NLRC5 inhibits neointima formation following vascular injury and directly interacts with PPARγ

Peipei Luan1,2, Weixia Jian2, Xu Xu1, Wenxin Kou1, Qing Yu1, Handan Hu3, Dali Li3, Wei Wang4, Mark W. Feinberg5, Jianhui Zhuang1, Yawei Xu1 & Wenhui Peng1

NLR Family CARD Domain Containing 5 (NLRC5), an important immune regulator in innate immunity, is involved in regulating inflammation and antigen presentation. However, the role of NLRC5 in vascular remodeling remains unknown. Here we report the role of NLRC5 on vascular remodeling and provide a better understanding of its underlying mechanism. Nlrc5 knockout (Nlr5−/−) mice exhibit more severe intimal hyperplasia compared with wild-type mice after carotid ligation. Ex vivo data shows that NLRC5 deficiency leads to increased proliferation and migration of human aortic smooth muscle cells (HASMCs). NLRC5 binds to PPARγ and inhibits HASMC dedifferentiation. NACHT domain of NLRC5 is essential for the interaction with PPARγ and stimulation of PPARγ activity. Pioglitazone significantly rescues excessive intimal hyperplasia in Nlr5−/− mice and attenuates the increased proliferation and dedifferentiation in NLRC5-deficient HASMCs. Our study demonstrates that NLRC5 regulates vascular remodeling by directly inhibiting SMC dysfunction via its interaction with PPARγ.

1 Department of Cardiology, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, China. 2 Department of Endocrinology, Xinhua Hospital, Shanghai Jiaotong University, School of Medicine, Shanghai 200092, China. 3 Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China. 4 Columbia Center for Translational Immunology, Columbia University Medical Center, New York 10032, USA. 5 Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA. Correspondence and requests for materials should be addressed to J.Z. (email: jh_zhuang@tongji.edu.cn) or to Y.X. (email: xuyawei@tongji.edu.cn) or to W.P. (email: pwenhui@tongji.edu.cn)
In response to injury, vascular smooth muscle cells (VSMCs) migrate and proliferate from the media into the intima. Such a process is called neointima formation or neointima hyperplasia, which leads to vascular remodeling followed by potential atherosclerosis progression, in-stent restenosis or vein bypass graft failure. Previous studies have shown that activation of both the innate and adaptive immune systems are involved in the pathogenesis of neointima hyperplasia and vascular remodeling. However, medical therapies for inhibiting intima hyperplasia are limited. This is largely because mechanisms through which these immune modulators regulate vascular remodeling are poorly understood.

Recent evidence highlights the importance of specific innate immunity signaling pathways activated in vascular dysfunction and repair. Innate immunity distinguishes a diversified set of extracellular and intracellular danger signals that primarily originate from microbes by groups of pattern recognition receptors including Toll-like receptors (TLRs) and Nod-like receptors (NLRs). NLRs are a group of evolutionarily conserved intracellular pattern recognition receptors that are useful in the detection of microbes and danger signals, and play a vital role in innate immunity and host physiology. Notably, mutations or single nucleotide polymorphisms in these genes associate with human diseases including auto-immune disease, gastric cancer, early-onset menopause, among others.

Among multiple members of NLRs family, NOD-like receptor family CARD domain containing 5 (NLRC5) has been reported to be critical in antigen presentation, inflammation, and tissue fibrosis. NLRC5 is abundantly expressed in immune cells in spleen, lymph node, and bone marrow. NLRC5 is also highly expressed in lung and intestine, suggesting that the functions of NLRC5 are not limited to pathogen recognition. NLRC5 shuttles between the cytoplasm and the nucleus in a cytokine response modifier A-dependent manner and acts as a key regulator of major histocompatibility complex (MHC) class I-dependent immune responses by cooperating with regulatory factor X5 (RFX5). In particular, it negatively regulates the NF-kB signaling, type I interferon activities, and the JAK2/STAT3-signaling pathway.

Likewise, our group has recently found that NLRC5 deficiency ameliorates diabetic nephropathy (DN) by alleviating chronic inflammation.

In this study, we demonstrate the protective role of NLRC5 in intimal hyperplasia, and the suppressive effect of NLRC5 on proliferation, migration, and dedifferentiation of VSMCs. Furthermore, we show a mechanistic link between NLRC5 and PPARy.

