaque area). Interestingly, both chimeric mouse groups (F→A and A→F) developed smaller plaques in the sinus and the aorta compared with A→A mice (Figure 6). Similarly, we observed a significant increase in collagen and smooth muscle α-actin content, and reduced macrophage content, within the plaques of mice lacking FHL2 in both the vascular and immune compartments (F→F mice), compared with A→A mice (Figure IX in the online-only Data Supplement). Collagen content was also significantly enhanced in the ApoE/FHL2<sup>−/−</sup> mice having received ApoE<sup>−/−</sup> marrow. These data suggest that absence of FHL2 in both the vascular and the myeloid compartments contributes to smaller, more stable plaques.

To further explain the differences in plaque size observed between A→A, chimeric, and F→F animals, we investigated leukocyte function. Monocyte adhesion to a monolayer of activated ECs was measured under physiological flow in vitro. Adhesion was greatest when ApoE<sup>−/−</sup> monocytes and ECs were paired (Figure X in the online-only Data Supplement). Substituting either the monocytes or the ECs, or both, for ApoE/FHL2<sup>−/−</sup> cells resulted in a ≈55% to 60% reduction of adhesion (P<0.05). These results clearly confirm the importance of FHL2 in monocytes and endothelial cells in the regulation of atheroprotective plaque formation.

**Figure 2.** ApoE/FHL2<sup>−/−</sup> mice develop more stable plaques. After 7 weeks of high-fat diet, lesion collagen content (Sirius red staining; A) and smooth muscle cell content (α-actin immunohistochemistry; B) were quantified in the aortic sinus of ApoE<sup>−/−</sup> and ApoE/FHL2<sup>−/−</sup> mice. Data are mean±SEM of n=8. *P<0.05 vs ApoE<sup>−/−</sup>. Scale bars, 50 µm.

**Figure 3.** Decreased macrophage content is associated with decreased adhesion molecule expression in ApoE/FHL2<sup>−/−</sup> mice. After 7 weeks of high-fat diet, lesion macrophage content was determined by MOMA-2 immunohistochemistry. A, Aortic mRNA expression of ICAM-1 and VCAM-1 (B) and lesion VCAM-1 expression determined by immunohistochemistry (C) in ApoE<sup>−/−</sup> and ApoE/FHL2<sup>−/−</sup> mice. Data are mean±SEM of n=8. **P<0.01 vs ApoE<sup>−/−</sup>, *P<0.05 vs ApoE<sup>−/−</sup>. Scale bars, 50 µm (A), 25 µm (C).
of plaque development. To verify whether FHL2 could also influence the homing of macrophages to established plaques in vivo, mice fed a HFD for 7 weeks were injected with equal parts macrophages from ApoE/FHL2−/− mice (stained with cell tracker red) and ApoE−/− mice (cell tracker green). Red and green cells within aortic sinus sections were visualized 48 hours later. Our data show that the homing of ApoE/FHL2−/− cells to atherosclerotic plaques was significantly reduced compared with ApoE−/− cells, regardless of recipient mouse genotype (Figure XI in the online-only Data Supplement). Regulatory T-cell numbers were also elevated in untransplanted ApoE/FHL2−/− mice compared with ApoE−/−. These combined data provide additional evidence to support the dual provenance of atheroprotective FHL2 knockout in ApoE−/− mice, the myeloid and vascular compartments.

Discussion

FHL2 is a ubiquitously expressed protein able to bind to many different proteins and function in a cell-type–dependent fashion. We have recently shown that FHL2 deficiency is protective, promoting early outgrowth cell function as well as endothelial cell survival and function. In the present study, we demonstrate that the absence of FHL2 in myeloid and vascular cells reduces plaque development in the ApoE−/− atherosclerotic mouse model. Moreover, we provide evidence that this atheroprotective effect could be, at least in part, because of reduced adhesion molecule expression in endothelial cells and because of a change in the chemokine/chemokine receptor expression balance leading to decreased recruitment of proinflammatory monocytes in ApoE/FHL2−/− mice compared with ApoE−/− mice.

