Elucidating the pathogenesis underlying bicuspid aortic valve disease using new disease models

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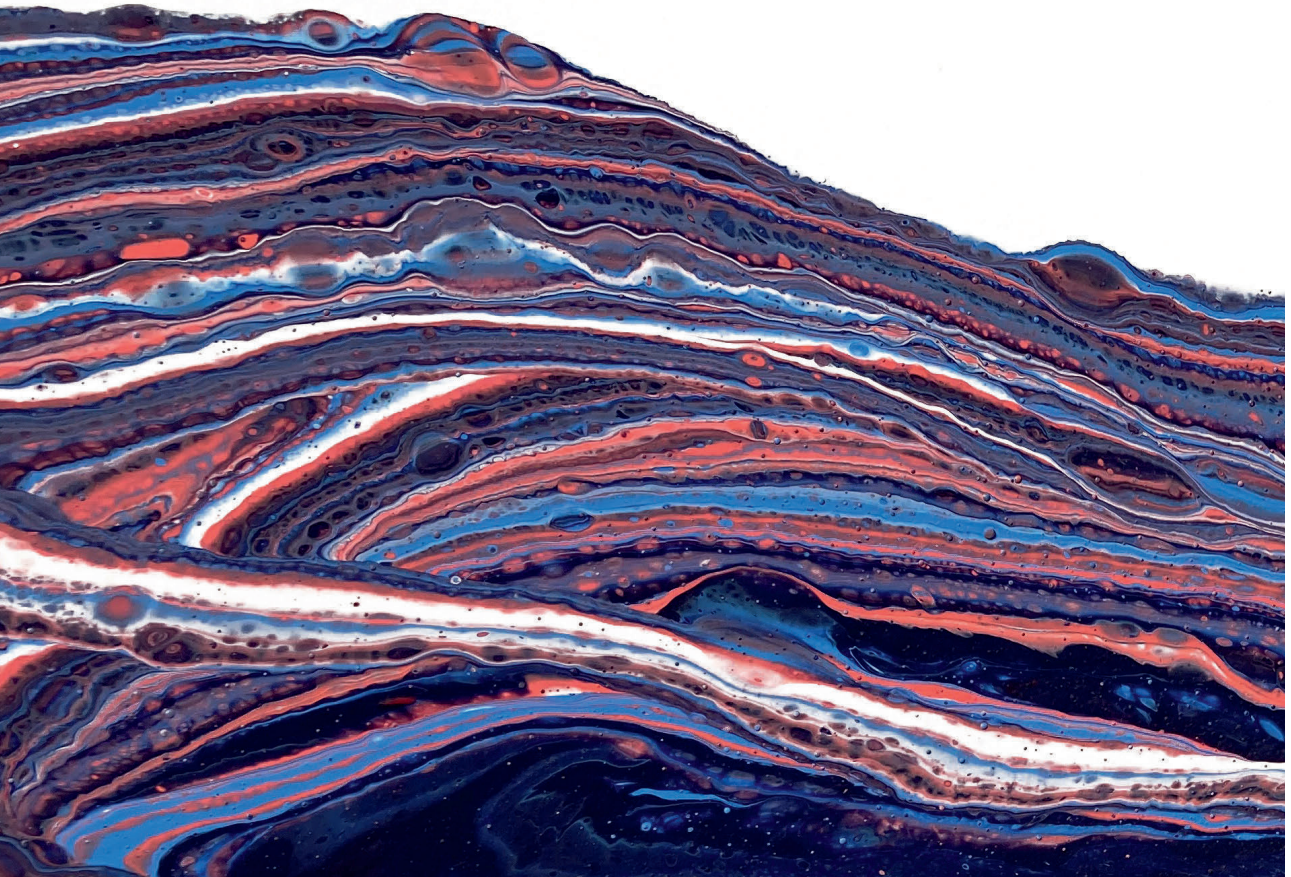

Chapter 9

LIM-only protein FHL2 attenuates inflammation in vascular smooth muscle cells through inhibition of the NFκB pathway

