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Disruption of the Novel Nested Gene Aff3ir Mediates Disturbed Flow-Induced Atherosclerosis in Mice

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eLife Assessment

The study presents **valuable** findings on the role of Aff3ir, a gene implicated in flow-induced atherosclerosis and regulating the inflammation-associated transcription factor, IRF5. The in vivo data are **solid** in providing evidence on the role of Aff3ir in shear stress and formation of atheromatous plaques. The work will be of interest to clinical researchers and biologists focusing on inflammation and atherosclerosis in cardiovascular disease with a broad eLife readership.

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Abstract

Disturbed shear stress-induced endothelial atherogenic responses are pivotal in the initiation and progression of atherosclerosis, contributing to the uneven distribution of atherosclerotic lesions. This study investigates the role of Aff3ir-ORF2, a novel nested gene variant, in disturbed flow-induced endothelial cell activation and atherosclerosis. We demonstrate that disturbed shear stress significantly reduces Aff3ir-ORF2 expression in athero-prone regions. Using three distinct mouse models with manipulated AFF3ir-ORF2 expression, we demonstrate that AFF3ir-ORF2 exerts potent anti-inflammatory and anti-atherosclerotic effects in $ApoE^{-/-}$ mice. RNA sequencing revealed that interferon regulatory factor 5 (IRF5), a key regulator of inflammatory processes, mediates inflammatory responses associated with AFF3ir-ORF2 deficiency. AFF3ir-ORF2 interacts with IRF5, promoting its retention in the cytoplasm, thereby inhibiting the IRF5-dependent inflammatory pathways. Notably, IRF5 knockdown in AFF3ir-ORF2 deficient mice almost completely rescues the aggravated atherosclerotic phenotype. Moreover, endothelial-specific AFF3ir-ORF2 supplementation using the CRISPR/Cas9 system significantly ameliorated endothelial activation and

atherosclerosis. These findings elucidate a novel role for AFF3ir-ORF2 in mitigating endothelial inflammation and atherosclerosis by acting as an inhibitor of IRF5, highlighting its potential as a valuable therapeutic approach for treating atherosclerosis.

Introduction

Atherosclerosis, characterized by the formation of fibrofatty lesions in the arterial wall, is a leading cause of morbidity and mortality worldwide, contributing to most myocardial infarctions and many strokes (Herrington, Lacey, Sherliker, Armitage, & Lewington, 2016; Libby et al., 2019). The activation of vascular endothelial cells (ECs), induced by various chemical and mechanical stimuli, such as lipopolysaccharide and shear stress, is an initial step in the development of atherosclerosis (Davignon & Ganz, 2004). Consequently, atherosclerotic lesions preferentially develop at the branches and curvatures of the arterial tree, where blood flow is disturbed (Davis, Earley, Li, & Chien, 2023). For decades, researchers have been interested in exploring the mechanisms underlying mechanotransduction during endothelial activation caused by disturbed flow (Davis et al., 2023). Several mechanosensitive proteins, such as YAP/TAZ (B. Li et al., 2019), Annexin A2 (Zhang et al., 2020), BMP4 (Sorescu et al., 2004), and NAD(P)H oxidase (Hwang et al., 2003; Jo, Song, & Mowbray, 2006), have been identified as key regulators of disturbed shear stress in ECs and have been implicated in the progression of atherosclerosis. Emerging evidence has revealed that pharmacological or genetic inhibition of endothelial YAP activation ameliorates the progression of atherosclerotic plaques in mice (B. Li et al., 2019; Wang et al., 2016; Yang et al., 2021), indicating that targeting disturbed flow-induced endothelial activation could be a promising therapeutic strategy for atherosclerosis. However, the precise mechanisms by which the disturbed flow exerts detrimental effects remain unclear.

The interferon regulatory factor (IRF) family of transcription factors, comprising nine members (IRF1-IRF9) in mammals, is primarily characterized by its role in mediating antiviral responses and type I interferon production (Sato, Taniguchi, & Tanaka, 2001). Although these members share a conserved DNA-binding domain in their N-terminal region that recognizes similar DNA sequences, IRF5 plays a central role in inflammation (Almuttaqi & Udalova, 2019; Takaoka et al., 2005). IRF5 mediates the production of proinflammatory cytokines, including IL-12b and IL-23a, and promotes the expression of inflammatory genes (Cai, Yao, & Li, 2017; Saliba et al., 2014; Weiss, Blazek, Byrne, Perocheau, & Udalova, 2013). It promotes inflammatory responses in various immune cells, including macrophages (Seneviratne et al., 2017), neutrophils (Weiss et al., 2015), and B cells (Savitsky, Yanai, Tamura, Taniguchi, & Honda, 2010). Global or myeloid-specific knockouts of IRF5 have been shown to exert anti-atherosclerotic effects (Leipner et al., 2021; Seneviratne et al., 2017). Despite the established importance of IRF5 in immune cells, its restrictively regulatory mechanism and role in shear stress-induced endothelial activation remain unknown.

We recently reported that a novel protein-coding nested gene, *Aff3ir*, contributes to endothelial maintenance by promoting the differentiation of vascular stem/progenitor cells (SPCs) into ECs (Zhao Y et al., 2024). AFF3ir-ORF2, encoded by the *Aff3ir* transcript variant 2, is predominantly expressed in the EC layer of the mouse aorta (Zhao Y et al., 2024). Notably, our recent study indicated that the overexpression of AFF3ir-ORF2 could enhance laminar flow-induced mRNA levels of essential EC markers in SPCs (Zhao Y et al., 2024), suggesting that AFF3ir-ORF2 may be a novel mechanotransduction protein in ECs. However, the regulation of Aff3ir-ORF2 under disturbed flow and its role in atherosclerosis remain unclear.

In this study, we aimed to elucidate the mechanism by which disturbed blood flow induces endothelial activation and atherosclerosis. Our study showed that disrupted Aff3ir-ORF2 expression in athero-prone regions led to inflammatory responses and development of

atherosclerosis. Aff3ir-ORF2, the expression of which is reduced by disturbed shear stress, exerts critical anti-inflammatory effects by binding to IRF5 and mitigating disturbed shear stress-induced IRF5 activation. Additionally, we demonstrated that endothelial-specific supplementation with Aff3ir-ORF2 significantly ameliorated disturbed flow-induced endothelial activation and the development of atherosclerotic plaques, highlighting its promising therapeutic potential for the treatment of atherosclerosis.

Results

Disturbed shear stress reduces the expression of AFF3ir-ORF2

Our recent study showed the active participation of *Aff3ir* in EC differentiation from vascular SPCs induced by laminar shear stress (Zhao Y et al., 2024), suggesting the potential involvement of this novel protein-encoding nested gene in mediating hemodynamic stimulation. To further elucidate the functional role of *Aff3ir* and its encoded proteins in disturbed shear stress-induced EC activation, we examined the expression of *AFF3* and *AFF3ir* in the intima of mouse aorta. We found that the mRNA level of *AFF3ir*, but not its parent gene *AFF3*, was significantly lower in the intima of the aortic arch, an area exposed to disturbed shear stress, compared to the intima of the thoracic aorta, which was exposed to steady unidirectional shear stress (B. Li et al., 2019) (**Figure 1A**). *Aff3ir* transcript variants can generate two proteins (Zhao Y et al., 2024), therefore, we measured the protein levels of AFF3ir-ORF1 and AFF3ir-ORF2. While AFF3ir-ORF1 and AFF3 showed comparable expression levels in the intima of aortic arch and thoracic aorta of mice, the expression of AFF3ir-ORF2 showed an 87% reduction in the intima of aortic arch compared to the intima of thoracic aorta (**Figure 1B, C**), suggesting that AFF3ir-ORF2 may be a novel mechanosensitive protein that responds to disturbed shear stress. Enface immunofluorescence staining confirmed a marked reduction in AFF3ir-ORF2 expression in the inner curvature of aortic arch compared to both the outer curvature of aortic arch and the thoracic aorta (**Figure 1D, E**). Moreover, to demonstrate the change in AFF3ir-ORF2 within the same visual field, we examined its expression in longitudinal sections of the mouse aorta (B. Li et al., 2019). We found that the expression of AFF3ir-ORF2, but not AFF3, was notably downregulated in athero-prone regions (the inner curvature of the aortic arch and bifurcation of the carotid artery) compared to that in the protective region in the outer curvature of the aortic arch (**Figure 1F**). Additionally, we found that the expression of AFF3, AFF3ir-ORF1, and AFF3ir-ORF2 in the media and adventitia was comparable between the aortic arch and the thoracic aorta (Figure S1A, B).

Next, we explored the impact of disturbed shear stress on AFF3ir-ORF2 expression *in vitro*. Mouse embryonic fibroblasts (MEFs) exhibit responses consistent with those of ECs (Chen et al., 2010; Wen et al., 2013), therefore, we investigated AFF3ir-ORF2 expression in MEFs from WT mice exposed to static or disturbed flow (0.5 ± 4 dyn/cm^2, 1 Hz). Consistent with our *in vivo* findings, while disturbed shear stress increased the expression of VCAM-1, a critical inflammatory marker of ECs (Nakashima, Raines, Plump, Breslow, & Ross, 1998), it significantly reduced both the protein and mRNA levels of AFF3ir-ORF2 (**Figure 1G–I**). The expression of AFF3 in response to the disturbed flow was minimally affected at both the mRNA and protein levels (**Figure 1G–I**). These results collectively demonstrate that disturbed shear stress induces a reduction in AFF3ir-ORF2 expression both *in vivo* and *in vitro*.

AFF3ir-ORF2 ameliorates disturbed shear stress-induced inflammation and atherosclerosis

Disturbed shear stress-induced atherogenic responses are initial events in atherosclerotic plaque formation (Davis et al., 2023). To elucidate the regulatory role of AFF3ir-ORF2 in disturbed shear stress-induced inflammation, we overexpressed AFF3ir-ORF2 in MEFs. AFF3ir-ORF2

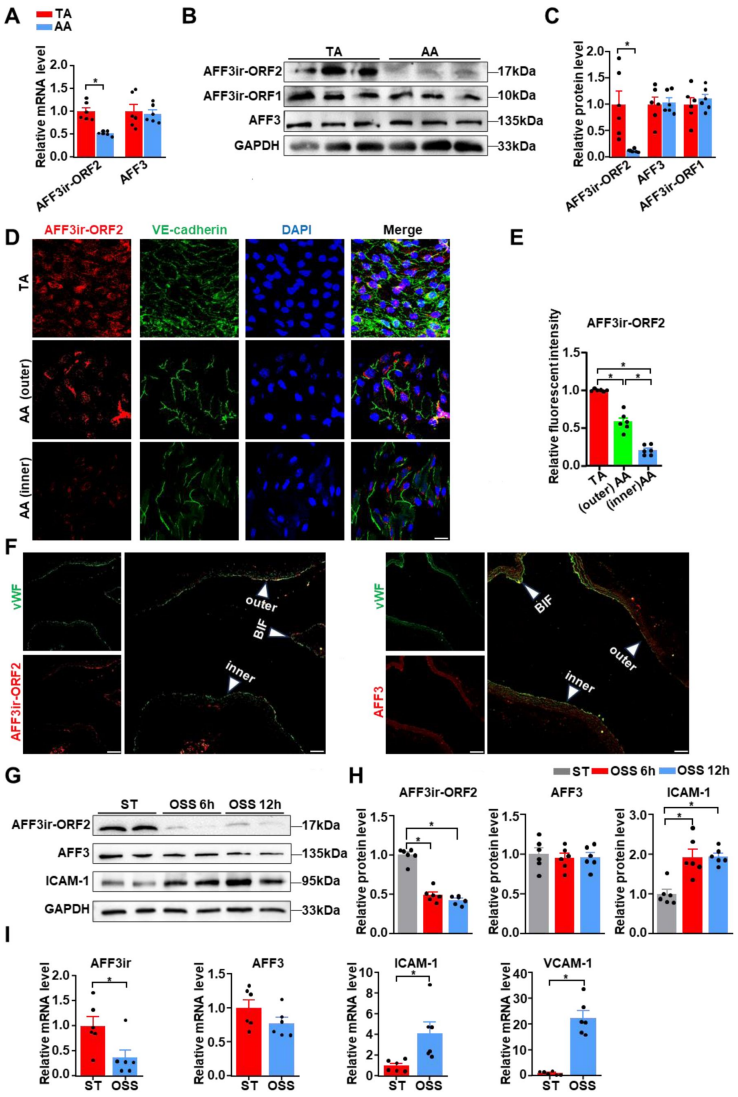

Figure 1.

Disturbed shear stress reduces the expression of AFF3ir-ORF2 *in vivo* and *in vitro*.