FHL2 has been shown to be expressed in different cell types present in the atherosclerotic plaque, such as endothelial cells, VSMCs,17,23 early outgrowth cells,24 dendritic cells,20 and macrophages.24 We verified that FHL2 is indeed highly expressed in the atherosclerotic plaque of ApoE−/− mice. Deletion of FHL2 was shown to preserve endothelial function in mice placed on a cholesterol-enriched diet, associated with resistance to atherogenesis. However, the study was not performed in an atherosclerotic mouse model, such that lesions were small and could not be characterized. This is an important point, because the study of FHL2 in relation to collagen synthesis has produced mixed results. FHL2 was found to be important for matrix protein assembly25 and collagen metabolism,26 but further studies showed that absence of FHL2 leads to greater collagen III accumulation.21 Similarly, FHL2 knockout reduced α-SMA expression27 and increased SMC proliferation28 in some conditions, but not in others.17 The effect of FHL2 knockout on collagen synthesis and SMC accumulation in the plaque was therefore not established. Our data, showing more staining for collagen and smooth muscle cells in the lesions of ApoE/FHL2−/− mice compared with ApoE−/− mice, suggest that the absence of FHL2 promotes a more stable plaque phenotype. Our in vitro data did not show any differences in aortic SMC proliferation and migration. However, we observed less Dil-LDL uptake by SMCs from ApoE/FHL2−/− than ApoE−/− mice. Because SMCs contribute to foam cell formation in the atherosclerotic plaque by taking up oxidized-LDL,29 our data point to a feature of SMCs that could account for smaller plaques, and proportionately higher SMC and collagen content, in ApoE/FHL2−/− mice.

Monocytes and macrophages play a critical role in initiation, progression, and stability of atherosclerotic plaques. In accordance with our finding that plaque area was smaller in ApoE/FHL2−/− mice than in ApoE−/− mice, we established that absolute monocyte/macrophage content was also diminished in the former group, suggestive of less monocyte...
infiltration in the ApoE/FHL2−/− lesions. Monocytes must first adhere to the endothelium via adhesion molecules before entering the plaque, and indeed we detected lower levels of VCAM-1 expression at the endothelial aspect of ApoE/FHL2−/− plaques. In the whole aorta, mRNA expression of ICAM-1 was significantly lower in ApoE/FHL2−/− than in ApoE−/− mice. These data are in line with a previous publication showing that FHL deletion downregulates VCAM-1 and ICAM-1 expression in mice.30 As a coactivator of nuclear factor κB,31,32 AP1,3 and cAMP response element-binding protein,33 FHL2 is likely to have a broad influence on inflammatory processes, including adhesion molecule expression. Furthermore, FHL2 has been shown to localize to cell adhesion complexes34 and it can associate with a number of integrins.35 These reports, together with our findings, suggest that the decrease in adhesion molecule expression and integrin function in the absence of FHL2 could be partly responsible for the reduced monocyte infiltration in the plaques of ApoE/FHL2−/− mice. Our in vitro data further uphold this concept. Although ApoE/FHL2−/− EC sprouting in matrigel was reduced, in accordance with lower neovascularization reported in absence of FHL2,36 ECs from ApoE/FHL2−/− mice displayed increased proliferation and reduced apoptosis compared with ApoE−/− ECs. This result is in line with our previous data showing an improved re-endothelialization capacity in FHL2−/− mice related to enhanced viability and migration of EC.18 In addition, we observed a lower adhesion capacity of monocytes incubated with ApoE/FHL2−/− ECs in a flow chamber. Hence, lower adhesive properties of ECs may well mitigate monocyte recruitment in ApoE/FHL2−/− mice.

In addition to lower cell adhesion molecule expression, we found that the absence of FHL2 in ApoE−/− mice altered the chemokine/chemokine receptor balance in the aorta and monocytes, in favor of CXCL1, CCL5, and CX3CR1. These data indicate that different monocyte subtypes are likely to be recruited in the plaques of ApoE−/− and ApoE/FHL2−/− mice. Indeed, 2 subsets of monocytes with different functions were distinguished based on variable levels of Ly6C, CX3CR1, and CCR2.3 Monocytes that express high levels of CX3CR1 are associated with a low expression of Ly6C marker, infiltrate via CCR5, and are known to be anti-inflammatory/patrolling. On the contrary, CCR2-expressing monocytes are deemed to be proinflammatory. In line with our mRNA data, FACS analysis of thioglycollate-elicited cells revealed that the number of monocytes expressing high levels of proinflammatory Ly6C was significantly smaller in ApoE/FHL2−/− than in ApoE−/− mice, despite an equivalent number of total monocytes/macrophages recruited in the peritoneal cavity. Many functions of these cells, including migration, proliferation, LDL uptake, or efferocytosis, were not affected by the absence of FHL2. However, we found that ApoE/FHL2−/− monocytes