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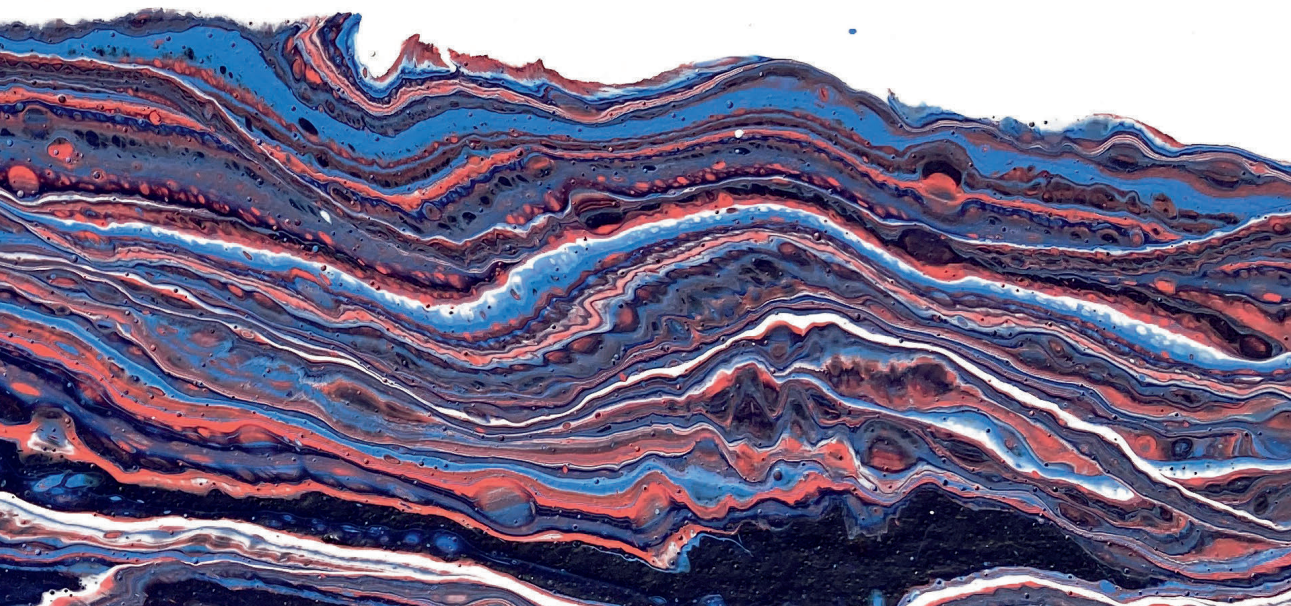

Abstract

Despite the advent of new-generation drug-eluting stents, in-stent restenosis remains a significant problem in patients with coronary artery disease. In- stent restenosis is defined as the gradual re-narrowing of a stented coronary artery lesion due to arterial damage with subsequent local inflammation of the vessel wall and excessive growth of the vascular smooth muscle cells (vSMCs). Four-and-a-half LIM-domain protein 2 (FHL2) is a scaffold protein involved in regulating vSMC function and inflammation. Previously we have demonstrated that FHL2 prevents vSMC proliferation in a murine carotid artery ligation model. However, the effect of FHL2 on the inflammatory response of the vSMCs is not investigated. Therefore, we studied the inflammatory response in the vessel wall of FHL2-deficient (-KO) mice after carotid artery ligation. We found that circulating cytokines and local macrophage infiltration in the ligated carotid vessels were increased in FHL2-KO mice after carotid artery ligation. Moreover, FHL2-KO vSMCs showed increased secretion of cytokines such as SDF-1α and RANTES, and enhanced activation of the NFκB pathway. Finally, we found that blocking the NFκB signaling pathway abrogated this pro-inflammatory state in FHL2-KO vSMCs. Taken together, our results demonstrate that FHL2 decreases the inflammatory response of vSMCs through inhibition of the NFkB-signaling pathway.

Introduction

With age, atherosclerotic plaques build up inside the human cardiovascular system, preferentially at sides with disturbed flow. Especially when occurring inside coronary arteries, these plaques can be life-threatening when reaching a point when the vessel is too narrow to supply the heart with sufficient blood. Furthermore, rupture of instable plaques can cause acute vessel occlusion and can lead to myocardial infarction. In many cases, a bare-metal stent is placed to widen the lumen of the narrowed artery and restore blood flow. Unfortunately, a large number of the patients (± 6-43%) develop in-stent restenosis due to growth of the intima/media into the lumen of the stent, which can already occur within 1 month after stent placement [1,2].