A, RT-PCR analysis of the mRNA levels of *AFF3ir-ORF2* and AF4/FMR2 family member 3 (*AFF3*) in the intima of thoracic aorta (TA) and aortic arch (AA) of C57BL/6 mice. Data are presented as mean ± SEM (n=6 mice per group). **P*<0.05, unpaired two-tailed *t*-test. **B, C,** Western blot analysis of the expression of the indicated proteins in the intima of TA and AA of C57BL/6 mice. Protein levels were normalized to those of GAPDH, and the relative expression values were compared to those of the TA group. Data are presented as mean ± SEM (n=6 mice per group). **P*<0.05, unpaired two-tailed *t*-test. **D, E,** En-face immunofluorescence staining of AFF3ir-ORF2, VE-cadherin, and DAPI, and quantification of AFF3ir-ORF2 expression in inner curvature of the AA (AA inner), outer curvature of the AA (AA outer), and TA of C57BL/6 mice. Scale bar, 20 μm. The immunofluorescence intensity of AFF3ir-ORF2 was normalized to that of DAPI, and the relative expression values were compared to that of the TA group. Data are presented as mean ± SEM (n=6 mice per group). **P*< 0.05, one-way ANOVA with Tukey post-test. **F,** Representative immunofluorescent staining for von Willebrand factor (vWF), AFF3ir-ORF2, and AFF3 in longitudinal aortic sections of C57BL/6 mice. n=6 mice per group. Scale bar, 25 μm. Inner, inner curvature of the AA; outer, outer curvature of the AA; BIF, Bifurcation. **G, H,** Mouse embryonic fibroblasts (MEFs) isolated from the embryo of C57BL/6 mice were subjected to static (ST) or oscillatory shear stress (OSS, 0.5 ± 4 dyn/cm^2, 1 Hz) for indicated time. Western blot analysis of the indicated proteins. Protein levels were normalized to GAPDH and the relative expression values were compared to that of the ST group. Data are mean ± SEM (n = 6 independent experiments). **P*< 0.05, one-way ANOVA with Tukey post-test. **I,** MEFs were subjected to ST or OSS treatment for 6 h. RT-PCR analysis of the mRNA levels of *AFF3ir*, *AFF3,* intercellular adhesion molecule 1 (*ICAM*-1), and vascular cell adhesion molecule 1 (*VCAM-1*) in MEFs. Data are mean ± SEM (n = 6 independent experiments). **P*< 0.05, unpaired two-tailed *t*-test.

overexpression attenuated ICAM-1 expression induced by disturbed shear stress at both the protein and mRNA levels (**Figure 2A-B**, Figure S2A). To further validate our findings in ECs, we overexpressed AFF3ir-ORF2 in human umbilical vein endothelial cells (HUVECs). Consistent with our previous results, AFF3ir-ORF2 overexpression reduced the protein level of ICAM-1 induced by disturbed shear stress in HUVECs (**Figure 2C-D**). Moreover, AFF3ir-ORF2 overexpression attenuated disturbed shear stress-induced expression of several inflammatory genes, including VCAM-1, IL-6, and IL-1β, in both MEFs and ECs. (Figure S2A-B). Interestingly, we found that AFF3ir-ORF2 overexpression did not affect the basal expression of these inflammatory genes under ST conditions (Figure S2A-B), likely due to the relatively low levels of inflammatory gene expression under ST compared to OSS conditions. Furthermore, we measured the concentrations of inflammatory factors, including IL-6 and IL- 1β, in the culture medium of MEFs. As expected, while AFF3ir-ORF2 overexpression had little effect on the concentrations of IL-6 and IL-1β under ST condition, it significantly reduced their release induced by disturbed shear stress (**Figure 2E**).

Given the anti-inflammatory effects of AFF3ir-ORF2, we speculated that it may ameliorate disturbed shear stress-induced inflammation and atherosclerosis *in vivo*. ApoE knockout ($ApoE^{-/-}$) mice were subjected to partial ligation surgery to induce disturbed flow in the left carotid arteries (LCAs). The endothelium of the LCAs was intravascularly infected with adenovirus (Ad-Scramble or Ad-AFF3ir-ORF2) prior to surgery (Nam et al., 2009; Zhang et al., 2020). Enface immunofluorescence staining confirmed the successful AFF3ir-ORF2 overexpression in the left carotid artery (Figure S2C-D). Mice infected with Ad-AFF3ir-ORF2 exhibited a significant decrease in lesion area in the LCAs compared to those infected with Ad-Scramble (23±17% vs 63±14%) (**Figure 2F-H**), with no obvious plaque formation observed in the right carotid arteries (Figure S2E). Overexpression of AFF3ir-ORF2 also attenuated disturbed flow-induced inflammatory responses, as evidenced by decreased VCAM-1 expression in the endothelium of LCAs (**Figure 2I-J**). These findings suggested that AFF3ir-ORF2 ameliorates shear stress-induced inflammation and atherosclerosis.

AFF3ir-ORF2 deficiency aggravates inflammation and atherosclerotic lesions in $ApoE^{-/-}$ mice

To explore the effects of AFF3ir-ORF2 on inflammation and atherosclerosis, we generated AFF3ir-ORF2 global knockout ($AFF3ir\text{-}ORF2^{-/-}$) mice. Genotyping PCR (Figure S3A) and western blot analysis of AFF3ir-ORF2 expression in mouse aortas (Figure S3B-C) confirmed the successful knockout. No obvious phenotypic abnormalities were observed in $AFF3ir\text{-}ORF2^{-/-}$ mice up to 20 weeks of age and monitoring was discontinued thereafter. Additionally, AFF3ir-ORF2 deficiency did not alter systolic blood pressure, diastolic blood pressure, or mean arterial pressure (Figure S3D), suggesting that AFF3ir-ORF2 is dispensable for physiological blood pressure maintenance. We then isolated MEFs from WT and $AFF3ir\text{-}ORF2^{-/-}$ mice. RT-PCR analysis confirmed the deficiency of AFF3ir-ORF2 in $AFF3ir\text{-}ORF2^{-/-}$ MEFs (Figure S3E). Interestingly, the AFF3ir-ORF2 knockdown efficiency showed discrepancies between the western blot (Figure S3B) and RT-PCR results (Figure S3E). In addition to the technical differences between PCR and western blot, the characteristics of AFF3ir-ORF2 may also contribute to this inconsistency. The parent gene, AFF3, is located in a genetically variable region, and it can be excised via intron 5 to form a replicable transposon that translocates to other chromosomes, potentially contributing to leukemia (Chinen et al., 2008; Hiwatari et al., 2003; Miller, Leventaki, Harker-Murray, Drendel, & Bone, 2022; von Bergh et al., 2002). AFF3ir, located in intron 6, exists within this transposon, which may complicate the measurement of its expression. Furthermore, we found that AFF3ir-ORF2 deficient MEFs displayed higher expression of inflammatory genes, including *ICAM-1*, *VCAM-1*, and *IL-1b*, compared to those in WT MEFs, under disturbed flow stimulation (Figure S3F).

Next, we crossed $AFF3ir\text{-}ORF2^{-/-}$ mice with $ApoE^{-/-}$ mice to generate double-knockout ($ApoE^{-/-}$ $AFF3ir\text{-}ORF2^{-/-}$) mice. Eight-week-old $ApoE^{-/-}$ and $ApoE^{-/-}AFF3ir\text{-}ORF2^{-/-}$ mice were fed a high-fat diet for 12 weeks to induce atherosclerosis (**Figure 3A**). *En face* Oil-Red O staining indicated that

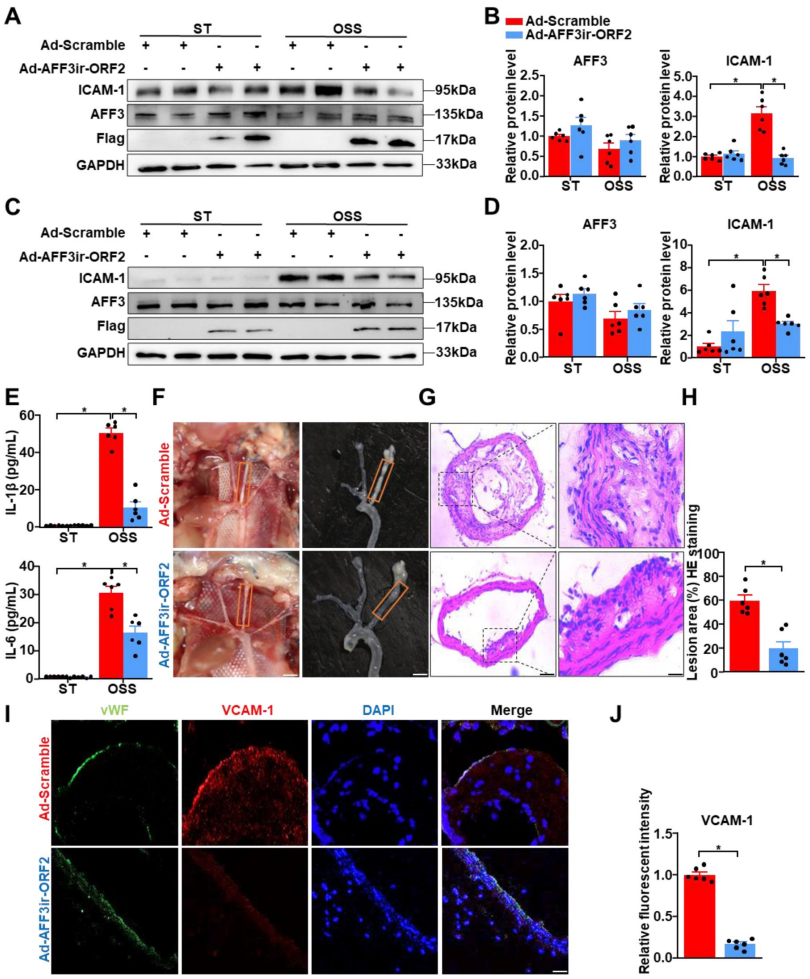

Figure 2.

AFF3ir-ORF2 overexpression alleviates disturbed flow-induced inflammation and atherosclerosis.

A-B, Mouse embryonic fibroblasts (MEFs) isolated from C57BL/6 mice were infected with indicated adenoviruses (Ad-Scramble or Ad-Aff3ir-ORF2) for 48 h and then exposed to static (ST) or oscillatory shear stress (OSS, 0.5 ± 4 dyn/cm^2, 1 Hz) for another 6 h. Western blot analysis of the indicated proteins and quantification of their relative expression levels are shown. The protein levels were normalized to GAPDH and the relative expression values were compared to MEFs infected with Ad-Scramble and treated with ST. Data are presented as mean ± SEM (n = 6 independent experiments). *P<0.05, two-way ANOVA with Tukey post-test. **C- D,** Human umbilical vein endothelial cells (HUVECs) were infected with Ad-Scramble or Ad- AFF3ir-ORF2 for 48 h and then exposed to ST or OSS for an additional 6 h. Western blot analysis of the indicated proteins and quantification of their relative expression levels are shown. The protein levels were normalized to GAPDH and the relative expression values were compared to HUVECs infected with Ad-Scramble and treated with ST. Data are presented as mean ± SEM (n = 6 independent experiments). *P<0.05, two-way ANOVA with Tukey post-test. **E,** MEFs were infected with Ad-Scramble or Ad-Aff3ir-ORF2 for 48 h and then exposed to ST or OSS for another 6 h. The concentration of IL-6 and IL-1β in cell culture medium were dectected with ELISA. The relative cytokines levels is relative to MEFs infected with Ad- Scramble and treated with ST. Data are presented as mean ± SEM (n = 6 independent experiments). *P< 0.05, two-way ANOVA with Tukey post-test. **F-J,** Eight-week-old male ApoE$^{-/-}$ mice were subjected to partial ligation of the carotid artery along with 10 μL of adenovirus suspension at 1 × 10^8 transducing units (TU)/mL was instilled into the left carotid artery (LCA). The mice were then fed high-fat diet for 4 weeks. **F,** Arterial tissues were isolated to examine the atherosclerotic lesions. Scale bar, 2 mm. **G, H,** LCAs were sectioned for hematoxylin and eosin staining. Quantification of the lesion area in LCAs was shown. Scale bar, 25 μm. Data are presented as mean ± SEM (n=6 mice per group). *P<0.05, unpaired two-tailed *t*-test. **I, J,** Immunofluorescence staining for vWF, VCAM-1, and DAPI in the LCAs, and quantification of the relative fluorescent intensity of VCAM-1. The immunofluorescence intensity of VCAM-1 was normalized to DAPI, and the relative expression values were compared to that of the Ad-Scramble group. Scale bar, 50 μm. Data are presented as mean ± SEM (n=6 mice per group). *P<0.05, unpaired two-tailed *t*-test.

AFF3ir-ORF2 deficiency accelerated the development of atherosclerosis in the entire aorta, AA, and TA. (**Figure 3B, C**). Furthermore, AFF3ir-ORF2 deletion increased the lesion area and lipid deposition in the aortic roots of ApoE$^{-/-}$ mice without altering the collagen fiber content (**Figure 3D, E**). Similar results were observed in distributing arteries (LCAs) (**Figure 3F, G**). Given that the expression of adhesion proteins, such as VCAM-1 in ECs is crucial for monocyte infiltration into plaques (Kobiyama & Ley, 2018), we assessed VCAM-1 expression in the aortic roots of these mice. We found that AFF3ir-ORF2 deletion increased VCAM-1 expression in the aortic roots of ApoE$^{-/-}$ mice (**Figure 3H, I**), indicating that the atherogenic effects of AFF3ir-ORF2 deletion may result from endothelial inflammation. Additionally, there were no significant differences between the two groups in body weight or triglyceride, total cholesterol, LDL cholesterol, and HDL cholesterol levels (Figure S4A, B), indicating that the atherogenic effect of AFF3ir-ORF2 silencing is unlikely to be related to lipid metabolism. Taken together, these results indicate that AFF3ir-ORF2 deficiency aggravates inflammation and atherosclerotic lesions in mice.