Figure 5. ApoE/FHL2−/− mice exhibit an altered balance of chemokines and receptors. A, After 7 weeks of high-fat diet the aorta of ApoE−/− and ApoE/FHL−/− was collected and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis was performed to detect the mRNA levels of chemokines. Data are mean±SEM of n=8. *P<0.05 vs ApoE−/−.

B, Cells were extracted from the peritoneal cavity of ApoE−/− and ApoE/FHL−/− mice 96 hours after injection of thioglycollate. RT-qPCR was performed to detect the mRNA levels of chemokine receptors. Data are mean±SEM of n=8. *P<0.05 vs ApoE−/−.

Figure 6. Absence of four-and-a-half LIM domain protein-2 (FHL2) from both the bone marrow and vascular cells participates in the atheroprotective effect observed in ApoE/FHL2−/− mice. ApoE−/− and ApoE/FHL2−/− mice were lethally irradiated and transplanted with the bone marrow from ApoE−/− (A→A, A→F) or ApoE/FHL2−/− (F→A, F→F) mice, respectively. After 7 weeks of high-fat diet lesion size was quantified in the aortic sinus (A) and the aorta (B). Data are mean±SEM of n=5 to 8. *P<0.05 and **P<0.001 vs A→A. Scale bars, 50 μm (A), 10 μm (B).
were less adherent to activated ECs in a flow chamber assay, and ApoE/FHL2−/− macrophages displayed reduced homing capacity in vivo compared with ApoE+/− macrophages. It has been reported that orphan nuclear receptor NR4A1 (Nur77) controls the differentiation and survival of patrolling Ly6C+ monocytes in the bone marrow.36 Nur77 deficiency polarizes monocytes toward the Ly6C− phenotype and enhances atherosclerosis.37 Conversely, Nur77 overexpression reduces inflammatory cytokine synthesis in macrophages.38 Because FHL2 can bind to Nur77 and repress its function,39 enhanced Nur77 activity in ApoE/FHL2−/− mice could account, at least in part, for the preferential recruitment of patrolling monocytes and reduced atherosclerotic lesion formation in these animals.

Finally, based on our data presented herein, identifying a role for FHL2 in adhesion molecule expression, cytokine and cytokine receptor expression, selective monocyte recruitment, as well as macrophage homing, and bolstered by a growing body of literature cited above reporting an adverse or proinflammatory role of FHL2 in monocytes, macrophages, and endothelial cells, we strongly suspected that the absence of FHL2 in both the vascular and the myeloid compartment might confer protection against atherosclerosis. Indeed, our irradiation-transplantation experiments showed that ApoE−/− mice receiving ApoE/FHL2−/− bone marrow and ApoE/FHL2−/− mice receiving ApoE−/− bone marrow developed significantly smaller and more stable plaques than ApoE−/− mice transplanted with ApoE−/− bone marrow (as did ApoE/FHL2−/− mice receiving ApoE/FHL2−/− bone marrow). In addition to effects on ECs, SMCs, monocytes, and macrophages, ApoE/FHL2−/− in either or both compartments was associated with enhanced regulatory T-cell numbers in the thymus. Regulatory T cells are known to reduce atherosclerotic plaque development through suppression of effector T cells and macrophages.40 Hence, the absence of FHL2 in myeloid cells as well as vascular cells plays an important role in the observed atheroprotective effect.

In summary, our data show for the first time that FHL2 is able to promote plaque formation by regulating proinflammatory chemokine production, recruitment of proinflammatory monocytes, and adhesion molecule expression. Further studies will be necessary to define the molecular mechanisms involved.

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Disclosures
None.

References
ameliorate atherosclerosis by acting on many cell types.