Placing a stent causes de-endothelialization of the underlying surface, initiating a wound healing response [3]. Due to the arterial damage, endothelial cells become dysfunctional and secrete several growth factors, cytokines and chemokines which in turn promote recruitment of inflammatory cells such as leukocytes and neutrophils into the injury site [4]. Furthermore, the release of chemokines such as stromal cell derived factor-1α (SDF-1α/CXCL12) induces a smooth muscle cell (SMC) phenotypic switch from contractile to synthetic phenotype allowing proliferation and migration of SMCs towards the damaged intima [5]. Moreover, macrophage infiltration and lipid deposition can create neoatherosclerosis within the restenotic tissue [3]. With time, these processes narrow the lumen of the artery requiring another intervention. Although it is well established that inflammation plays a pivotal role in the pathogenesis of in-stent restenosis [6,7], the factors that regulate the inflammatory process are still largely unknown.

Four-and-a-half LIM-domain 2 (FHL2) is a LIM-only protein that can act as a scaffold protein. By binding other proteins, it can affect signaling of pathways controlling many cellular processes such as inflammation, apoptosis and migration [8,9]. FHL2 has been shown to act as a cofactor for several transcription factors including Nur77, androgen receptor, LXR and NFκB [9-11]. FHL2-KO mice do not present with cardiovascular pathologies, but when triggered by for example a Western-Type diet, adrenergic stimulation or carotid artery ligation, the importance of FHL2 for homeostasis of the cardiovascular system becomes very apparent [12-14]. Carotid artery ligation in mice induces a vascular healing response similar to in-stent restenosis, involving intimal thickening, inflammation and extensive SMC proliferation [15]. We demonstrated previously that SMC proliferation and the formation of SMC-rich lesions are increased in FHL2-KO mice after carotid artery ligation, demonstrating that absence of FHL2 enhances disease progression [12].

As a scaffolding protein, FHL2 interacts with various proteins and modulates several pathways in a cell- and context-dependent fashion. For example, FHL2 has been shown to induce expression of IL-6 and IL-8 via activation of the NFκB signaling pathway in liver and muscle cells [16]. In line with this, bone marrow derived macrophages from FHL2-KO mice exhibited decreased levels of IL-6 and TNFα in response to LPS [16]. In contrast, bone marrow derived macrophages from FHL2-KO also showed increased expression of TNFα, MCP-1 and SDF-1α following LPS stimulation [17]. Interestingly, peritoneal macrophages from FHL2-KO mice displayed no change in pro-inflammatory cytokine expression following LPS stimulation [18]. However, under basal conditions, FHL2-KO mice exhibited higher serum protein levels of S100A8/A9, a well-established pro-inflammatory marker [18,19]. Taken together, these data suggests that FHL2 regulates the inflammatory response in a highly context- and tissue-dependent manner. Therefore, we investigated the role of FHL2 in the inflammatory response of vascular SMCs *in vitro* and in a murine carotid artery ligation model. In this study, we found that both systemic and local vascular inflammation is increased in FHL2-KO mice after carotid artery ligation. In addition, FHL2 deficient SMCs secreted increased amounts of chemokines and cytokines, which was decreased to baseline when FHL2 levels were exogenously increased. Finally, we show that pharmacological inhibition of the NFκB pathway significantly attenuated the increased inflammatory status of FHL2 deficient SMCs signifying that FHL2 interferes with this specific signaling pathway.

Results

FHL deficiency increases inflammation in carotid artery ligation

Previous studies showed that carotid artery ligation causes an increase in SMC-rich lesion area in FHL2-deficient mice compared to control mice [12]. Since inflammation is an important trigger for intimal hyperplasia, we determined the plasma levels of the pro-inflammatory cytokine SDF-1α in FHL2-KO and WT mice 4 weeks after ligation. We found a significant increase of SDF-1α in plasma of FHL2-KO mice compared to WT control (Figure 1A). In addition, baseline plasma levels of SDF-1α were elevated in FHL2-KO mice compared to WT animals (Figure 1B). An increase in plasma SDF-1α levels reflects a systemic increase in inflammation that may drive monocyte recruitment from the bone-marrow [4,20].