**Results**

**NLRC5 increased during vascular remodeling.** Vascular remodeling is considered a major feature in vascular diseases, such as in-stent restenosis, Kawasaki disease, and atherosclerosis. Therefore, we first examined the expression of NLRC5 in coronary arteries from patients with Kawasaki disease and in coronary plaques from patients undergoing coronary artery bypass graft surgery. Compared with normal coronary arteries, NLRC5 expression was more abundant in VSMCs in both Kawasaki disease (Supplementary Fig. 1) and coronary plaques; however, its expression was also ubiquitous, rather than localized in the proliferative medial layer (Fig. 1a–c and Supplementary Fig. 2). We further investigated the expression of Nlr5 in a vascular injury model of complete carotid ligation. Similar to what we observed in human diseased arteries, Nlr5 expression dramatically increased in carotid arteries after injury (Fig. 1d, e). Notably, Nlr5 expression was abundant in the neointima rather than the media in the injured artery, and located more in the nucleus rather than in the cytoplasm of α-SMA positive VSMCs. Despite a fraction of Nlr5 co-localizing with endothelial cells marker CD31, there was no difference in endothelial Nlr5 expression between sham and ligation groups (Supplementary Fig. 3). Using western blot, we further confirmed that Nlr5 expression started to increase in carotid arteries at 1 week after the ligation and its expression was profoundly high at 2 weeks later, reaching approximately six-fold higher compared to the sham group, suggesting that Nlr5 expression was induced in VSMCs during neointima formation.

We next treated HAMScs with PDGF-BB (10 ng/ml), a potent stimulator of VSMCs and a key mediator in vascular injury, for different periods of time. NLRC5 expression started to slightly increase 1 h after stimulation and remained at a sustainable higher level after 12 h of stimulation (Fig. 1h). Since we observed that NLRC5 located in the nuclei of VSMCs in mouse carotid tissues in response to injury, we next examined NLRC5 expression in the cytoplasmic and nuclear fractions of PDGF-BB-stimulated HAMScs. NLRC5 increased at 6 h in the nucleus and was maintained up to 12 h following PDGF-BB stimulation (10 ng/ml). In contrast, the expression of NLRC5 in the cytoplasm of HAMScs was at a very low level and remained unchanged following PDGF-BB stimulation (10 ng/ml). Although there was rare cytoplasmic localization, NLRC5 was predominantly expressed in the nuclei of HAMScs and increased in response to PDGF-BB treatment (10 ng/ml) (Fig. 1i).

**NLRC5 attenuated neointimal formation in vivo.** Given that NLRC5 was upregulated in VSMCs after carotid ligation, we hypothesized that NLRC5 contributed to neointimal formation after vascular injury. To test this hypothesis, we studied Nlr5 whole body knockout mice (Nlr5<−/−>). The strategy of knockout mouse generation is summarized in Fig. 2a and genotyping was performed in Nlr5<−/−/> and littermate Nlr5<+/+/> mice. To investigate the effect of NLRC5 on neointimal formation, Nlr5<−/−/> and Nlr5<+/+/> mice were subjected to vascular injury by carotid ligation for 3 weeks. We verified the success of Nlr5 deletion in Nlr5<−/−/> mice and tested the specificity of Nlr5 staining in ligated carotid arteries (Supplementary Fig. 4). NLRC5 deficiency significantly aggravated neointimal formation reflected by enlarged intima areas (Nlr5<+/+/> 2.86 ± 0.45 × 10⁴ μm² vs. Nlr5<−/−/> 5.39 ± 0.86 × 10⁴ μm², P < 0.01 by Student’s t-test) and increased intima/media ratios (Nlr5<+/+/> 0.40 ± 0.10 vs. Nlr5<−/−/> 0.82 ± 0.10, P < 0.01 by Student’s t-test, Fig. 2b–d). Furthermore, NLRC5 deficiency dramatically enhanced vascular hyperplasia reflected by the increased percentage of PCNA-positive nuclei, a marker of proliferation (Nlr5<+/+/> 20.27 ± 1.83% vs. Nlr5<−/−/> 37.42 ± 2.95%, P < 0.01 by Student’s t-test, Fig. 2e, f).

We also measured systolic blood pressure (BP), heart rate, and analyzed the lipid profile to exclude potential confounding factors associated with vascular remodeling. Tail-cuff BP measurement showed no difference in systolic BP (Nlr5<+/+/> 113.68 ± 9.45 mmHg vs. Nlr5<−/−/> 116.09 ± 11.46 mmHg) and heart rate between Nlr5<−/−/> and Nlr5<+/+/> mice after 3-week complete carotid ligation (Supplementary Fig. 5A and B). Plasma total cholesterol, triglyceride, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and fasting glucose levels were similar between two groups (Supplementary Fig. 5C and D).