AFF3ir-ORF2 mitigates disturbed shear stress-induced inflammation by interacting with IRF5 and retaining it within the cytosol

To explore the mechanism by which AFF3ir-ORF2 mitigates atherogenesis, we performed RNA sequencing (RNA-seq) on MEFs from WT and AFF3ir-ORF2$^{-/-}$ mice. The Principal component analysis plot depicted clear clustering of WT versus AFF3ir-ORF2$^{-/-}$ samples (**Figure 4A**). We identified 1,167 upregulated and 310 downregulated genes in the AFF3ir-ORF2$^{-/-}$ group, with a criterion of 1.5-fold change and P<0.05 (**Figure 4B**, Figure S5A). All the differentially expressed genes were subjected to bioinformatics enrichment analysis using Gene Ontology (GO) databases. GO analysis showed that these genes were mainly enriched in processes, including leukocyte cell-cell adhesion, regulation of cell−cell adhesion, and leukocyte activation involved in immune response (**Figure 4C**), which is highly consistent with the phenotypes observed in AFF3ir-ORF2$^{-/-}$ mice. To further investigate the function features of these differentially expressed genes in the context of the atherosclerotic microenvironment, we mapped the differential gene list onto the atherosclerosis-related gene dataset (Rouillard et al., 2016), resulting in 363 overlapping genes. GO analysis of these genes revealed enrichment in processes related to cell−cell adhesion and leukocyte activation involved in immune response (Figure S5B), which is highly consistent with the observed effects of AFF3ir-ORF2 on VCAM- 1 expression.

To further identify the upstream transcriptional regulators of these genes, we used the list of differentially expressed genes from the RNA-seq data to predict upstream transcription factors using the ChEA3 database (Keenan et al., 2019). Then, the top 20 transcription factors obtained from the ChEA3 database were mapped to the atherosclerotic disease-related gene list in the Disgenet database (Pinero et al., 2017). Interferon regulatory factor 5 (IRF5) and IRF8 were identified as key upstream regulators (**Figure 4D**). IRF5 and IRF8, which are members of the same family of transcription factors originally implicated in interferon production, have been identified as critical regulators of the inflammatory response and contribute to the pathogenesis of various inflammatory diseases (Almuttaqi & Udalova, 2019; Salem, Salem, & Gros, 2020). However, their potential roles in disturbed shear stress-induced inflammation remain unclear. We speculated that AFF3ir-ORF2 interacts with IRF5 and/or IRF8. Coimmunoprecipitation assays indicated that endogenous AFF3ir-ORF2 could bind to both IRF5 and IRF8 (**Figure 4E**). To determine which transcription factor that mediates the inflammatory effects of AFF3ir-ORF2 deficiency, we silenced IRF5 and IRF8 in WT and AFF3ir-ORF2$^{-/-}$ MEFs exposed to disturbed flow. Notably, silencing IRF5, but not IRF8, blunted the upregulation of inflammatory genes, including *ICAM-1*, *VCAM-1*, *IL-6*, and *IL-1β* (**Figure 4F**), suggesting that IRF5 was the predominant factor mediating the anti-inflammatory effects of AFF3ir-ORF2 in the context of disturbed shear stress. Consistently, we found that IRF5 silencing significantly inhibited the upregulation of ICAM-1 protein levels induced by AFF3ir-ORF2 deficiency under disturbed shear stress (**Figure 4G, H**).

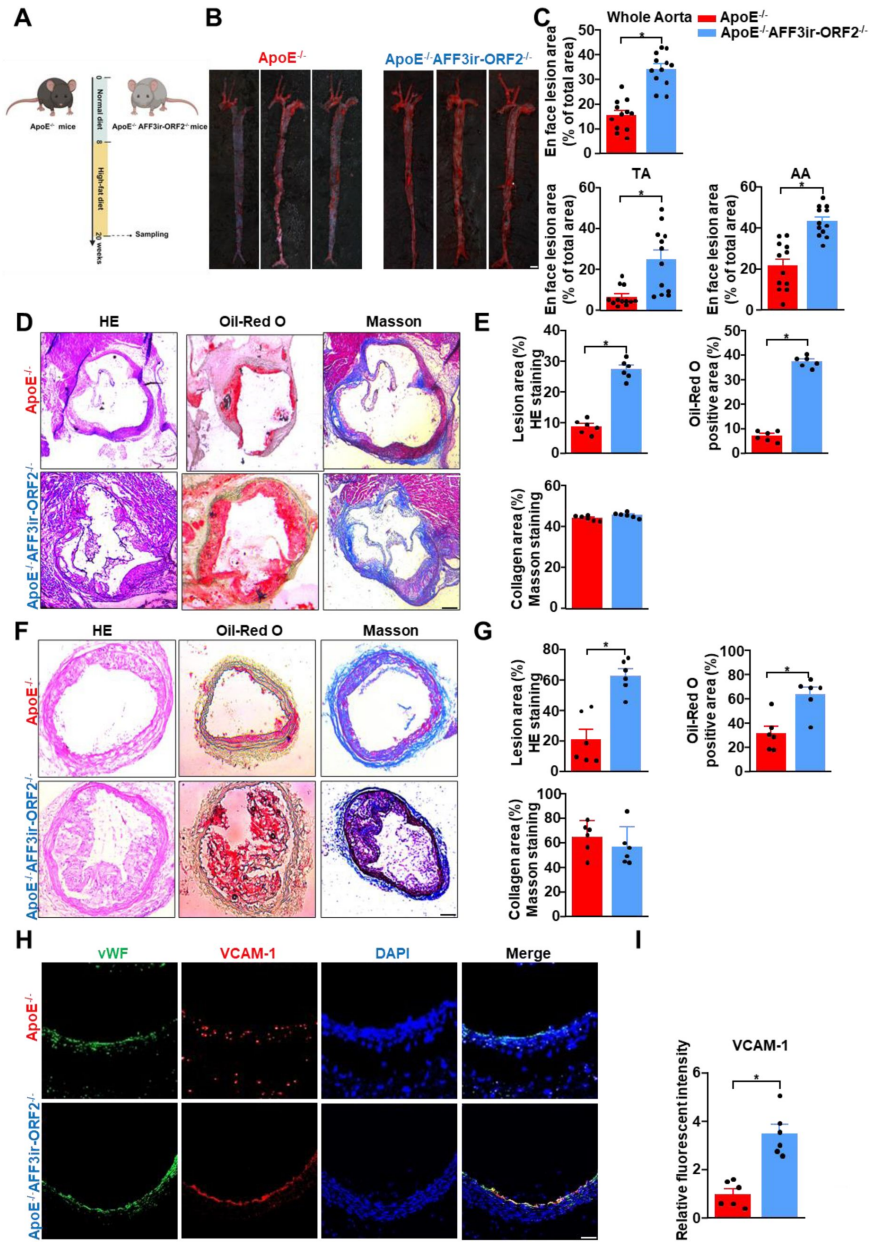

Figure 3.

AFF3ir-ORF2 deletion aggravates inflammation and atherosclerotic lesions in ApoE$^{-/-}$ mice.

Eight-week-old male ApoE$^{-/-}$ and ApoE$^{-/-}$AFF3ir-ORF2$^{-/-}$ mice were fed a high-fat diet for 12 weeks. Arterial tissues and aortic roots were isolated to examine atherosclerotic lesions. **A,** Schematic of experimental strategy. **B,** Representative images of en face Oil-Red O staining of the aortas. Scale bar, 4 mm. **C,** Quantification of the plaque area in the whole aorta, aortic arch (AA), and thoracic aorta (TA). Data are presented as mean ± SEM (n=12 mice per group). *P<0.05, unpaired two-tailed t-test. **D,** Oil-Red O, hematoxylin and eosin (HE), and Masson staining of the aortic roots. Scale bars, 500 μm. **E,** Quantification of plaque size, Oil-Red O- positive area, and collagen fiber content in aortic root sections. Data are presented as mean ± SEM (n=6 mice per group). *P< 0.05, unpaired two-tailed t-test. **F**, LCAs were sectioned and stained with Oil-Red O, HE, and Masson's trichrome. Scale bars, 500 μm. **G,** Quantification of plaque size, Oil-Red O-positive area, and collagen fiber content in the LCA sections. Data are presented as mean ± SEM (n=6 mice per group). *P<0.05, unpaired two-tailed t-test. **H,** Representative immunofluorescence images of vWF, VCAM-1, and DAPI in the aortic roots. Scale bar, 500 μm. **I**, Quantification of the relative fluorescence intensity of VCAM-1. The immunofluorescence intensity of VCAM-1 was normalized to that of DAPI, and the relative expression values were compared to that of the ApoE$^{-/-}$ group. Data are presented as mean ± SEM (n=6 mice per group). *P<0.05, unpaired two-tailed t-test.

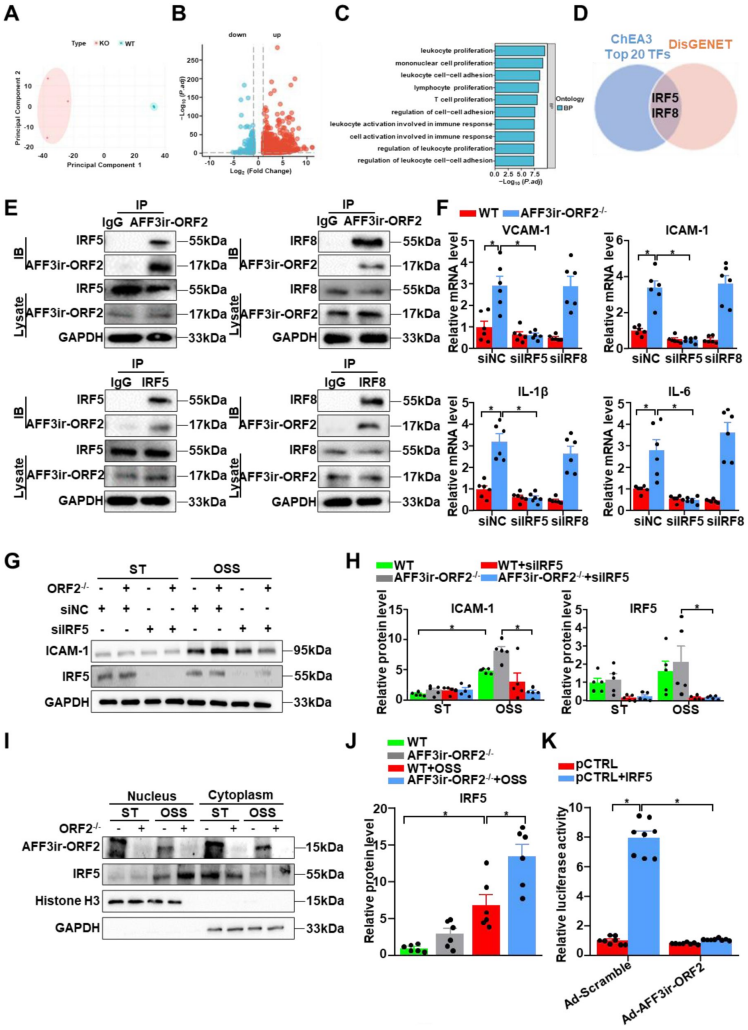

Figure 4.

AFF3ir-ORF2 mitigates disturbed shear stress-induced inflammation by interacting with IRF5 and retaining it within the cytosol.

A–D, Mouse embryonic fibroblasts (MEFs) were isolated from wild-type (WT) and AFF3ir-ORF2$^{-/-}$ mice. **A,** Principal component analysis (PCA) analysis of RNA-seq data to visualize sample to sample variation. **B**, Volcano map showing mRNA profiles of WT and AFF3ir-ORF2$^{-/-}$ MEFs (n=3). **C,** Gene Ontology enrichment pathway analysis of the differentially expressed genes. **D**, Venn diagrams of the top 20 transcription factors from the ChEA3 and DisGENET analysis related to atherosclerosis. **E,** Immunoprecipitation performed using antibodies against AFF3ir-ORF2, IRF5, and IRF8. n = 3 independent experiments. **F–H,** WT and AFF3ir-ORF2$^{-/-}$ MEFs were subjected to silence of Control (siNC), IRF5 (siIRF5), or IRF8 (siIRF8) with siRNAs for 24 h, followed by exposure to static (ST) or oscillatory shear stress (OSS, 0.5 ± 4 dyn/cm^2, 1 Hz) for another 6 h. **F**, RT-PCR analysis of the mRNA levels of VCAM-1, ICAM-1, IL-6, and IL-1β. The relative expression values were compared to WT MEFs transfected with siNC and treated with ST. Data are mean ± SEM (n = 6 independent experiments). *P< 0.05, two-way ANOVA with Tukey post-test. **G, H**, Representative western blots of IRF5 and ICAM-1 expression. Data are mean ±SEM (n = 5 independent experiments). *P< 0.05, two-way ANOVA with Tukey post-test. **I, J**, WT, and AFF3ir-ORF2$^{-/-}$ MEFs were exposed to ST or OSS for 6 h. Nuclear and cytoplasmic proteins were extracted from the cells. Representative western blots of the indicated proteins and quantification of IRF5 expression in nucleus are shown. The expression of these proteins was relative to the level of nuclear IRF5 in ST-treated WT MEFs. Data are mean ± SEM (n = 6 independent experiments). *P< 0.05, one-way ANOVA with Tukey post-test. **K,** HEK293 cells were transfected with the firefly luciferase reporter plasmid containing the IRF5-responsive ZNF217 promoter along with a β- galactosidase reporter plasmid for 24 hours. Cells were infected with the indicated adenoviruses (Ad-Scramble or Ad-Aff3ir-ORF2) for 24 h. Promoter activity was measured using luciferase, which was normalized to β-gal. Data are mean ±SEM (n = 6 independent experiments). *P< 0.05, two-way ANOVA with Tukey post-test.