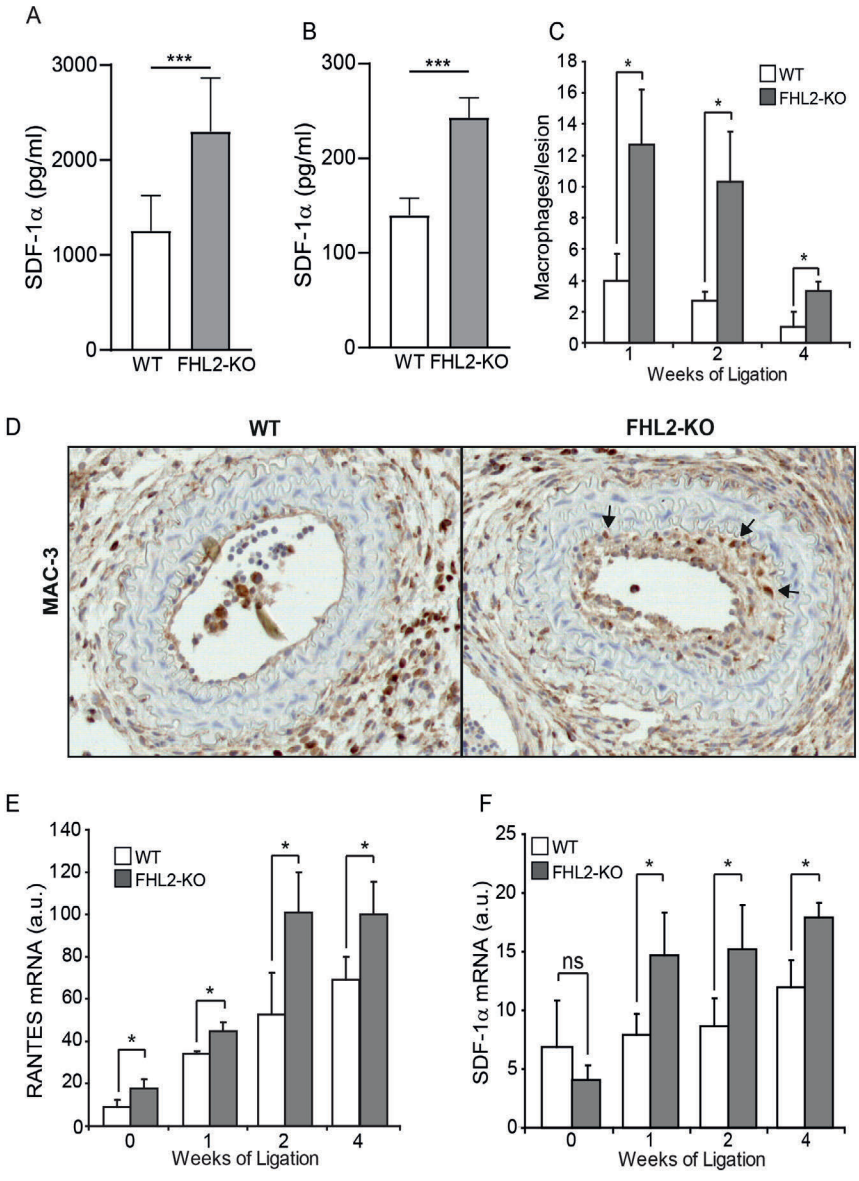

Figure 1. FHL2 deficiency increases inflammatory protein expression. A. Plasma levels of SDF-1α in the WT and FHL2-KO mice after 4 weeks of carotid artery ligation. B. Plasma levels of SDF-1α in the WT and FHL2-KO mice at baseline. C. Quantification of MAC-3 immunostaining on WT and FHL2-KO carotid arteries ligated for 1,2 or 3 weeks. D. Representative images of MAC-3 staining of WT and FHL2-KO carotid arteries ligated for 2 weeks. Arrows indicate positive cells. E,F. Graphs showing results of qRT-PCR for mRNA expression of RANTES (E) and SDF-1α (F) in the ligated vessels from WT and FHL2-KO mice for the indicated time periods. Data represent means±SD, *p<0.05, ***p<0.005.

To determine if there was an influx of macrophages in the local microenvironment, we stained carotid lesions for MAC-3, a protein present on differentiating and activated macrophages, allowing the number of macrophages in the vessel to be quantified. In the first week after ligation the number of macrophages were the highest, and decreased over time in both WT and FHL2-KO mice (Figure 1C,D). However, at all timepoints analysed (1, 2 and 4 weeks after ligation), the number of macrophages was significantly increased in the FHL2-KO mice compared to WT animals. Next we determined mRNA levels of RANTES and SDF-1α in ligated carotid artery tissue at these different timepoints. Both inflammatory mediators showed increasing expression over time in FHL2-KO and WT mice (Figure 1E-F), but the expression levels of RANTES were significantly higher in FHL2-KO mice compared to WT animals under both basal and ligated conditions. Although SDF-1α levels were unchanged at baseline in FHL2-KO mice, carotid artery ligation resulted in significantly higher the levels of SDF-1α in FHL2-KO mice compared to WT counter parts.