In the hematopoietic system, NLRC5 is highly expressed in lymphocytes and myeloid cells, and NLRC5 deletion resulted in decreased CD8+ T cells number. Based on these findings, we further analyzed T cells and myeloid cells after carotid ligation in Nlr5<−/−/> mice. Consistent with previous reports, flow cytometric analysis showed a significant reduction in the percentage of CD8+ T cells in splenocytes (Nlr5<+/+/> 10.7 ± 1.2% vs. Nlr5<−/−/> 5.7 ± 0.6%, P = 0.010 by Student’s t-test, Fig. 2f, g).
Supplementary Fig. 6A), and in peripheral blood (Nlrc5+/+ 8.8 ± 0.3% vs. Nlrc5−/− 7.2 ± 0.3%, P = 0.011 by Student’s t-test, Supplementary Fig. 6B) of Nlrc5−/− mice. No differences in CD45+CD11b+Gr1+ myeloid cells were found in bone marrow, spleen, and peripheral blood between Nlrc5−/− and Nlrc5+/+ mice (Supplementary Fig. 7A, B). Considering the fact that CD45+ leukocytes infiltrated carotid arteries and modulated neointimal formation after vascular injury27, we assessed the presence of CD45+ cell population in ligated carotid arteries. There was no difference in the number of CD45+ leukocytes in ligated vs. sham ligated carotid arteries (Supplementary Fig. 8A, B).
**Fig. 1** NLRCS is upregulated in human coronary plaque and in mouse ligated carotids. a Representative images of hematoxylin/eosin-stained normal coronary artery and coronary plaque. Scale bar: 100 μm. b Immunofluorescence staining shows that NLRCS (green) is upregulated in vascular smooth muscle cells (VSMCs, stained in red) residing in coronary plaque. Scale bar: 100 μm (upper) and 20 μm (lower). c Quantitative analysis of the percentages of NLRCS-positive stained VSMCs in coronary arteries (n = 3 per group). Five fields per section from each sample are analyzed. d Immunofluorescence staining shows that NLRCS (red) is constitutively colocalized with VSMCs (labeled with α-SMA in green) in the neointima layer following carotid ligation. Normal IgG isotype serves as negative control. Scale bar: 50 μm and 20 μm.

**Fig. 2** NLRCS attenuates neointimal formation in vivo. a Schematic diagram of generating Nlrc5 knockout construct. Recombination of the Nlrc5−/− allele is examined by PCR in the Nlrc5+/+, Nlrc5+/−, and Nlrc5−/− mice. b Representative images of hematoxylin/eosin-stained Nlrc5−/− and Nlrc5+/− mice carotid arteries at 3 weeks after carotid ligation. Scale bar: 100 μm (upper) and 50 μm (lower). c, d Quantification of the intima area and intima/media ratio in the histological sections (n = 7 per group). e, f Immunohistochemistry staining (red arrow) and quantitative analysis of PCNA-positive cells in the carotid arteries at 3 weeks after carotid ligation. n = 8 per group. Five fields per section from each sample are analyzed. Scale bar: 100 and 20 μm. g Western blotting of Flag tag and β-actin in the ligated Ad-Control- and Ad-Nlrc5-transduced carotids at 5–10 days following carotid ligation. h Representative images of hematoxylin/eosin-stained carotid arteries transduced with Ad-Control or Ad-Nlrc5 at 3 weeks after carotid ligation. Scale bar: 50 μm (upper) and 20 μm (lower). i, j Quantification of the intima area and intima/media ratio in the histological sections (n = 6 per group). k, l Immunohistochemistry staining (red arrow) and quantitative analysis of PCNA-positive cells in the carotids transduced with Ad-Control or Ad-Nlrc5 (n = 6 per group). Five fields per section from each sample are analyzed. Scale bar: 20 μm. Data are presented as mean ± SD. Two-tailed Student’s t-test is used to compare two groups (c, e, g, j, k, and l). *P < 0.05. Original magnification, × 200 (b and h) and × 400 (b, e, h, and k). Source data are provided as a Source Data file.
ligated carotid arteries between Nlrc5−/− and Nlrc5+/+ mice, implicating that depletion of NLRC5 did not affect leukocyte recruitment after carotid ligation (Supplementary Fig. 8).

In an opposite approach, we evaluated whether Nlrc5 overexpression suppresses neointimal thickening. Nlrc5 overexpression was induced by local transduction of NLRC5 using adenoviruses. Overexpression of Nlrc5 was verified by both western blot analyses and immunofluorescence staining (Fig. 2g and Supplementary Fig. 9). Ad-Nlrc5 mice exhibited decreased intima areas (Ad-Control 2.59 ± 0.47 × 10⁵ μm² vs. Ad-Nlrc5 1.07 ± 0.60 × 10⁵ μm², P < 0.01 by Student’s t-test) and intimal/media ratios (Ad-Control 5.04 ± 0.12 vs. Ad-Nlrc5 0.18 ± 0.06, P < 0.01 by Student’s t-test) 28 days after carotid ligation (Fig. 2h–i). The proliferation marker PCNA was remarkably decreased in carotid arteries after ligation with Nlrc5-overexpression (Ad-Control 38.24 ± 2.83% vs. Ad-Nlrc5 21.06 ± 3.96%, P < 0.01 by Student’s t-test, Fig. 2k, l).