In addition, neither IRF5 nor IRF8 expression levels were affected by AFF3ir-ORF2 deficiency (Figure S5C). However, we found that AFF3ir-ORF2 deficiency significantly increased the expression of IRF5-targeted genes (predicted by the ChEA3 database), including ICAM-1, CCL5, and CXCL10 (Figure S5D). Notably, the protein level of IRF5 was not significantly affected by disturbed shear stress (**Figure 4G, H**). Consistently, the mRNA levels of IRF5 were previously reported to be barely changed in the context of disturbed shear stress (GSE276195, Figure S5E) or the atherosclerotic environment (GSE222583, Figure S5F). Given that the transcriptional activity of IRF5 depends on its nuclear translocation (Lv et al., 2024), we next explored whether AFF3ir-ORF2 affects the subcellular localization of IRF5. Subcellular fractionation assays indicated that IRF5 was predominantly localized in the cytoplasm under static conditions, but exhibited obvious nuclear localization when exposed to disturbed shear stress (**Figure 4I, J**). While the total expression of IRF5 was barely affected by AFF3ir-ORF2 deficiency or overexpression, nuclear localization of IRF5 increased with AFF3ir-ORF2 deficiency (**Figure 4I, J**). To further ascertain the role of AFF3ir-ORF2 in regulating the transcriptional activity of IRF5, we performed a luciferase reporter assay (Qiao, Lv, Qiao, Wang, & Miao, 2022). AFF3ir-ORF2 overexpression significantly decreased the transcriptional activity of IRF5 (**Figure 4K**). In summary, these results suggested that AFF3ir-ORF2 acts as an endogenous inhibitor of IRF5 and exerts anti-inflammatory effects by retaining IRF5 in the cytosol.

IRF5 knockdown prevents the aggravation of atherosclerosis induced by ORF2 deficiency

Next, we investigated the role of IRF5 in disturbed flow-induced atherosclerosis *in vivo* and whether it mediates the atherogenic phenotype associated with AFF3ir-ORF2 deficiency. ApoE$^{-/-}$ and ApoE$^{-/-}$AFF3ir-ORF2$^{-/-}$ mice were subjected to partial ligation surgery in the LCAs and intravascularly infected with lentiviruses expressing either IRF5-specific shRNA (lenti-shIRF5) or Scramble shRNA (lenti-shScramble). En-face immunofluorescence staining confirmed successful IRF5 deletion in the left carotid artery (Figure S6A). After a 4-week high-fat diet challenge, IRF5 deletion resulted in an approximately 60% reduction in plaque area in the LCAs of ApoE$^{-/-}$ mice (**Figure 5A, B**). In addition, IRF5 deletion attenuated endothelial activation, as evidenced by reduced VCAM-1 expression in the endothelium of LCAs (**Figure 5C, D**). Notably, although ApoE$^{-/-}$AFF3ir-ORF2$^{-/-}$ mice exhibited an increased plaque area in the LCAs compared to ApoE$^{-/-}$ mice, IRF5 deletion almost completely abolished these differences, reducing both the plaque area and VCAM-1 expression in the endothelium of the LCAs (**Figure 5A–D**). These findings provide in vivo evidence that AFF3ir-ORF2 deficiency-induced atherosclerosis is mediated by endothelial IRF5.

Endothelial-specific AFF3ir-ORF2 supplementation ameliorates EC activation and atherosclerosis in mice

Given the significant anti-inflammatory effects of AFF3ir-ORF2 on endothelial activation and atherosclerosis, we explored the potential use of gene therapy targeting AFF3ir-ORF2 to treat atherosclerosis. Endothelial-specific AFF3ir-ORF2 overexpression was achieved using an EC-enhanced AAV-mediated CRISPR/Cas9 genome-editing system controlled by an EC-specific ICAM2 promoter as we previously reported (Z. Y. Li et al., 2024; Swiech et al., 2015). ApoE$^{-/-}$ mice infected with AAV-ICAM2-Control or AAV-ICAM2- AFF3ir-ORF2 were fed a high-fat diet for 12 weeks (**Figure 6A**). En-face immunofluorescence staining confirmed successful AFF3ir-ORF2 overexpression in ECs (Figure S7A-B). Endothelial-specific AFF3ir-ORF2 overexpression had a minimal effect on triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol levels in the plasma of mice (Figure S7C). However, compared to the negative control, endothelial-specific AFF3ir-ORF2 overexpression significantly reduced the Oil-red O- positive lesion area in the whole aortas of ApoE$^{-/-}$ mice (19±5% vs 54±8%) (**Figure 6B, C**). Moreover, endothelial-specific AFF3ir-ORF2 overexpression reduced the lesion area and lipid deposition in the aortic roots of ApoE$^{-/-}$

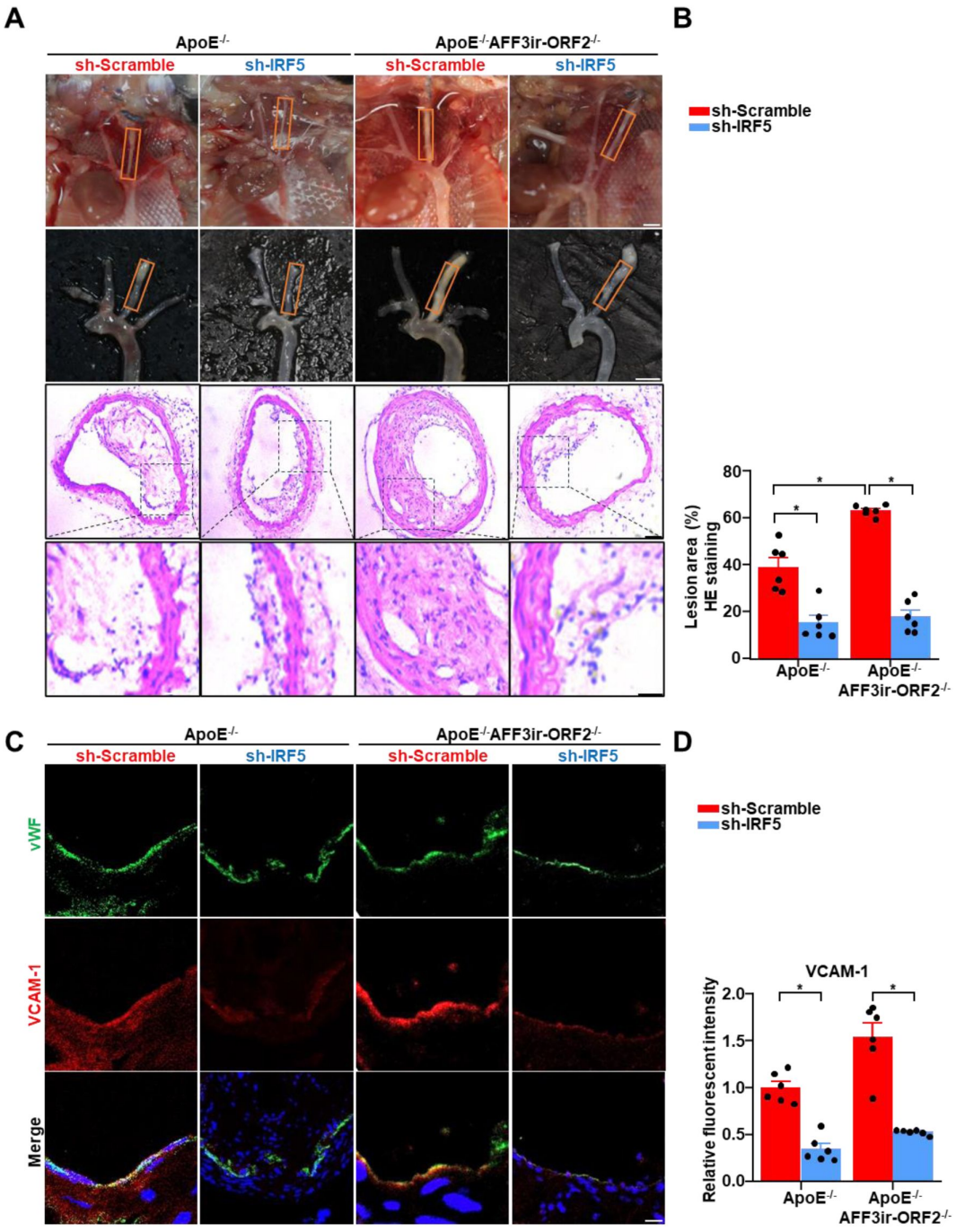

Figure 5.

IRF5 knockdown prevents the aggravation of atherosclerosis in AFF3ir-ORF2 deficienct mice.

Eight-week-old male ApoE$^{-/-}$ mice were subjected to partial ligation of the left carotid artery (LCA) along with 10 μL of lentivirus suspension at 1 × 10^{8} transducing units (TU)/mL was instilled into the LCA. The mice were then fed a high-fat diet for 4 weeks. **A-B,** Arterial tissues were isolated to examine the atherosclerotic lesions. LCAs were sectioned for hematoxylin and eosin staining. Quantification of the lesion area in LCAs was shown. Data are mean ± SEM (n=6 mice per group). *P< 0.05, two-way ANOVA with Tukey post-test. Scale bar: 2 mm for gross images, 25 μm for staining images. **C, D,** Immunofluorescence staining for vWF, VCAM- 1, and DAPI in the LCAs and quantification of the relative fluorescent intensity of VCAM-1. Scale bar, 50 μm. The immunofluorescence intensity of VCAM-1 was normalized to DAPI, and the relative expression values were compared to that of the group of ApoE$^{-/-}$ mice infected with Ad-scramble. Data are presented as mean ± SEM (n=6 mice per group). *P< 0.05, two-way ANOVA with Tukey post-test.

mice without altering the collagen fiber content (**Figure 6D, E**). In addition, AFF3ir-ORF2 overexpression effectively suppressed VCAM-1 expression in the endothelium of the aortic roots of ApoE$^{-/-}$ mice (**Figure 6F, G**). Collectively, these results suggest that supplementation with AFF3ir-ORF2 was effective in preventing atherosclerosis development.

Discussion

Endothelial activation is a critical initial event in the development of atherosclerosis, and emerging evidence suggests that targeting disturbed shear stress-induced endothelial activation is a promising therapeutic strategy. In the present study, we elucidated the role of the novel nested gene-encoded protein, AFF3ir-ORF2, in sensing disturbed shear stress. Moreover, we demonstrated that AFF3ir-ORF2 acts as an endogenous inhibitor of IRF5, a key regulator of the inflammatory response, thereby exerting potent anti-inflammatory and anti-atherogenic effects (**Figure 7**).

Using three mouse models (global AFF3ir-ORF2 knockout, locally AFF3ir-ORF2 endothelial expression, and endothelial-specific AFF3ir-ORF2 overexpression), we demonstrated that AFF3ir-ORF2 exerted potent anti-inflammatory and anti-atherosclerosis effects in ApoE$^{-/-}$ mice. Notably, while AFF3ir-ORF2 knockout increased VCAM-1 expression in the endothelium and enlarged the plaque area in the aortic roots, it had a minimal effect on collagen deposition within the plaques. This discrepancy may be attributed to the differential expression patterns of IRF5 across various cell types (Roberts, Collado, & Barnes, 2024). Phenotypically modulated vascular smooth muscle cells (VSMCs) within the fibrous cap produce extracellular matrix molecules critical for plaque composition and stabilization (Bennett, Sinha, & Owens, 2016). A previous study has shown minimal colocalization between IRF5 and the VSMC marker, α- smooth muscle actin, in aortic root lesions of ApoE$^{-/-}$ mice (Seneviratne et al., 2017), indicating a relatively low IRF5 expression level in VSMCs. Consequently, AFF3ir-ORF2 likely exerts its anti-inflammatory effects primarily through endothelial IRF5. Consistently, endothelial-specific AFF3ir-ORF2 overexpression reduced the aortic plaque area, but had minimal effects on collagen deposition. Our findings establish the potent anti-atherosclerotic role of AFF3ir-ORF2 in early and advanced atherosclerosis mouse models. However, given the multiple critical roles of ECs throughout the initiation and progression of atherosclerosis, further investigations are needed to explore the potential role of AFF3ir-ORF2 in other atherosclerotic processes, including endothelial-to-mesenchymal transition, plaque rupture, and atherothrombotic occlusion. Additionally, we found that disturbed shear stress transcriptional downregulated the expression of AFF3ir. However, the protein levels of AFF3ir-ORF2, but not those of AFF3ir-ORF1, were reduced by disturbed shear stress. Since both AFF3ir-ORF1 and AFF3ir-ORF2 are derived from AFF3ir, different translation mechanisms may be involved in the production of AFF3ir-ORF1/2.