FHL2-KO SMCs express increased inflammatory markers in vitro

Activated SMCs can produce an array of inflammatory cytokines that are instrumental in progression of vascular diseases, including restenosis [21,22]. Therefore, to further substantiate our *in vivo* observations, we isolated aortic SMCs from FHL-KO and WT mice. Upon serum-starvation, we measured the mRNA levels of SDF-1α in cells and protein levels in the supernatants of WT and FHL2-KO SMCs. Consistent with the *in vivo* findings, both mRNA expression and protein levels of SDF-1α were significantly increased in FHL2-KO SMCs compared to WT SMCs (Figure 2A,B). Moreover, FHL2-
KO SMCs showed a significant increase in expression of several other proinflammatory cytokines, namely protein expression of IL-6 and MCP-1, and mRNA expression of RANTES and CXCL-1 compared to WT SMCs (Figure 2C-D). Altogether, FHL2-KO SMCs exhibit a pro-inflammatory phenotype *in vitro*.

FHL2 attenuates inflammation via inhibition of the NFκB pathway

Previous reports showed that activation of NFκB pathway plays a crucial role in neointima formation and cytokine production [6,23,24]. Since FHL2 can act as a cofactor for NFκB in different cell types, we determined mRNA levels of the inhibitor of NFκB signal transduction, IκBα, and found that IκBα levels were slightly decreased in serum-starved FHL2-KO SMCs compared to WT cells. Interestingly, stimulation with serum decreased IκBα expression only in FHL2-KO SMCs, suggesting that the pro-inflammatory phenotype of the FHL2-KO SMCs is regulated via activation of the NFκB pathway (Figure 3A). To further study

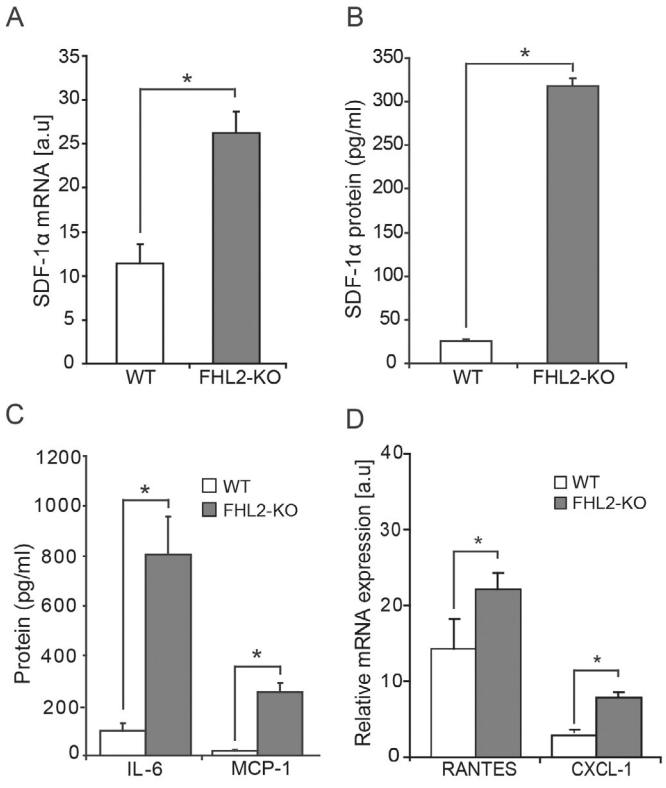

Figure 2. FHL2-KO smooth muscle cells show increased inflammatory markers in FHL2-KO *in vitro*. A. Semiquantitative RT-PCR was performed to assess mRNA expression of SDF-1α in serum-starved SMCs (n=2). B. Protein levels in supernatants of serum-starved SMCs measured by ELISA (n=3). C. Graph showing ELISA results of IL-6 and MCP-1 protein expression (C) or semi-quantitative RT-PCR for mRNA of RANTES and CXCL1 (D) of serum-starved SMCs stimulated with FCS for 8h (n=2). Data represent means±SD, *p<0.05.

the effect of FHL2 on the NFκB signaling pathway, we determined NFκB transcription in FHL2-deficient SMCs by using two distinct NFκB luciferase-reporter constructs. As expected, in normal culture conditions, NFκB transcription was significantly higher in FHL2-KO SMCs versus WT SMCs (Figure 3B). Moreover, NFκB activity is markedly higher in FHL2-KO SMCs under TNFα stimulated conditions as demonstrated by both NFκB reporter plasmids (Figure 3C). To further elucidate the effect of FHL2 on NFκB signaling in SMCs, we made use of a pharmacological inhibitor of NFκB, BAY11-7085, which inhibits of IκBα phosphorylation, thereby keeping the NFκB dimer sequestered in the cytoplasm [25]. Inhibition of NFκB with BAY11-7085 decreased mRNA expression of IL6, MCP-1, RANTES and SDF-1α significantly in the FHL2-KO SMCs (Figure 3D).