In this article, we report for the first time that the absence of four-and-a-half LIM domain protein-2 in myeloid and vascular cells reduces atherosclerotic plaque formation in apolipoprotein E–deficient mice significantly. This atheroprotective effect could be explained partly by decreased proinflammatory chemokine production and proinflammatory monocyte recruitment, as well as regulation of adhesion molecule expression. Absence of four-and-a-half LIM domain protein-2 in myeloid and vascular cells is also associated with a more stable plaque phenotype. These novel findings support the notion that four-and-a-half LIM domain protein-2 could be a potential therapeutic target to ameliorate atherosclerosis by acting on many cell types.
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Online Figure I. No differences in body weight and plasma lipid levels in ApoE-/- and ApoE/FHL2-/- mice. A: Body weight of ApoE-/- and ApoE/FHL2-/- mice was measured before and after 7 weeks of high fat diet (HFD). B: Plasma cholesterol, HDL, and triglycerides were measured after 7 weeks of high fat diet. Data are mean ±SEM of n=8.
Online Figure II. Equivalent ROS generation in the atherosclerotic plaques of the brachiocephalic artery of ApoE/- and ApoE/FHL2/- mice. ROS generation was analyzed by dihydroethidium staining (DHE). Data are mean ±SEM of n=5. Scale bar = 50 µm.
Online Figure III. No differences in spleen monocyte and macrophage numbers between ApoE/- and ApoE/FHL2/- mice. After 7 weeks of high fat diet, spleen monocyte and macrophage count was assessed by flow cytometry. Data are mean ±SEM of n=8.
Online Figure IV. Equivalent function of peritoneal monocytic cells from ApoE-/- and ApoE/FHL2-/- mice. Cells were extracted from the peritoneal cavity of ApoE-/- and ApoE/FHL2-/- mice 96 hours after injection of thioglycollate. A: Boyden chamber assays were used to investigate MCP-1-induced migration. B: Cell proliferation was assessed by Ki67 immunostaining. C: LDL uptake was measured by flow cytometry. D: Efferocytosis was assessed by immunocytochemistry. Data are mean ±SEM of n=8.
Online Figure V. No differences in interleukin or TNF-α protein levels between ApoE-/- and ApoE/FHL2-/-.