In addition to ECs, various other cell types, particularly immune cells, play crucial roles in the progression of atherosclerotic plaques (Wolf & Ley, 2019). Although our results indicated the potent anti-inflammatory role of AFF3ir-ORF2 in ECs, the potential contributions of other cell types may also be involved, which could be further elucidated using AFF3ir-ORF2 tissue-specific knockout or overexpression mouse models. Macrophage polarization and inflammatory responses accelerate plaque development, leading to an increase in necrotic core and vulnerable plaques (Krausgruber et al., 2011). Elevated IRF5 expression and nuclear localization have been observed in macrophages within plaques of ApoE$^{-/-}$ mice (Seneviratne et al., 2017). IRF5 has been demonstrated to drive macrophages towards a pro-inflammatory state, thereby affecting plaque stability (Seneviratne et al., 2017). Global or myeloid cell-specific deletion of IRF5 stabilizes atherosclerotic plaques by suppressing the inflammatory phenotypes of macrophages (Leipner et al., 2021; Seneviratne et al., 2017). Despite these findings, the role of IRF5 in shear stress-induced endothelial activation remains largely unknown. Our study provides evidence that disturbed shear stress is sufficient to induce IRF5 nuclear translocation and activation in ECs.

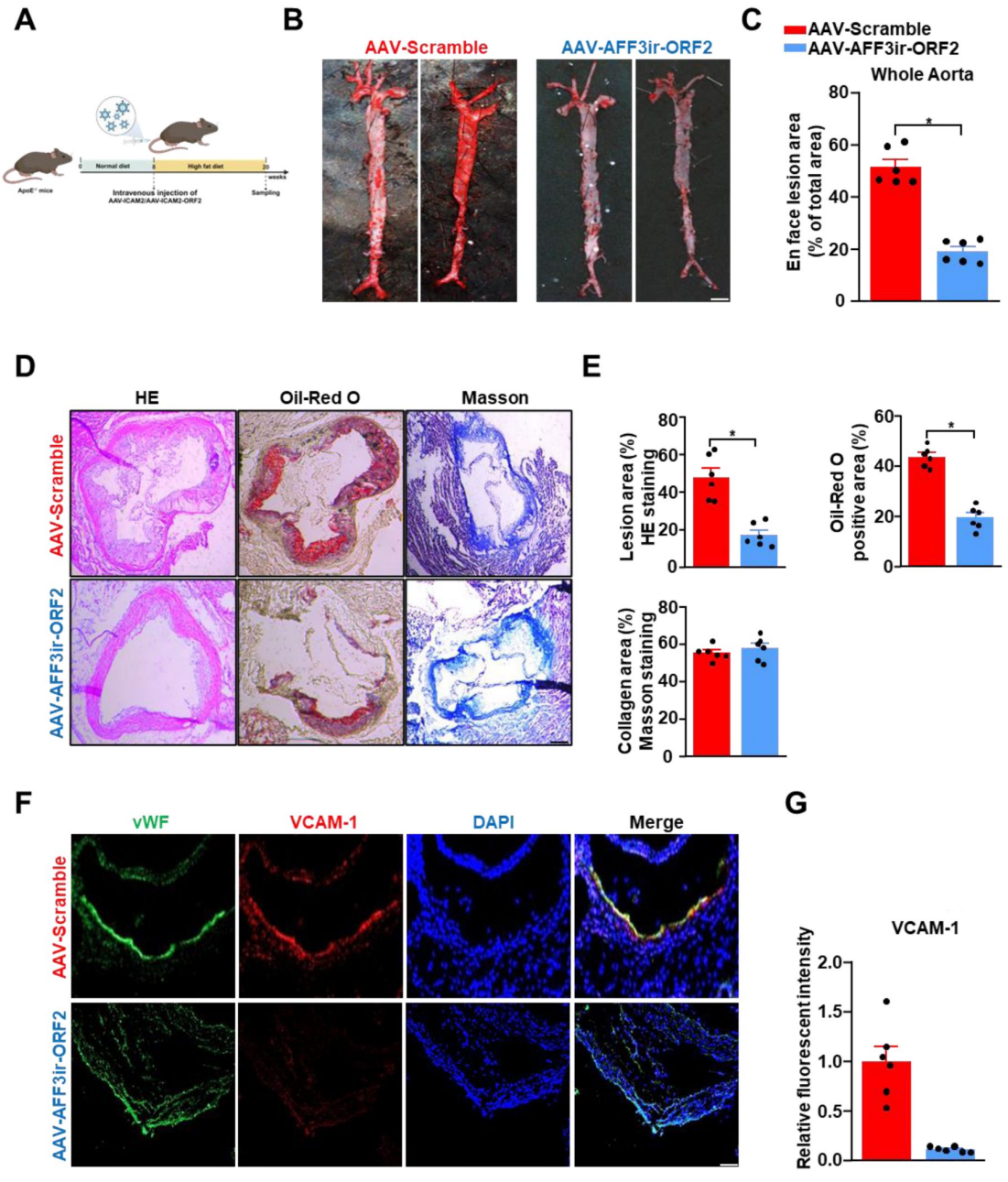

Figure 6.

Endothelial-specific AFF3ir-ORF2 supplementation alleviates EC activation and atherosclerosis in ApoE$^{-/-}$ mice.

Eight-week old ApoE$^{-/-}$ male mice were infused with the indicated adeno-associated virus (AAV) and then fed a high-fat diet for 12 weeks. **A**, Schematic of the experimental strategy. **B**, Representative images of enface Oil-Red O staining of the aortas. Scale bar, 4 mm. **C,** Quantification of the plaque area in the entire aortas. Data are presented as mean ± SEM (n=6 mice per group). *P< 0.05, unpaired two-tailed t-test. **D,** Hematoxylin and eosin (HE), Oil-Red O, and Masson staining of the aortic roots. Scale bars, 500 μm. **E,** Quantification of plaque size, Oil-Red O-positive area, and collagen fiber content in aortic root sections. Data are presented as mean ± SEM (n=6 mice per group). *P< 0.05, unpaired two-tailed t-test. **F**, Representative immunofluorescence image of vWF, VCAM-1, and DAPI in the aortic roots. Scale bar, 500 μm. **G**, Quantification of the relative fluorescent intensity of VCAM-1. The immunofluorescence intensity of VCAM-1 was normalized to that of DAPI, and the relative expression values were compared to that of the AAV-Scramble group. Data are presented as mean ± SEM (n=6 mice per group). *P< 0.05, unpaired two-tailed t-test.

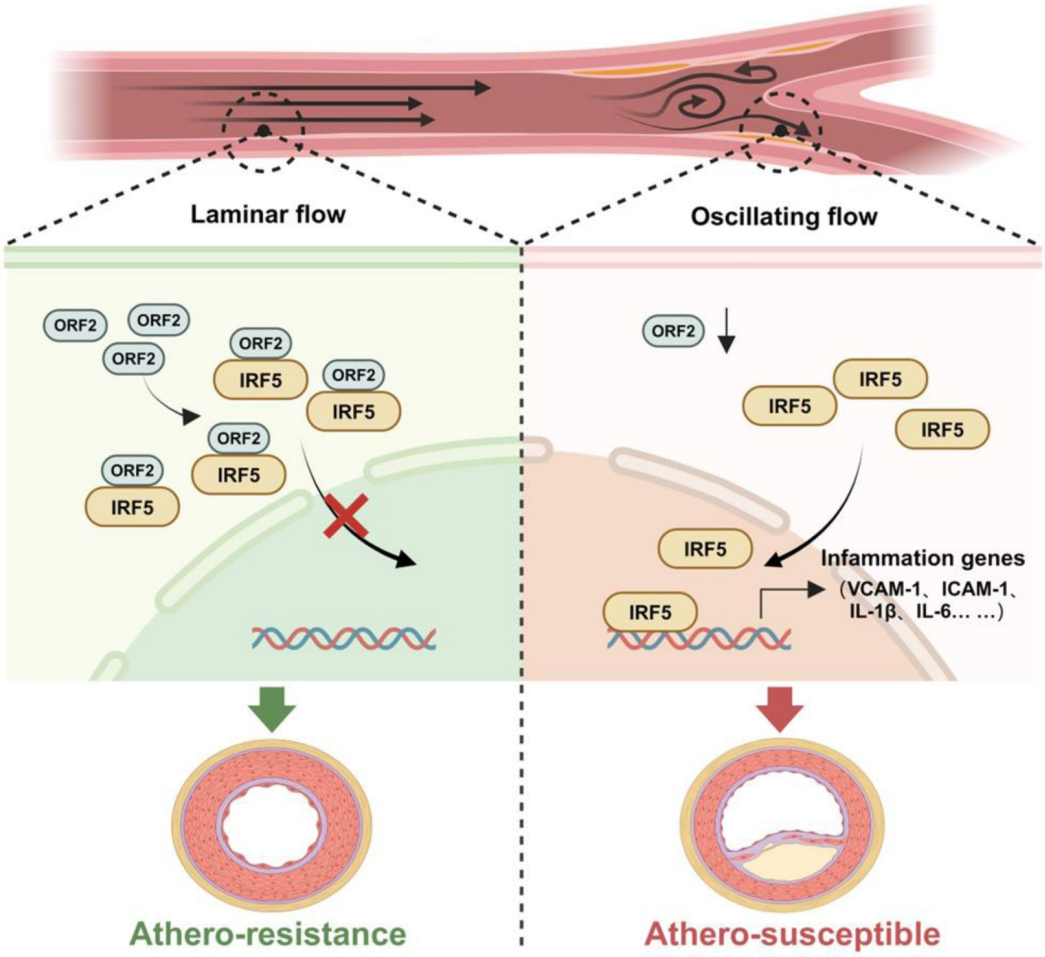

Figure 7.

Schematic illustration of the AFF3ir-ORF2/IRF5 cascade in disturbed flow-induced endothelial activation and atherosclerosis.

Disturbed flow induced a down-regulation of AFF3ir-ORF2, which could interact with IRF5 and promote the latter's retention in the cytoplasm, thereby boosting IRF5-dependent inflammatory pathways in endothelial cells and leading to atherogenesis.

Furthermore, IRF5 knockdown in ECs significantly reduced disturbed flow-induced plaque formation in LCAs. These findings suggest that targeting endothelial IRF5 may be an effective strategy for combating the early stages of atherosclerosis.

Given the key role of IRF5 in mediating inflammatory responses, it has been considered as an attractive therapeutic target, and various strategies have been developed to study and modulate its function (Almuttaqi & Udalova, 2019). For example, nanoparticle-delivered siRNA targeting IRF5 in macrophages promotes inflammation resolution, improves infarct healing, and attenuates post-myocardial infarction remodeling (Courties et al., 2014). Additionally, manipulating IRF5 protein levels through the E3 ubiquitin ligase, TRIM21, has been explored as a strategy for modulating its activity (Lazzari et al., 2014). Given the crucial physiological role of IRF5, strategies aimed at suppressing its pathophysiological activation without altering basal levels may offer additional benefits. Our study introduces a novel approach to inhibit IRF5 activation. We found that the novel nested gene-encoded protein, AFF3ir-ORF2, interacts with IRF5, leading to cytoplasmic retention and inactivation under disturbed shear stress conditions. Importantly, endothelium-specific supplementation with AFF3ir-ORF2 effectively attenuated endothelial activation and reduced the atherosclerotic plaque area in ApoE$^{-/-}$ mice, suggesting that targeting endothelial IRF5 activation with AFF3ir-ORF2 holds promise for the treatment of atherosclerosis. Furthermore, as emerging studies have highlighted the substantial contributions of IRF5 to autoimmune diseases (Graham et al., 2006), neuropathic pain (Masuda et al., 2014), obesity (Dalmas et al., 2015), and hepatic fibrosis (Alzaid et al., 2016), future research should investigate whether AFF3ir-ORF2 has beneficial effects in these contexts.

Conclusion

In conclusion, this study provides novel evidence that the disruption of AFF3ir-ORF2 expression under disturbed flow promotes endothelial inflammatory responses and atherosclerosis. AFF3ir-ORF2 serves as an endogenous inhibitor of IRF5 by binding to IRF5 and preventing its nuclear translocation. Supplementation with endothelial AFF3ir-ORF2 may be a promising therapeutic strategy for treating atherosclerosis.