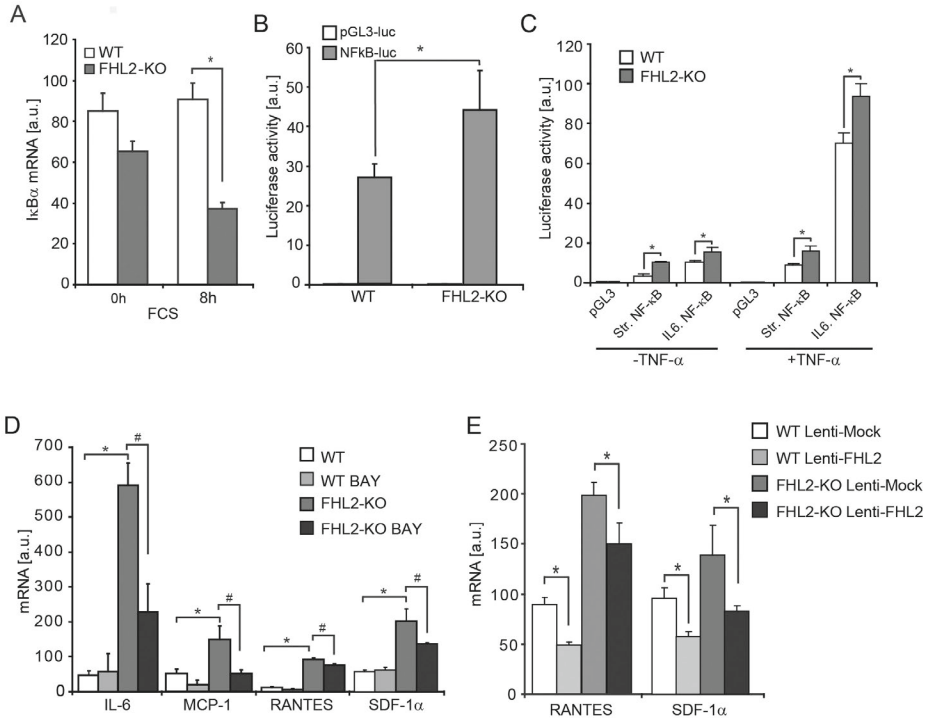

Figure 3. FHL2 attenuates inflammation via the NFκB-pathway. A. Graph showing qRT-PCR results for mRNA expression of IκBα in serum-starved SMCs stimulated with FCS. B. Graph showing NFκB-response element reporter-plasmid luciferase activity in serum-starved SMCs stimulated with TNF-α. C. Graphs showing the activity of NF-κB was monitored in serum-starved SMCs transfected with a construct containing the NF-κB response element of the Stratagene (Str.) or IL-6 promoter stimulated with or without TNF-α. D. Graph showing qRT-PCR results for mRNA expression of IL-6, MCP-1, RANTES and SDF1-α in serum-starved SMCs treated with or without BAY11-7085. E. Graph showing qRT-PCR results of mRNA expression of RANTES and SDF1-α, in WT and FHL2-KO SMCs transduced with FHL2-lentivirus. Data represent means±SD. *p<0.05 for FHL2-KO versus WT. #p<0.05 for BAY or PD versus control.

Finally, when FHL2 levels were restored in FHL2-KO SMCs using FHL2 lentivirus, the expression levels of RANTES and SDF-1α were significantly decreased (Figure 3E). Taken together, these data support that FHL2-KO SMCs exhibit a high pro-inflammatory phenotype via sustained activation of the NFκB pathway. In Figure 4 we summarized our data in a schematic representations.