Macrophages were extracted from the peritoneal cavity of ApoE-/- and ApoE/FHL2-/- mice 96 hours after injection of thioglycollate. A. RT-qPCR was performed to detect the mRNA levels of indicated chemokines. B. IL-10 levels secreted by macrophages during 24 hours were measured by Elisa. Data are mean ±SEM of n=8.
Online Figure VI. Variable function of endothelial cells from ApoE-/ and ApoE/FHL2-/mice. A: Endothelial cell (EC) proliferation was measured using Ki67 immunostaining (green) (n=6). *p<0.05 vs ApoE-/-.
B: Microvessel sprouting from ApoE-/ and ApoE/FHL2-/aortic rings into matrigel was measured after 8 days of culture (n=5). *p<0.05 vs ApoE-/-.
C: EC apoptosis under basal conditions (Veh) or in the presence of TNF-α (50ng/ml) + cycloheximide (Cyclo., 25µg/ml), was measured using AnnexinV/PI flow cytometry (n=4). Wilcoxon followed by Steel post test was performed. *p<0.05 vs ApoE-/- Veh, #p<0.05 vs (ApoE-/- TNF-α+Cyclo).
D: EC migration was measured using Boyden chambers (n=4). Data are mean ±SEM. Scale bars = 50 µm.
Online Figure VII. Variable function of vascular smooth muscle cells from ApoE/- and ApoE/FHL2/- mice. A: Smooth muscle cell proliferation was measured using Ki67 immunostaining (n=6). B: SMC migration was measured using a scratch assay (n=6). C: Dil-LDL-uptake was measured by immunostaining (n=3). Data are mean ±SEM. ***p<0.0001 vs ApoE/-. Scale bar = 50 µm.
Online Figure VIII. Body weight and plasma lipid levels in ApoE/- and ApoE/FHL2/- mice transplanted with ApoE/- or ApoE/FHL2/- bone marrow. A: Body weight of the different groups of mice as measured before and after 7 weeks of high fat diet (HFD). B: Plasma cholesterol, HDL and triglycerides were measured in the different groups after 7 weeks of high fat diet. Data are mean ±SEM of n=4-7. *P<0.05 vs A→A.
Online Figure IX. Plaque characterization at the aortic sinus of ApoE-/- and ApoE/FHL2-/- mice transplanted with ApoE-/- or ApoE/FHL2-/- bone marrow. ApoE-/- and ApoE/FHL2-/- mice were lethally irradiated and transplanted with the bone marrow of ApoE-/- (A→A, A→F) or ApoE/FHL2-/- (F→A, F→F) mice, respectively. After 7 weeks of high fat diet, lesion collagen content (Sirius red staining) (A), smooth muscle cell content (alpha-actin immunohistochemistry) (B), and macrophage content (Moma-2 immunohistochemistry) (C) were quantified in the aortic sinus of mice. Data are mean ±SEM of n=3-5. *p<0.05 and **p<0.01 vs A→A.
Online Figure X. Adhesion of bone marrow-derived monocytes from ApoE-/− and ApoE/FHL2-/− mice. Monocytes were extracted from the bone marrow of ApoE-/− and ApoE/FHL2-/− mice. Endothelial cells (EC) from ApoE-/− (A) or ApoE/FHL2-/− mice (F) were stimulated with TNF-α (25 ng/ml, 3h). Thereafter, adhesion of monocytes (Mono) from ApoE-/− or ApoE/FHL2-/− mice to the ECs was quantified under flow conditions (2 dynes/cm²). Data are mean ±SEM of n=4. *p<0.05 vs MonoA/ECa.
Online Figure XI. Reduced homing ApoE/FHL2-/- macrophages to atherosclerotic plaques of mice. Peritoneal thioglycollate-elicited macrophages from ApoE-/- and ApoE/FHL2-/- mice were stained with fluorescent cell markers (ApoE-/- cells: green; ApoE/FHL2-/- cells: red). Thereafter 5x10^6 cells of both genotypes were injected through the tail vein into ApoE-/- or ApoE/FHL2-/- mice fed a high fat diet during 7 weeks. Red and green fluorescent cells were counted 48 hours later within the atherosclerotic plaques of the aortic sinus. Data are mean ±SEM of n=3. Wilcoxon followed by Steel post test was performed.
Online Figure XII. Differences in immune T cell count between ApoE-/- and ApoE/FHL2-/- mice. ApoE-/- and ApoE/FHL2-/- mice were lethally irradiated and transplanted with the bone marrow of ApoE-/- (A→A, A→F) or ApoE/FHL2-/- (F→A, F→F) mice, respectively. After 7 weeks of high fat diet, thymic CD69+ (A) and FoxP3+ regulatory T cell (B) count among CD4+/CD8- T cells was assessed by flow cytometry. Data are mean ±SEM of n=3-5. *P<0.05 vs ApoE-/- or A→A.
SUPPLEMENTAL MATERIAL AND METHODS

Absence of Four-and-a-Half LIM domain protein 2 decreases atherosclerosis in ApoE-/- mice

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Material & methods:

Animal housing and diet.

B6.129P2-Apoεtm1Unc/J (ApoE−/−) male mice were obtained from Jackson laboratory (Bar Harbor, ME). FHL2 knockout (FHL2−/−) mice, initially provided by Dr. Büttner, University of Bonn, Bonn, Germany; were bread into ApoE−/− background for more than ten generations to obtain double knockout mice for ApoE and FHL-2 (ApoE/FHL2−/−). The McGill University animal care committee approved the experimental protocol, and animals were handled in accordance with institutional guidelines.

To evaluate the role of FHL2 in atherosclerosis, 12 week old ApoE−/− and ApoE/FHL2−/− male mice were given a high fat diet (TD.02028; Harlan Laboratories Inc., WI) for 7 weeks containing 21.2% fat including 1.25% cholesterol and 0.5% cholic acid.

To determine the origin of cells involved in the atheroprotective effect in the absence of FHL2, we performed irradiation-transplantation experiments. 8 week old ApoE−/− and ApoE/FHL2−/− male mice were lethally irradiated with a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International, Hamburg) 1 day before the transplantation. Bone marrow was isolated by flushing the femurs and tibias of male ApoE−/− or ApoE/FHL2−/− mice with RPMI 10% serum. Irradiated recipients received 10×10⁶ bone marrow cells by tail vein injection. After a four week recovery period, they were fed a high fat diet for 7 weeks.