Materials and methods

Animals

C57BL/6 and Apolipoprotein E-null (ApoE$^{-/-}$) mice were purchased from the Experimental Animal Centre of Military Medical Science Academy (Beijing, China). AFF3ir-ORF2- heterozygote (AFF3ir-ORF2$^{+/-}$) mice were acquired from Dr. Lingfang Zeng's Laboratory at King's College London. Briefly, gRNAs targeting the mouse AFF3ir-ORF2 locus (gRNA1: GCAACCCACGGAGTTGCAGTTGG; gRNA2: GTCATTAACTCCTTTAATATAGG; gRNA3: TGCAACTCCGTGGGTTGCTGTGG; gRNA4: GACCACACATAACAGTGAATAGG) and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted knockout offspring. F0 founder animals were identified by PCR followed by sequence analysis, and then bred to WT mice to test germline transmission and produce F1 animals. Heterozygous targeted mice were intercrossed to generate homozygous targeted mice. The genotyping primer used were: 5'- GGAAAGACCACAGAATCAATGACA-3', 5'-AACATTGCTATACCCCACTATA-3'. To generate ApoE and AFF3ir-ORF2 double knockout mice (ApoE$^{-/-}$ AFF3ir-ORF2$^{-/-}$), ApoE$^{-/-}$ mice were crossed with AFF3ir-ORF2$^{-/-}$ mice. The animals were maintained at 21 ± 1°C under a 12-hour light/dark cycle (lights on at 07:00, lights off at 19:00) with ad libitum access to water and standard chow unless specified otherwise. This study adhered to the *Guide for the Care and Use of Laboratory Animals* of the US National Institutes of Health (NIH Publication No. 85- 23, revised 2011). All study protocols were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University.

Carotid artery partial ligation surgery

The surgery was performed as we previously described (Yang et al., 2021). Briefly, mice were anesthetized with isoflurane (2-3%). A ventral midline incision (4-5 mm) was made in the neck and the left carotid artery was exposed through blunt dissection of subcutaneous fat and muscle tissue. The left external carotid, internal carotid, and occipital arteries were ligated with a 6-0 silk suture, leaving the superior thyroid artery intact. For adenovirus and lentivirus infection studies, adenovirus (Ad-ORF2 or Ad-Scramble) or lentivirus (lenti-shRNA-IRF5 or lenti-shRNA-Scramble) was introduced into the lumen of the left carotid artery and kept inside for 40 min. After infection, the adenovirus or lentivirus was released, and blood flow to the common carotid artery was restored. Mice were fed with a high-fat diet (TD88137, ENVIGO, USA) immediately after surgery and continued for 4 weeks.

Endothelial AFF3ir-ORF2 overexpression in mice

Endothelial-specific adeno-associated virus (AAV)-mediated CRISPR/Cas9 shuttle plasmid was constructed by Cell & Gene Therapy (Shanghai, China) as previously reported (Wang et al., 2016). $ApoE^{-/-}$ mice received a single tail vein injection of recombinant AAV containing an endothelial-specific human ICAM-2 promoter driving AFF3ir-ORF2 overexpression (AAV- AFF3ir-ORF2) or a control empty vector (AAV-Scramble), with a dose of 1×10^{11} viral genomes in a 200 μL volume of sterile PBS. Subsequently, the mice were fed with a high-fat diet (TD88137, ENVIGO, USA) for 3 months.

Oil-Red O staining for atherosclerotic plaques in mouse aorta

The $ApoE^{-/-}$, $ApoE^{-/-}$AFF3ir-$ORF2^{-/-}$, and EC-specific AFF3ir-ORF2 overexpression mice were anaesthetized by inhalation of 2% isoflurane and euthanized by cervical dislocation. The aortas were dissected in 1×PBS and opened to expose the atherosclerotic plaques. After fixation in 4% formaldehyde for 1 hat 4 °C, the tissues were rinsed in water for 10 min, followed by 60% isopropanol. The aortas were then stained with Oil-Red O for 30 min with gentle shaking, rinsed again in 60% isopropanol, and subsequently rinsed in water three times. The samples were mounted on wax with the endothelial surface facing upwards. Images were captured using an HP Scanjet G4050. Plaque areas were quantified using NIH ImageJ software by calculating the plaque area relative to the total vascular area.

Immunofluorescence staining

MEFs slides or frozen sections were fixed in 4% paraformaldehyde for 30 min, then permeabilized in 0.1% Triton X-100 (in PBS) and blocked with 1% bovine serum albumin for 30 min at room temperature. Sections were incubated overnight at 4°C with primary antibodies (1:100). AFF3ir-ORF2 (Cat. No. C0302HL300-4) antibody was from GenScript (Piscataway, NJ, USA). The vWF (Cat. No. ab11713) and VE-Cadherin (Cat. No. ab33168) antibodies were obtained from Abcam (Cambridge, UK). VCAM-1 (Cat. No. sc-13160) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Following primary antibody incubation, sections were treated with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (1:200, Thermo Fisher Scientific, Grand Island, NY, USA) at room temperature for 1 hour. Slides were then mounted with DAPI-containing mounting medium. Antibody specificity and target staining authenticity were verified using negative controls. Immunofluorescence micrographs were acquired using a Leica confocal laser scanning microscope. Representative images were randomly selected from each group.

Histological analysis of atherosclerotic lesions

Harvested carotid arteries and cross-sections of aortic roots were fixed in 4% paraformaldehyde and embedded in optimal cutting temperature compound (OCT). OCT-embedded tissues were sectioned at a thickness of 7 μm. Slides were immersed in 1×PBS for 5 min to remove OCT, and

subsequently stained with Oil-Red O, haematoxylin and eosin (HE), and Masson's trichrome stain to assess lipid accumulation, lesion area, and collagen deposition, respectively (B. Li et al., 2019). Images were acquired using microscopy.

Quantification of plasma lipid levels

Blood samples were obtained via cardiac puncture, rinsed with heparin, and collected in 1.5 mL Eppendorf tubes. Total plasma cholesterol, triglycerides, LDL cholesterol, and HDL cholesterol levels were measured enzymatically using an automated clinical chemistry analyser kit (Biosino Biotech, Beijing, China).

Cell culture, transfection, and shear stress experiments

Mouse Embryonic Fibroblasts (MEFs) were obtained and cultured as previously described (Ferreira & Hein, 2023). Cell passages 4 to 7 were used in all experiments. MEFs and HEK293 cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were incubated at 37°C in a humidified environment containing 5% CO_2 and grown to 70-80% confluence before treatment.

Small interfering RNA against IRF5 or IRF8 were synthesized from General Biosystems (Hefei, China). The sequences of siRNAs are shown in the Table S1. The MEFs were passaged to 6- well plated and transfected with 20 nmol/L siRNA per well using the Lipofectamine RNA iMAX Reagent (Invitrogen, Carlsbad, CA, USA).

For flow experiments, confluent monolayers of MEFs were seeded onto glass slides, and a parallel plate flow system was used to launch oscillatory flow (0.5 ± 4 dyn/cm^2). The flow system was enclosed in a chamber (Frangos, Eskin, McIntire, & Ives, 1985; Fu et al., 2011).

Adenovirus and lentivirus production and infection

AFF3ir-ORF2 sequences were inserted into the GV138 vector (CMV-MCS-3FLAG) to generate recombinant adenovirus (Ad-AFF3ir-ORF2). The short hairpin RNA (shRNA) sequences targeting mouse IRF5 were 5'-GGGACAACACCATCTTCAAGG-3', 5'GGTTGCTGCTGGAGATGTTCT-3', and 5'-GCCTAGAGCAGTTTCTCAATG-3'. The control shRNA was 5'-GCGTGATCTTCACCGACAAGA-3'. These shRNAs were constructed and cloned into pLV-U6-shRNA-CMV-EGFP to generate recombinant lentivirus (lenti-shRNA-IRF5 or lenti-shCtrl). MEFs were infected with adenovirus or lentivirus at a multiplicity of infection (MOI) of 10, with no detectable cellular toxicity observed.

Western blot analysis

Whole-cell lysates were prepared in a lysis buffer containing a complete protease inhibitor cocktail, PhosSTOP, and PMSF (Roche, Mannheim, Germany). Cytoplasmic and nuclear proteins were extracted from wild-type and AFF3ir-ORF2$^{-/-}$ MEFs using a protein extraction kits (Invent Biotechnologies, SC-003, Beijing, China). Protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (Cat. No. 10600001; GE Healthcare; Chicago, IL, USA). The membranes were incubated with primary antibodies. IRF5 (Cat. No. 96527), IRF8 (Cat. No. 98344), and Flag (Cat. No. 14793) antibodies were from Cell Signaling Technology (Danvers, MA, USA). ICAM-1 (Cat. No. ab222736) antibodies were from Abcam (Cambridge, UK). AFF3ir-ORF2 (Cat. No. C0302HL300-4) and AFF3ir-ORF1 (Cat. No. C0302HL300) antibodies were from GenScript (Piscataway, NJ, USA). GAPDH (Cat. No. 60004-1-Ig) antibody was from Proteintech (Wuhan, China). AFF3 (Cat. No. PA5-68961) antibody was from Thermo Fisher Scientific (Waltham, MA, USA).

After incubation with horseradish peroxidase-conjugated secondary antibodies, the proteins were visualized using enhanced chemiluminescence reagents in a ChemiScope3600 Mini chemiluminescence imaging system (Clinx Science Instruments; Shanghai, China). Protein levels

were quantified by measuring integrated density with NIH Image J software (*https://imagej.nih.gov/ij/*), using GAPDH as a loading control for normalization.

Co-immunoprecipitation

Whole-cell lysates were prepared by lysing cells in a 1% NP-40 lysis buffer containing 50 mM Tris-HCl, 1% Nonidet-P40, 0.1% SDS, and 150 mM NaCl, supplemented with a complete protease inhibitor cocktail (Cat. No. 04693132001; Roche, Indianapolis, IN, USA), a phosphatase inhibitor (PhosSTOP; Cat. No. 04906845001; Roche), and PMSF (Cat. No. IP0280; Solarbio Life Sciences; Beijing, China). Samples were incubated on ice for 30 minutes, then centrifuged at 12,000 g for 10 minutes, and the supernatant was transferred to a new tube. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Grand Island, NY, USA).

For immunoprecipitation, 1000 μg of protein was incubated with specific antibodies at 4 °C for 12 hour with constant rotation. Subsequently, 50 μL of 50% Protein A/G PLUS-Agarose beads was added, and the incubation continued for an additional 2 hr. Beads were washed five times with the lysis buffer and collected by centrifugation at 12,000 g for 2 min at 4°C. After the final wash, the supernatant was removed and discarded. Precipitated proteins were eluted by resuspending the beads in 2×SDS-PAGE loading buffer and boiling for 5 min. The eluates from immunoprecipitation were subjected to Western blot analysis.

ELISA

The concentrations of IL-6 (EM0121) and IL-1β (EM0109) in cell culture supernatant were measured using ELISA kit (FineTest, Wuhan, China). The experiments were conducted according to the protocols provided by the manufacturer.

Total RNA extraction and real-time quantitative PCR analysis

Total RNA was extracted from cells using an RNA extraction kits (Transgen Biotech, ER501- 01, Beijing, China). Reverse transcription was performed with a reverse transcription kit (Thermo Fisher Scientific, Grand Island, NY, USA). Quantitative PCR was conducted using SYBR Select (Thermo Fisher Scientific) according to the manufacturer's protocol, with GAPDH serving as the internal control. The primers for quantitative real-time PCR are listed in Supplementary Table 2.

Luciferase reporter assay

The IRF5-binding motif and the full-length ZNF217 promoter were ligated into pGl3-based plasmids (Genechem, Shanghai, China), as previously described (Qiao et al., 2022). HEK293T cells were seeded into 24-well plates and grown to 70-80% confluency. Cells were transfected with the firefly luciferase reporter plasmid containing the IRF5-responsive ZNF217 promoter along with a β-galactosidase reporter plasmid (Promega, Madison, WI, USA) for 24 hour. Subsequently, cells were then infected with adenovirus (Ad-ORF2 or Ad-Scramble) for an additional 24 hour. Relative luciferase activity was measured using a luciferase assay and normalized to β-galactosidase activity as determined by the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA).

RNA-sequencing (RNA-seq)

RNA-seq was performed as we previously described (Z. Y. Li et al., 2024). Wild-type (WT) and AFF3ir-ORF2$^{-/-}$ MEFs were harvested, and RNA was extracted using the MagicPure Total RNA Kit (TransGen, Beijing, China). Whole transcriptome RNA-seq analysis were conducted by the Beijing Genomics Institute (BGI). Paired-end sequencing in 150-bp length was performed using the DNBSEQ-G400 platform. Raw data was filtrated using SOAPnuke (v1.5.6). Differential gene

expression analysis, with thresholds set at $P < 0.05$ and fold change ≥1.5, was performed via the BGI website (*http://omiscribe.bgi.com*). Pathway enrichment analysis was carried out using DAVID tools.

Statistical analysis

Statistics analyses were performed using GraphPad Prism 8.0. No sample outliers were excluded. At least six independent experiments were performed for all biochemical experiments and the representative images were shown. Unpaired Student's *t* test (2-tailed), 1-way ANOVA or 2-way ANOVA with Bonferroni multiple comparison post hoc test were used for analyses, as appropriate. Sample size, statistical method, and statistical significance are specified in Figures and Figure Legends. Levels of probabilities less than 0.05 were regarded as significant.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The RNA-sequencing data has been submitted to GEO datasets (GSE286206). Source data are provided with this paper.