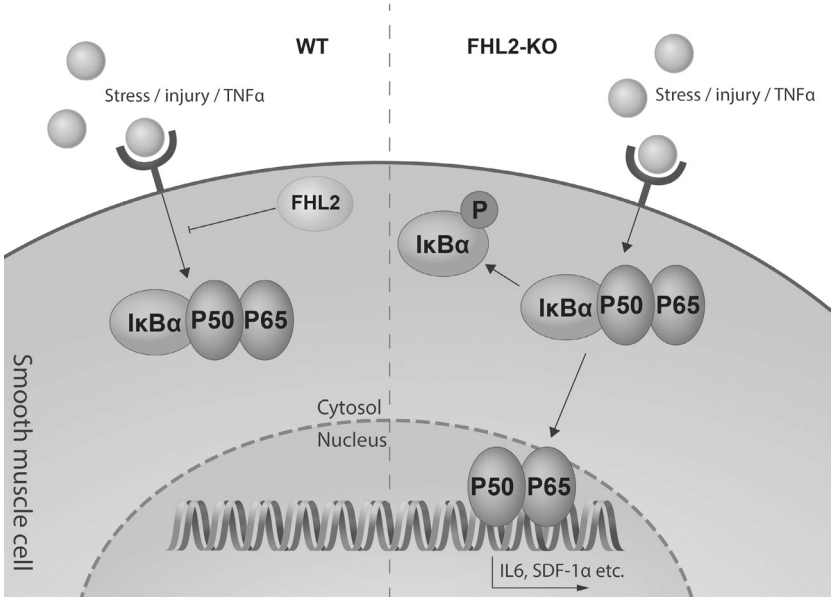

Figure 4. FHL2 regulates inflammation in vascular smooth muscle cells through inhibition of NFkB pathway. The left panel shows the normal situation where FHL2 inhibits NFkB pathway in the cytosol, whereas the right panel represents the effect of FHL2 deficiency resulting in enhanced activity of NFkB pathway.

Discussion

Numerous studies have demonstrated that neointima formation is associated with increased cytokine and chemokine production by SMCs [26-28]. In this study we found that FHL2 deficiency increases both local and systemic inflammation after ligation of the carotid artery. Moreover, FHL2-deficiency induces the secretion of multiple inflammatory cytokines by SMCs, among which IL-6, MCP-1, CXCL1, RANTES and SDF-1α. Furthermore, we show that blocking the NFκB pathway, the inflammatory phenotype of FHL2-KO SMCs is reverted.

The NFκB signaling pathway is a central regulator of inflammatory events associated with neointima formation. FHL2 has previously been shown to modulate NFκB activity in osteoclasts, even though FHL2 does not directly interact with NFκB [29]. We found that NFκB transcriptional activity is also constitutively enhanced in FHL2 depleted SMCs and consistent with this, we observed diminished IκBα expression in FHL2-KO SMCs. These data indicate that the function of endogenous FHL2 involves inhibition of the inflammatory response of SMCs. It has been shown that FHL2-KO mice show higher levels of inflammation in the serum under basal conditions [18]. In the current study, we also found that higher

levels of SDF-1α in the serum of FHL2-KO mice compared to WT mice under basal conditions. Furthermore, we demonstrated that FHL2 deficient SMCs exhibit a proinflammatory phenotype compared to WT SMCs through a sustained activation of NFκB pathway.

Previously it has been reported that FHL2 can induce or attenuate inflammation depending on the cell type involved in various disease models [16-19]. Therefore, FHL2 as a scaffold protein,

can regulate inflammatory responses of multiple cell types in a cell-and context-dependent manner. Further research is warranted to delineate the molecular mechanism of FHL2 on inflammation in various cell types in different disease contexts.

We have reported previously that FHL-KO mice showed an increased SMC-rich lesion area in the ligated vessels *in vivo*, and increased proliferation of FHL2-KO SMCs was also observed *in vitro* [12]. Interestingly, our results concerning SDF-1α complement the explanation for increased lesion area upon FHL2-deficiency. It has been shown that SDF-1α is abundantly expressed after vascular injury and was identified in a genome-wide association study of myocardial infarction [30] and also associates with carotid artery disease [4,31]. After vascular injury, platelets account for short-term SDF-1α release whereas SMCs mediate long-term SDF-1α release to contribute to the process of vascular remodeling and repair [32-34]. SDF-1α is also known to recruit circulating SMC progenitor cells into the vessel wall, but this aspect has not been addressed in the current study [33]. We show SDF-1α expression is markedly higher in the plasma of FHL2-KO mice than of WT mice. Furthermore, FHL2-KO SMCs displayed higher levels of SDF-1α expression and overexpression of FHL2 restored SDF-1α expression in FHL2-KO SMCs. Hence, we propose that enhanced SDF-1α levels along with other inflammatory cytokines in FHL2-KO mice may contribute to neointima formation by promoting platelet activation, possibly by recruiting SM-progenitor cells, and by enhancing proliferation and migration of SMCs.