To investigate the role of FHL2 in the recruitment of macrophages in the atherosclerotic plaques, we performed a macrophage homing assay. ApoE−/− and ApoE/FHL2−/− mice were fed a high fat diet for 7 weeks. Forty-eight hours before sacrifice, mice were injected with equal parts thioglycollate-elicited macrophages from ApoE/FHL2−/− mice (stained with red PHK26, Sigma Aldrich, MO) and ApoE−/− mice (green PKH2). Red and green cells within aortic sinus sections were visualized with a fluorescent microscope.

Plasma and blood analyses.

Blood was removed by cardiac puncture and was collected in in EDTA-coated tubes (Sarstedt, Germany). Plasma total cholesterol, high-density lipoprotein (HDL) and triglycerides were determined by the Montreal Clinical Research Institute. Blood cells enumeration was performed with a hematology analyzer (scil vet ABC™).

Atherosclerotic lesion characterization.

The entire aorta, from the heart to the iliac arteries, was removed and rinsed with PBS and fixed in 4% paraformaldehyde. Periadventitial tissue was removed and the aorta was cut longitudinally. The aortic surface was stained en face with oil red O (Electronic Microscopy Sciences, PA). The lesion area of the aortic arch, as defined as the region from ascending arch to the first intercostal arteries, was quantified as a fraction of the total vessel area using Image J software (National Institute of Health). The heart and the brachiocephalic artery (BCA) were removed, rinsed, fixed in 4% paraformaldehyde and incubated overnight in a 30% sucrose solution.¹ The tissues were frozen in Tissue Tek OCT medium (Sakura, CA), and serial cryosections of 6-µm thickness were cut from the aortic sinus and the BCA. Sections spanning
the entire aortic sinus or BCA (5-7 sections per animal) were stained with oil red O, and the mean lesion area measured in mm² was calculated using ImageJ software. The collagen content of the plaque was evaluated similarly after staining with picrosirius red (Polysciences, PA).

**Immunofluorescence and immunohistochemistry.**

FHL2 and macrophage content within plaques of the aortic sinus were determined by immunofluorescence. Briefly, the sections were incubated with 3% bovine serum albumin (Sigma, MO). The aortic sinus sections were then incubated with polyclonal anti-FHL2 (Santa Cruz, TX) or anti-moma-2 (Abcam, MA) primary antibodies. Sections were rinsed, and further incubated with fluorescently labeled secondary antibodies (1:500) (Invitrogen, ON). Sections were mounted with medium containing DAPI (Vector labs). Smooth muscle cell content of the plaques was evaluated by immunohistochemistry. Sections were incubated with 5% bovine serum albumin, incubated with monoclonal anti-α-smooth muscle cell actin (Sigma), and positive staining was revealed using DAB (Sigma). At least five sections per animal were stained. Negative controls were performed using sections incubated with secondary antibodies only.

**Different cell type extractions**

- **Thioglycollate-elicited monocytes/macrophages.**

In order to obtain peritoneal cells, 1.5 ml of 4% thioglycollate solution (Criterion, CA) was injected IP in ApoE−/− and ApoE/FHL2−/− mice. Ninety-six hours later, the ascitic fluid containing elicited monocytes and macrophages were collected.² FACS analysis and gene expression analysis was then performed on these cells. For macrophage homing experiments, after extraction, cells from ApoE/FHL2−/− mice were stained with PKH26 (red fluorescence) and cells from ApoE−/− mice with PKH2 (green fluorescence).

- **Bone-marrow derived monocytes**

Primary bone marrow-derived monocytes were obtained by flushing the bone marrow of both femurs and tibias of ApoE−/− and ApoE/FHL2−/− mice with RPMI. Cells were centrifuged and re-suspended in RPMI containing serum (10% FBS).

- **Endothelial cell extraction**

Endothelial cells were obtained from the lungs of ApoE−/− and ApoE/FHL2−/− mice. The lungs were rapidly excised and diced into 1-mm-sized fragments with sterile scissors in RPMI 1640. The lung fragments were digested with 0.1% collagenase A (Roche Diagnostics, Mannheim), and the resulting cell suspension was plated on a gelatin-coated flask and grown in Dulbecco's Modified Eagle Medium (DMEM)/F12 + 20% FBS + 1% ECGS. For 2 successive passages, endothelial cells were detached with trypsin and isolated using a CD102 antibody (BD Bioscience, Missisauga) coupled to Dynal beads (Invitrogen, CA).