Acknowledgements

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Additional files

supplemental figure 1-7 and supplemental table 1-2

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Reviewer #1 (Public review):

Summary:

The authors report the role of a novel gene Aff3ir-ORF2 in flow induced atherosclerosis. They show that the gene is anti-inflammatory in nature. It inhibits the IRF5 mediated athero-progression by inhibiting the causal factor (IRF5). Furthermore, authors show a significant connection between shear stress and Aff3ir-ORF2 and its connection to IRF5 mediated athero-progression in different established mice models which further validates the ex vivo findings.

Strengths:

(1) Adequate number of replicates were used for this study.
(2) Both in vitro and in vivo validation was done.
(3) Figures are well presented
(4) In vivo causality is checked with cleverly designed experiments

Weaknesses:

(1) Inflammatory proteins must be measured with standard methods e.g ELISA as mRNA level and protein level does not always correlate.
(2) RNA seq analysis has to be done very carefully. How does the euclidean distance correlate with the differential expression of genes. Do they represent neighborhood? If they do how does this correlation affect the conclusion of the paper?
(3) Volcano plot does not indicate q value of the shown genes. It is advisable to calculate q

value for each of the genes which represents the FDR probability of the identified genes.
(4) GO enrichment was done against Global gene set or local geneset? Authors should provide more detailed information about the analysis.
(5) If the analysis was performed against global gene set. How does that connect with this specific atherosclerotic microenvironment?
(6) what was the basal expression of genes and how does the DGE (differential gene expression) values differ?
(7) How did IRF5 picked from GO analysis? was it within 20 most significant genes?
(8) Microscopic studies should be done more carefully? There seems to be a global expression present on the vascular wall for Aff3ir-ORF2 and the expression seems to be similar like AFF3 in fig 1.

Comments on Revision:

The authors have adequately addressed my concerns.

https://doi.org/10.7554/eLife.103413.2.sa2

Reviewer #2 (Public review):

Summary:

The authors recently uncovered a novel nested gene, Aff3ir, and this work sets out to study its function in endothelial cells further. Based on differences in expression correlating with areas of altered shear stress, they investigate a role for the isoform Aff3ir-ORF2 in endothelial activation and development of atherosclerosis downstream of disturbed shear stress. Using a knockout mouse model and in vivo overexpression experiments, they demonstrate a strong potential for Aff3ir-ORF2 to alleviate atherosclerosis. They find that Aff3ir-ORF2 interacts with the pro-inflammatory transcription factor IRF5 and retains it in the cytoplasm, hence preventing upregulation of inflammation-associated genes. The data expands our knowledge of IRF5 regulation which could be relevant to researchers studying various inflammatory diseases as well as adding to our understand of atherosclerosis development.

Strengths:

The in vivo data is convincing using immunofluorescence staining to assess AFF3ir-ORF2 expression, a knockout mouse model, overexpression and knockdown studies and rescue experiments in combination with two atherosclerotic models to demonstrate that Aff3ir-ORF2 can lessen atherosclerotic plaque formation in ApoE-/- mice.

Weaknesses:

The effect on atherosclerosis is clear and there is sufficient evidence to conclude that this is the result of reduced endothelial cell activation. However, other cell types such as smooth muscle cells or macrophages could be contributing to the effects observed. The mouse model is a global knockout and the shRNA knockdowns (Fig. 5) and overexpression data in Figure 2 are not cell type-specific. Only the overexpression construct in Figure 6 uses an ICAM-2 promoter construct, which drives expression in endothelial cells, though leaky expression of this promoter has been reported in the literature.

The in vitro experiments are solidly executed, but most experiments are performed in mouse embryonic fibroblasts (MEFs) and results extrapolated to endothelial cell responses. However, several key experiments are repeated in HUVEC, thereby making a solid case that Aff3ir-ORF2 can regulate IRF5 in both MEFs and HUVEC. It is important to note that the sequence of AFF3ir-ORF2 is not conserved in humans and lacks an initiation codon, hence the regulatory pathway is not conserved. However, the overexpression studies in HUVEC suggest

that mouse AFF3ir-ORF2 can also regulate human IRF5 and hence the mechanism retains relevance for possible human health interventions.

Overall, the paper succeeds in demonstrating a link between Aff3ir-ORF2 and atherosclerosis. The study shows a functional interaction between Aff3ir-ORF2 and IRF5 in embryonic fibroblasts, but makes a solid case that this mechanism is relevant for atherosclerosis development via endothelial cell activation.

https://doi.org/10.7554/eLife.103413.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The authors report the role of a novel gene Aff3ir-ORF2 in flow-induced atherosclerosis. They show that the gene is anti-inflammatory in nature. It inhibits the IRF5-mediated athero-progression by inhibiting the causal factor (IRF5). Furthermore, the authors show a significant connection between shear stress and Aff3ir-ORF2 and its connection to IRF5 mediated athero-progression in different established mice models which further validates the ex vivo findings.*
>
> *Strengths:*
>
> *(1) An adequate number of replicates were used for this study.*
>
> *(2) Both in vitro and in vivo validation was done.*
>
> *(3) The figures are well presented.*
>
> *(4) In vivo causality is checked with cleverly designed experiments.*

We thank you for your positive remarks.

> *Weaknesses:*
>
> *(1) Inflammatory proteins must be measured with standard methods e.g ELISA as mRNA level and protein level does not always correlate.*

Thanks. We have followed your advice and performed ELISA experiments to measure the concentrations of inflammatory cytokines, including IL-6 and IL-1β. The newly acquired results have been included in Figure 2E (Line 160-163) in the revised manuscript.

> *(2) RNA seq analysis has to be done very carefully. How does the euclidean distance correlate with the differential expression of genes. Do they represent the neighborhood?*

If they do how does this correlation affect the conclusion of the paper?

We thank the reviewer for this professional comments and apologize for the confusion. The heatmap using Euclidean distance was generated based on the expression levels of all differentially expressed genes (calculated with deseq2). Since its interpretation overlaps with the volcano plot presented in Figure 4B, we have moved the heatmap to Figure S5A in the

revised manuscript and provided a detailed description in the figure legend (Lines 106-108 in the supporting information). Additionally, to better illustrate the variation among all samples, we have performed PCA analysis and included the new results in Figure 4A of the revised manuscript.

> *(3) The volcano plot does not indicate the q value of the shown genes. It is advisable to calculate the q value for each of the genes which represents the FDR probability of the identified genes.*

Thank you for your careful review. We apologize for the incorrect labeling.

It was *P*.adj value. The label for Figure 4B has been corrected in the revised manuscript.

> *(4) GO enrichment was done against the Global gene set or a local geneset? The authors should provide more detailed information about the analysis.*

Thank you. We performed GO enrichment analysis against the global gene set. The description of the results has been updated in the revised manuscript (Lines 222–224).

> *(5) If the analysis was performed against a global gene set. How does that connect with this specific atherosclerotic microenvironment?*

Thank you for your insightful comments. We have followed your advice and investigated the functional characteristics of these differentially expressed genes in the context of the atherosclerotic microenvironment. The RNA-seq differential gene list was further mapped onto the atherosclerosis-related gene dataset (PMID: 27374120), resulting in 363 overlapping genes. The 363 genes were subjected to bioinformatics enrichment analysis using Gene Ontology (GO) databases. GO analysis of these genes revealed enrichment in processes related to cell−cell adhesion and leukocyte activation involved in immune response (Figure S5B), which is highly consistent with the observed effects of AFF3ir-ORF2 on VCAM-1 expression. The newly acquired data are presented in Figure S5B and the description of the results is included in the revised manuscript (Line 227-233).

> *(6) What was the basal expression of genes and how did the DGE (differential gene expression) values differ?*

Thanks for the comments. The RNA-sequencing data has been submitted to GEO datasets (GSE286206), making the basal gene expression data available to readers.

The differential expression analysis was performed using DESeq2 (v1.4.5) (PMID: 25516281) with a criterion of 1.5-fold change and $P<0.05$. We has included the description in the revised manuscript in Lines 220-222 and Lines 575-576.

> *(7) How was IRF5 picked from GO analysis? was it within the 20 most significant genes?*

Sorry for the confusion. IRF5 was not identified through GO analysis. To determine the upstream transcriptional regulators, we used the ChEA3 database to predict potential upstream transcription factors based on all differentially expressed genes. The top 20 transcription factors were selected based on their scores. To further explore their relationship with atherosclerosis, these top 20 transcription factors were mapped to the atherosclerosis-related gene list in the DisGeNET database. IRF5 and IRF8 were the only two overlapping genes. To clarify this process, we have included a more detailed description of the IRF prediction approach in the revised manuscript (Lines 234–239).

> *(8) Microscopic studies should be done more carefully? There seems to be a global expression present on the vascular wall for Aff3ir-ORF2 and the expression seems to be similar to AFF3 in Figure 1.*

We thank the reviewer for the valuable suggestion. We have followed your advice and provided the more representative images in Figure 1F.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The authors recently uncovered a novel nested gene, Aff3ir, and this work sets out to study its function in endothelial cells further. Based on differences in expression correlating with areas of altered shear stress, they investigate a role for the isoform Aff3ir-ORF2 in endothelial activation and development of atherosclerosis downstream of disturbed shear stress. Using a knockout mouse model and in vivo overexpression experiments, they demonstrate a strong potential for Aff3ir-ORF2 to alleviate atherosclerosis. They find that Aff3ir-ORF2 interacts with the pro-inflammatory transcription factor IRF5 and retains it in the cytoplasm, hence preventing upregulation of inflammation-associated genes. The data expands our knowledge of IRF5 regulation which could be relevant to researchers studying various inflammatory diseases as well as adding to our understanding of atherosclerosis development.*
>
> *Strengths:*
>
> *The in vivo data is solid using immunofluorescence staining to assess AFF3ir-ORF2 expression, a knockout mouse model, overexpression and knockdown studies, and rescue experiments in combination with two atherosclerotic models to demonstrate that Aff3ir-ORF2 can lessen atherosclerotic plaque formation in ApoE$^{-/-}$ mice.*

We thank you for your positive remarks.

> *Weaknesses:*
>
> *While the in vivo data is generally convincing, a few data panels have issues and will need addressing. Also, the knockout mouse model will need to be described, since the paper referred to in the manuscript does not actually report any knockout mouse model. Hence it is unclear how Aff3ir-ORF2 is targeted, but Figure S2B shows that targeting is partial, since about 30% expression remains at the RNA level in MEFs isolated from the knockout mice.*

We thank you for the valuable comments.

First, we have followed your advice and included detailed information regarding the animal construction in the revised manuscript in Line 405-415. Additionally, the genotyping results have been included in new Figure S3A.

Second, we acknowledge your concern about the knockout efficiency of ORF2 in mice. While the PCR assay indicated approximately 30% residual expression, our Western blot analysis of aorta samples demonstrated that ORF2 protein was barely detectable in knockout mice, as shown in new Figure S3B-C. Besides, our *in vivo* experiments using MEF from WT and AFF3ir-ORF2$^{-/-}$ mice (Figure 4I) further confirmed successful knockout.

Third, we have included a discussion addressing the discrepancies between PCR and Western blot results. In addition to technical differences between the two methods, the nature of AFF3ir-ORF2 may also contribute to these inconsistencies. The parent gene AFF3 is located in

a genetically variable region and can be excised via intron 5 to form a replicable transposon, which translocates to other chromosomes and has been linked to leukemia (PMID: 34995897, 12203795, 12743608, and 17968322). AFF3ir is located in the intron 6, thus it exists in the transposon, which may complicate the measurement of its expression. Replicable transposons can exist as extrachromosomal elements, allowing them to be inherited across generations. We have included these discussion in the revised manuscript in Line 188-196.

> *While the effect on atherosclerosis is clear, the conclusion that this is the result of reduced endothelial cell activation is not supported by the data. The mouse model is described as a global knockout and the shRNA knockdowns (Figure 5) and overexpression data in Figure 2 are not cell type-specific. Only the overexpression construct in Figure 6 uses an ICAM-2 promoter construct, which drives expression in endothelial cells, though leaky expression of this promoter has been reported in the literature. Therefore, other cell types such as smooth muscle cells or macrophages could be responsible for the effects observed.*

Thank you for your critical comment. To address your concern, we have made the following three revisions:

First, we have analyzed the expression of AFF3ir-ORF2 in the vascular wall with or without intima in WT and AFF3ir-ORF2 knockout mice. As shown in Figure 1B and Figure S1A, while the expression of AFF3ir-ORF2 was notably downregulated in the aortic intima of athero-prone regions compared to the protective region, it remained largely unchanged in the aortic wall without intima across different regions of the aorta. This suggested that AFF3ir-ORF2 might play a predominant role in endothelial cells rather than other cell types in the context of shear stress.

Second, we have used human endothelial cells (HUVECs) to further confirm our findings. As shown in Figure 2C and Figure S2B, we found that AFF3ir-ORF2 overexpression could attenuate disturbed shear stress-induced IRF5 nuclear translocation and the expression of inflammatory genes in HUVECs, suggesting the potential anti-inflammatory effects of AFF3ir-ORF2 in endothelial cells.