Previous research on ApoE-/- mice showed a decrease in atherosclerotic lesion size in absence of FHL2 [13]. Moreover, FHL2-/- mice on a cholesterol-enriched diet show the same decrease [35]. Atherosclerosis is primarily initiated by lipid accumulation and activation of mononuclear cells whereas carotid artery ligation induces a SMC based pathology. This might be at the root of the differences in vascular pathogenesis in $FHL2^{-/-}$ mice.

In conclusion, FHL2 deficiency causes an increased inflammatory response after carotid artery ligation. *In vitro*, FHL2-KO SMCs secrete increased amounts of cytokines and show enhanced activation of NFκB pathway. Exogenous FHL2 efficiently blocked the production of these cytokines. These results indicate that

FHL2 inhibits the inflammatory response of vascular SMCs through attenuating NFkB-signaling.

Methods

Animals and Left carotid artery ligation

All mice experiments were approved by the local animal ethic committee of the Amsterdam Medical Center, University of Amsterdam, The Netherlands (DBC102226) and were carried out according to the guidelines issued by the Dutch government. The procedure of murine left carotid artery ligation has been described previously [12].

Isolation and culturing of mouse aortic SMCs

Aortas from age and sex-matched WT and FHL2-KO mice were harvested and aortic SMCs were prepared as described previously [12].

Lentiviral transduction and Luciferase reporter assays in SMCs

Recombinant lentiviral particles encoding FHL2 were produced, concentrated, and titrated as described previously [11,12]. Transduction of SMCs was described previously [11,12]. Transient transfection and reporter assays were carried out in serum starved SMCs with the NFκB luciferase reporter plasmids using Fugene6 transfection reagent (Roche) according to the manufacturer's protocol and described previously [12].

Cytokines measurement by ELISA

Cytokine levels in mouse serum and cell supernatants were measured using the Cytometric Bead Array mouse inflammation kit (BD Biosciences, San Diego, CA). SDF-1α secretion was measured by ELISA (RayBiotech).

qPCR

RNA was isolated from cells using the Total RNA mini kit (Bio-Rad) and from tissue using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was made using the iScript cDNA synthesis kit (Bio-Rad). Real-time reverse transcription PCR was performed using the MyIQ system (Bio-Rad) and the following mouse primers: SDF-1α forw: 5'-CCTCGGTGTCCTCTTGCTGTCC-3', SDF-1α rev: 5'-GGCTCTGGCGATGTGGCTCTC-3', IL6 forw: 5'-GTTCTCTGGGAAATCGTGGA -3', IL6 rev: 5'-GGAAATTGGGGTAGGAAGGA-3', MCP-1 forw: 5'-AGCAGGTGTCCCAAAGAAGC-3', MCP-1

rev: 5′-TGAAGACCTTAGGGCAGATGC-3′. RANTES forw: 5′-TCGTGCCCACGTCAAGGAGTATTT-3′,, RANTES rev: 5′-TCTTCTCTGGGTTGGCACACACTT-3′,; CXCL-1 forw: 5′-GCTGGGATTCACCTCAAGAA-3′, CXCL-1 rev: 5′- AGGTGCCATCAGAGCAGTCT-3′. As an internal control for cDNA content of the samples, acidic ribosomal phosphoprotein P0 was measured (P0 forw: 5′-GGACCCGAGAAGACCTCCTT-3′, P0 rev: 5′-GCACATCACTCAGAATTTCAATGG-3′).

Immunostaining

Mouse carotid sections were fixed and stained as previously described [12]. Briefly, Paraffin sections were deparaffinized and rehydrated. Sections were boiled and blocked with 1% BSA in 0.1% Tween-PBS. Then sections were incubated overnight at 4°C with primary antibodies directed against MAC3 (1:1000;M3/84, BD Pharmingen) followed by an HRP-conjugated secondary goat anti-rabbit antibody. DAB substrate was used for detection. After counterstaining with hematoxylin all the sections were embedded in pertex (HistoLab).

Statistical analysis

Statistical assays were performed using Graph Pad Prism (version 7). All tests were analyzed using Students t-test. Data are reported as mean±SD unless otherwise specified. P values <0.05 were considered as statistically significant.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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