- **Vascular smooth muscle cell extraction**

VSMCs were derived from aortas of ApoE−/− and ApoE/FHL2−/− mice. Briefly, aortas were cleaned of adipose and connective tissue and VSMCs were dissociated by enzymatic digestion. The tissue was filtered and the cell suspension centrifuged and re-suspended in DMEM/F12
containing 10% FBS, 20 mmol/L HEPES, 2 mmol/L L-glutamine, 1000 U/mL penicillin, and 1 mg/mL streptomycin. VSMCs were cultured in culture media and maintained at 37°C in a humidified incubator (5% CO₂/95% air). Cells were used after 3-6 passages.

**Analysis of aortic and monocytic cell gene expression by real-time PCR.**

Total RNA from aorta and thioglycollate elicited monocytic cells was isolated using a Total RNA Mini Kit (Geneaid, Taipei). RNA was quantified on a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, DE). cDNA was synthesized from 1µg RNA by using qScript cDNA SuperMix kit (Quanta biosciences, MD). The expression levels of Icam1, Vcam1, Cxcl3, Ccl2, Ccl5, I-10, Il1-β, Il12-β, TNF-α, Cxcr3, Ccr2, and Ccr5 were analyzed by quantitative real time polymerase chain reaction (RT-qPCR) using 7500 Fast PCR (Applied Biosystems, CA) under standard conditions of 60°C annealing temperature for 40 cycles. All primers (Table) were designed using Primer 3 Plus software. SYBR green chemistry (SYBR Green FastMix, Low ROX, Quanta biosciences) was used with specific primers (listed in Supplementary Table) for all genes. Results were analyzed using the ΔΔCt method as calibrator samples. The analyzed genes were expressed relative to the murine Rps16 housekeeping gene.

**Elisa**

IL-10 levels were measured in the supernatant of thioglycollate elicited monocytic cells using a solid phase sandwich Elisa kit (My Bio Source, CA) according to the manufacturer’s protocol.

**Flow cytometry**

Spleens were harvested and single-cell suspensions of splenocytes were prepared by gently mincing the spleen and passing through a cell strainer (100µm pores, BD Biosciences). Spleen and peritoneal cells were centrifuged for 5 minutes at 1500 rpm, resuspended in 2% normal mouse serum in PBS, and stained for the surface markers CD11B, Gr1, Ly6C, F4/80 (eBioscience, CA). Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences, CA). Fluorescence minus one controls was used to determine fluorescence background and positively. Data analysis was performed using Flow Jo software (Tree Star Inc., OR).

Gating was first performed on forward versus side scatter to remove cell debris and doublets before selection of live cells based on exclusion of a viability dye. Monocytes were selected based on their expression of CD11b marker after exclusion of Gr1+ cells (granulocytes). Among monocytes, inflammatory subtypes were detected based on their expression of Ly6C hi marker, and macrophages were selected based on their expression of F4/80 marker. Each cell population was considered positive or negative compared with degree of fluorescence when stained with appropriate isotype control antibody and using fluorescence minus one.

**Cell migration**

Migratory capacity of peritoneal monocytic cells, endothelial cells was evaluated by using modified Boyden chambers. An uncoated polycarbonate filter with 8-µm pore size (BD Biosciences) was placed between the upper and lower chamber. Ninety six hours after thioglycollate injection, the ascitic fluid containing elicited monocytes and macrophages was collected. 100000 cells were re-suspended in RPMI (0.5% FBS) and placed in the upper chamber, the lower chamber was filled with RPMI (0.5% FBS) containing 100 ng/ml MCP-1 (R &
D). Migration was evaluated 18 hours later by the mean number of DAPI-positive cells counted in at least 5 high-power fields per filter.

Migratory capacity of aortic SMCs was evaluated using the scratch assay. Cells were seeded into 24-well plates and grown to 100% confluence. The cells were then serum starved for 6 hours and wounded once by scratching across the maximum diameter of each well. Pictures were taken immediately after scratching as well as 24 hours later using an inverted microscope fitted with a Leica digital camera. Images were analyzed using Image J.