Third, we agree with the reviewer's comment that we cannot completely exclude the potential involvement of other cell types. Hence, we have included a limitation statement in the discussion part in Lines 341-344.

> *The weakest part of the manuscript is the in vitro experiment using some nonidentifiable expression differences. The data is used to hypothesise on a role for IRF5 in the effects observed with Aff3ir-ORF2 knockout.*

Thank you for the comments. To address your concerns, we have made the following two changes:

First, we have further investigated the functional features of the differential genes from the RNA-seq in the context of atherosclerotic microenvironment. The differential gene list was mapped onto the atherosclerosis-related gene dataset (PMID: 27374120), and a total of 363 genes overlapped. These 363 genes were subjected to bioinformatics enrichment analysis using Gene Ontology (GO) databases. GO analysis showed that these genes were mainly enriched in cell−cell adhesion and leukocyte activation involved in immune response, which aligns with the expression of VCAM-1 affected by AFF3ir-ORF2. The newly acquired data are presented in Figure S5B and the description of the results has been updated in the revised manuscript (Line 227-233).

Second, we have further verified the RNA-seq results *in vitro*. Several classical inflammatory factors, including ICAM-1, CCL5, and CXCL10, which mRNA levels were significantly

downregulated in RNA-seq and were also identified as target genes of IRF5, were analyzed. We found that AFF3ir-ORF2 deficiency aggravated, while AFF3ir-ORF2 overexpression attenuated, the expression of ICAM-1, CCL5, and CXCL10 induced by disturbed shear stress (New Figure S5D). Besides, the regulation of ICAM-1 by AFF3ir-ORF2 was confirmed at both protein and mRNA levels in HUVECs (Figure 2C-D and Figure S2B).

> *Overall, the paper succeeds in demonstrating a link between Aff3ir-ORF2 and atherosclerosis, but the cell types involved and mechanisms remain unclear. The study also shows a functional interaction between Aff3ir-ORF2 and IRF5 in embryonic fibroblasts, but any relevance of this mechanism for atherosclerosis or any cell types involved in the development of this disease remains largely speculative.*

Thank you for all the valuable comments. The specific responses have been provided above. Briefly, we have followed your advice and further confirmed the regulation of AFF3ir-ORF2 on IRF5 in endothelial cells. Besides, the RNA-seq results have been further analyzed, and partial results have been verified in endothelial cells to support the anti-inflammatory role of AFF3ir-ORF2. We greatly appreciate the reviewer's insightful comments, which guided our revisions and contributed to significantly improving the paper.

> ***Reviewer #3 (Public review):***
>
> *This study is to demonstrate the role of Aff3ir-ORF2 in the atheroprone flow-induced EC dysfunction and ensuing atherosclerosis in mouse models. Overall, the data quality and comprehensiveness are convincing. In silico, in vitro, and in vivo experiments and several atherosclerosis were well executed. To strengthen further, the authors can address human EC relevance.*

We thank you for your positive remarks and insightful comments.

> *Major comments:*
>
> *(1) The tissue source in Figures 1A and 1B should be clarified, the whole aortic segments or intima? If aortic segment was used, the authors should repeat the experiments using intima, due to the focus of the current study on the endothelium.*

We thank you for the suggestion. The tissue used in Figures 1A and 1B was from aortic intima. The description has been updated for clarity in the revised manuscript on Lines 114-125.

> *(2) Why were MEFs used exclusively in the in vitro experiments? Can the authors repeat some of the critical experiments in mouse or human ECs?*

Thank you for this insightful comment. Isolation and culture of mouse primary aortic ECs were notorious technically difficult and shear stress experiment require a large number of cells. Considering MEFs exhibit responses consistent with those of ECs, which has been delicately proved (PMID: 23754392), we used MEFs in our in vitro experiments.

However, following your valuable advice, we have now employed human ECs (HUVECs) to confirm our findings. Consistent with our results in MEFs, we found that AFF3ir-ORF2 overexpression reduced the expression of inflammatory genes induced by disturbed shear stress at both protein and mRNA levels in HUVECs (Figure 2C, Figure S2B). Notably, despite the significant anti-inflammatory effects of AFF3irORF2, the sequence of this gene is not conserved in Homo sapiens and lacks an initiation codon, which is why we did not further proceed with the loss-of-function experiments.

> *(3) The authors should explain why AFF3ir-ORF2 overexpression did not affect the basal level expression of ICAM-1, VCAM-1, IL-1b, and IL-6 under ST conditions (Figure 2A-C).*

We thank you for raising this critical question. Indeed, we found that AFF3ir-ORF2 overexpression did not affect the basal level of inflammatory genes under ST conditions, while it exerted anti-inflammatory effects under OSS conditions. One underlying reason might be the relative low level of expression of inflammatory genes under ST compared to OSS conditions. Additionally, as our findings suggested, AFF3ir-ORF2 exerted its anti-inflammatory role by binding to IRF5 and inhibiting IRF5 nuclear translocation. However, as shown in Figure 4I, IRF5 might be predominantly localized in the cytoplasm rather than the nucleus under ST conditions.

We have included the description in the revised manuscript on Lines 157-163.

> *(4) Please include data from sham controls, i.e., right carotid artery in Figure 2E.*

Thank you for the suggestion. We have followed your advice and included sham controls (staining of the right carotid arteries) in Figure S2E.

> *(5) Given that the merit of the study lies in the effect of different flow patterns, the legion areas in AA and TA (Figure 3B, 3C) should be separately compared.*

We have followed your valuable suggestion and included the additional statistical results in Figure 3C in the revised manuscript.

> *(6) For confirmatory purposes for the variations of IRF5 and IRF8, can the authors mine available RNA-seq or even scRNA-seq data on human or mouse atherosclerosis? This approach is important and could complement the current results that are lacking EC data.*

Thank you for your valuable suggestion. In the present study, we found that disturbed flow did not alter the protein level of IRF5 but promoted its nuclear translocation. Following your advice, we analyzed the expression of IRF5 in human ECs (GSE276195) and atherosclerotic mouse arteries (GSE222583) using public databases. Consistently, IRF5 did not show significant changes in mRNA levels under these conditions (Figure S5E-F), suggesting that the regulation of IRF5 in the context of disturbed flow or atherosclerosis is primarily post-translational.

> *(7) With the efficacy of using AAV-ICAM2-AFF3ir-ORF2 in atherosclerosis reduction (Figure 6), the authors are encouraged to use lung ECs isolated from the AFF3ir-ORF2/-mice to recapitulate its regulation of IRF5.*

We greatly appreciate your valuable suggestion to use lung ECs from mice. We have observed that AFF3ir-ORF2 deficiency enhanced the nuclear translocation of IRF5 induced by OSS. Noteworthy, the transcriptional levels of IRF5 were minimally affected by AFF3ir-ORF2 deficiency. Hence, to recapitulate the regulation of IRF5 with lung ECs isolated from the AFF3ir-ORF2$^{-/-}$ mice, it would require treating lung ECs with OSS followed by isolation of subcellular components. However, both in vitro shear stress treatment and subcellular fraction isolation require a large number of cells, and mouse lung ECs are difficult to culture and pass through several passages. Therefore, we hope the reviewer understands that these experiments were not performed. As an alternative, we have confirmed the transcriptional activity changes of IRF5 due to AFF3ir-ORF2 manipulation by analyzing the expression of its target genes indicated from RNA-seq results in both the intima of mouse aorta (Figure S5C-D)

and HUVECs (Figure 2C-D and Figure S2B). Our findings show that AFF3ir-ORF2 deficiency increases, while its overexpression decreases, the expression levels of IRF5-targeted genes in endothelial cells.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations for the authors):***
>
> *Figure 2H - As I understand it, this is MFI measurement of VCAM. Please change accordingly.*

Thanks. Corrected.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *My major concern is the use of MEFs for all in vitro experiments. All experiments should be done in endothelial cells if the aim is to show a mechanism relevant to endothelial activation and atherosclerosis. Lines 314-316 of the conclusion are absolutely not supported by the data.*

Thank you for the insightful comment. Following your advice, we have employed human ECs (HUVECs) to confirm our findings. Consistent with the findings in MEFs, we found that AFF3ir-ORF2 decreased the expression of inflammatory genes induced by disturbed shear stress, both at protein and mRNA levels in HUVECs (Figure 2C, Figure S2B).

> *Since the in vivo experiments are not cell type-specific, it would be important to test and compare the expression of Aff3ir-ORF2 in endothelial cells as well as smooth muscle and macrophages to support any claim of cell type involvement in the effects observed.*

We thank you for the valuable suggestion. In the revised manuscript, we have followed your suggestion and analyzed the expression pattern of AFF3ir-ORF2 in different regions of the aorta with or without endothelium. We observed a marked reduction in AFF3ir-ORF2 expression in the intima of the aortic arch compared to that in the intima of the thoracic aorta (Figure 1B-C). In contrast, the expression of AFF3irORF2 in the media and adventitia was comparable between the aortic arch and thoracic aorta (Figure S1A-B). These findings provide further evidence supporting the predominant role of endothelial cells. The description has been modified accordingly in the revised manuscript on Lines 121-134.

> *The results of the RNA-seq experiment should be disclosed. The experiment should be deposited on GEO or similar and a table of differentially expressed genes added to the manuscript.*

Thank you for the suggestion. We have followed your advice and submitted the RNA-sequencing data to GEO datasets (GSE286206). Besides, a table of differentially expressed genes has been included in the revised manuscript as Table S3.

> *Minor comments:*
>
> *(1) Figure 1A. Missing the labels of the target.*

Thanks. Corrected.

> *(2) Figure 1D. Cell alignment in AA compared to TA suggests that the image is of the outer curvature, but Figure 1F is showing that the outer curvature is expressing more ORF2 than the inner. Why was the outer curvature chosen for this panel and is it true to*

> *conclude on that assumption that expression of ORF2 compares as TA > Outer > Inner curvature?*

We thank you for the insightful suggestion. We have followed your advice and performed en-face immunofluorescence staining of AFF3ir-ORF2 and quantification of AFF3ir-ORF2 expression in AA inner, AA outer, and TA regions. As shown in new Figure 1D-E, the results indeed indicated that expression of AFF3irORF2 compares as TA > AA outer > AA inner.

> *(3) Figure 2H. Target mislabelled as ICAM-1 instead of VCAM-.*

Thanks. Corrected.

> *(4) Figure S1A. VE-cad staining and cell shape differ between control and overexpression. Is this a phenotype or are different areas of the vasculature shown, which would make it hard to interpret since Aff3ir-ORF2 levels differ in different vessel areas?*

We thank the reviewer for raising this important question. For Figure S1A, only common carotid arteries were used for the staining. The potential differences in cell shape observed might be due to variations in the procedure during immunofluorescence staining. To avoid any misinterpretation, more representative images have been provided in the revised Figure S2C.

> *(5) Figure 3D-G. Images are not representative of the quantification results.*

Thank you. More representative images have been replaced in the revised Figure 3D and Figure 3F.

> *(6) Line 220. Data for IRF8 are not shown in the figure to support this claim.*

Thank you for pointing this out. The expression level of IRF8 has been included in Figure S5C.

> *(7) Figure 6F. AAV-AFF3ir-ORF2 panel order inverted.*

Thanks. Corrected.

> *(8) Line 401. Type "hat" instead of "h at".*

Sorry for the typo. Corrected.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *Minor comments:*
>
> *(1) The rationale for the following sentence (lines 126-128) is lacking: "Moreover, 126 we observed the expression of AFF3ir-ORF2 in longitudinal sections of the mouse aorta (B. 127 Li et al., 2019)".*

Thanks. The rationale for these experiments have been included in the revised manuscript on Line 127-129.

> *(2) The source of antibodies against AFF3ir-ORF1 and AFF3ir-ORF2 used in western blot and immunostaining experiments were not mentioned in the manuscript.*

Thanks. The antibody information has been included in the method part on Line 456-457, 510-511.

> *(3) The rationale and data interpretation is not clear for the following sentence (lines 220-221): "In addition, neither IRF5 nor IRF8 expression was regulated by AFF3irORF2 220 (Figure 4F)".*

Thank you for pointing this out. The expression level of IRF8 has been included in Figure S5C. The sentence has been modified accordingly on Lines 253254.

> *(4) The quality of AFF3ir-ORF2 blot in Figure 4I needs improvement.*

Thanks. More representative images have been included in Figure 4I.

> *(5) It appears that AFF3ir-ORF2 was present in both cytoplasm and nucleus. Does AFF3ir-ORF2 have a nuclear entry peptide? Also, the nuclear entry of AFF3ir-ORF2 can be enhanced by an immunofluorescence staining experiment.*

Thank you for your insightful comments. Indeed, although we did not observe any significant subcellular changes in the localization of AFF3ir-ORF2 under shear stress conditions, our immunostaining results revealed that AFF3ir-ORF2 is localized in both the cytoplasm and nucleus. To explore whether AFF3ir-ORF2 contains nuclear localization signals, we utilized the NLStradamus tool (http://www.moseslab.csb.utoronto.ca/NLStradamus/) to analyze its sequence. The predication indicated that AFF3ir-ORF2 lacks a nuclear localization signal.

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