**Cell proliferation**

Peritoneal elicited cells, endothelial cells and VSMCs were cultured in RPMI (10% FBS) or DMEM/F12 (10% FBS) and starved for 18 hours. Then cells were fixed (PFA2%), permeabilized (0.5% triton) and incubated with BSA (3%), Ki67 primary antibody (1/50, Abcam) and a CY3-coupled secondary antibody (Invitrogen). Nuclei were stained with Dapi. Fluorescence was visualized with a DM2000 microscope (Leica Microsystems).

**LDL-uptake**

Peritoneal elicited cells were suspended in 2% normal mouse serum in PBS and stained for Dil-Ac LDL (5µg/ml) (Invitrogen). Flow cytometry was performed on the BD LSR Fortessa (BD Biosciences) for peritoneal cells. Data analysis was performed using Flow Jo software (Tree Star Inc.). Aortic SMCs were incubated with Dil-Ac LDL (5µg/ml) for 4 hours. Then cells were washed, fixed (PFA2%) and the nuclei were stained with Dapi. Fluorescence was visualized with a DM2000 microscope (Leica Microsystems).

**Efferocytosis**

Peritoneal elicited cells were cultured for 24 hours in RPMI (1% FBS). Raw264 macrophages were pre-stained with 10µM red cell tracker (Invitrogen), and placed under UV for 30 minutes followed by 24h incubation at 37°C to induce apoptosis. Apoptotic raw macrophages were co-incubated with peritoneal macrophages for 1 hour at a 5:1 ratio. Cells were washed, fixed and nuclei were stained with DAPI. Fluorescence was visualized with a DM2000 microscope.

**Cell apoptosis**

Apoptosis of endothelial cells from ApoE-/- and ApoE/FHL2-/- mice was evaluated using Annexin- PI staining. Briefly, cells were starved for 6 hours and stimulated with vehicle or TNF-α (50ng/ml) and cycloheximide (25µg/ml) for 16 hours. Then the treated cells were harvested and re-suspended in 100 µl binding buffer. After adding 5µl Annexin V–FITC (BD Biosciences) and 10µl PI (BD Biosciences) into the cell suspension, the samples were incubated for 15 min at room temperature in the dark. The apoptotic index was immediately determined by flow cytometry.

**Micro vessel sprouting**

Aortae from ApoE-/- and ApoE/FHL2-/- mice were excised, cut into 1 mm rings and embedded in Matrigel (BD Biosciences) in a 96 well plate. Matrigel was polymerized for 30 minutes at 37°C. Embedded rings were covered with of endothelial cell growth medium and cultivated for 8 days. Pictures were taken using an inverted microscope fitted with a Leica digital camera, and analyzed using Image J.
Flow adhesion assay

A flow adhesion assay was performed as previously described. Briefly, endothelial cells from ApoE-/- and ApoE/FHL2-/- mice were seeded on gelatin-coated µ-Slides VI0.4 (Ibidi, Germany) and confluent monolayers were stimulated with TNF-α (10 ng/ml; R&D Systems, UK) overnight. Bone marrow-derived monocytes were freshly isolated from ApoE-/- and ApoE/FHL2-/- mice. Monocytes were perfused over endothelial monolayers during 5 minutes at a constant shear stress of 2 dynes/cm² using a syringe pump. Adherent monocytes were counted thereafter using an inverted microscope fitted with a Zeiss digital camera.

Reactive oxygen species (ROS) production

ROS production within the atherosclerotic plaques was assessed on cryosections of the BCA. Sections were incubated with the ROS-sensitive fluorescent dye DHE (2 µM), in the dark, for 5 min. Fluorescence was imaged with fluorescent microscope fitted with a Leica digital camera. DHE fluorescence intensity per total plaque surface area was quantified with ImageJ.

Statistical analysis

Results are expressed as mean±SEM. All data was first evaluated for normal distribution using the Shapiro-Wilks test. When the distribution was normal, we performed a Student’s t-test to compare two groups, or we performed a two way ANOVA followed by the Dunnett’s post test for multiple group comparisons. When the groups were small the normal distribution test could not be performed (online figures VI-XII), we performed the non parametric Mann-Whitney U test to compare two groups, or the Friedman test followed by the Steel post test for multiple group comparisons. A probability value of p<0.05 was considered to be statistically significant using GraphPad Prism software and JMP10.

References:


Table. Oligonucleotide sequences

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