**NLRC5 prevented VSMC dysfunction in vitro.** VSMCs dedifferentiation, together with proliferation and migration, is a critical process for neointimal formation. The MTS and Edu incorporation assays demonstrated that NLRC5 knockdown promoted HASMCs proliferation upon PDGF-BB stimulation (10 ng/ml) (Supplementary Fig. 10A). To examine the effects of NLRC5 deficiency on VSMC migration, we performed scratch assay in HASMCs by silencing NLRC5. Migration of HASMCs was markedly enhanced after NLRC5 knockdown with 12-h PDGF stimulation (Fig. 3c, d), while much fewer Ad-NLRC5-overexpressing HASMCs migrated through scratch (Supplementary Fig. 10B and C).

Accumulating studies highlight that contractile VSMCs are capable of dedifferentiating into synthetic VSMCs in response to vascular injury and several extracellular stimuli. VSMC phenotype switching coordinates with VSMC proliferation and migration. Myosin, α-SMA, and Calponin are considered as VSMC differentiation markers. Consistent with the in vivo findings, NLRC5 knockdown reduced the expression of α-SMA, Calponin, and Myosin concomitant with increased expression of proliferative markers PCNA and Cyclin D1. These data indicated that NLRC5 depletion promoted VSMC dedifferentiation, a process of switching from a contractile to proliferative phenotype (Fig. 3e, f). In contrast, NLRC5 overexpression led to increased expression of α-SMA, Calponin, and Myosin, but decreased expression of PCNA and Cyclin D1 in HASMCs upon PDGF-BB stimulation (10 ng/ml) (Supplementary Fig. 10D and E).

Since VSMCs apoptosis was involved in neointimal formation, we also examined the influence of NLRC5 on apoptosis in HASMCs. No significant difference in early or late apoptosis was observed between scramble and NLRC5 siRNA groups (Supplementary Fig. 11A and B). In vivo TUNEL experiments also displayed similar apoptosis ratios in ligated carotid arteries between Nlrc5−/− and Nlrc5+/+ mice (Supplementary Fig. 11C and 11D). In summary, these data implied that NLRC5 suppressed excessive VSMC proliferation, migration, and dedifferentiation upon PDGF-BB stimulation.

**Interaction between NLRC5 and PPARγ.** Current literature suggests a controversial role of NLRC5 in regulating inflammation via NF-κB-signaling pathway. Interestingly, NLRC5 knockdown in HASMCs failed to regulate the phosphorylation of IkBα under the stimulation of PDGF-BB (10 ng/ml) (Supplementary Fig. 12A), which was consistent with the results that the NLRC5 was rarely expressed in the cytoplasm (Fig. 1i). This led us to explore other possible signaling pathways downstream of NLRC5. It is reported that HLA is ubiquitously expressed in human tissues and cells, and NLRC5 acted as a transcriptional coactivator of MHC-I/HLA through recruitment of enhanceosome component RXF5 to SXY module. Thus, we checked the expression of RXF5 and the concomitant changes in classical and uncoupled HLA expression. RXF5 expression remained unchanged upon PDGF-BB stimulation (10 ng/ml) on the mRNA level and it was significantly lower in HASMCs than that in the human mononuclear cell line THP-1, which served as a positive control (Supplementary Fig. 12B). It should be noted that, unlike MHC-II that is found only on antigen-presenting cells, MHC-I/HLA is constitutively expressed on the surface of all nucleated cells in mammalians. Compared with control group, neither classical HLA (HLA-A and HLA-B) nor uncoupled HLA (HLA-E) expression level changed in HASMCs transfected with NLRC5 siRNA (Supplementary Fig. 12C–F). Based on the human inflammatory cytokine array, we found that NLRC5 silencing did induce substantial alterations in the expression of MIF and Serpin E1 under the induction of PDGF-BB (Supplementary Fig. 12G–I). In contrast to the protective effect of NLRC5 in vascular remodeling, our prior study found that NLRC5 played a contradictory role in diabetic mice that loss of NLRC5 ameliorated DN. Therefore, we hypothesized that different environments or stimuli may influence the function of NLRC5. To prove this, we stimulated VSMCs with high glucose or PDGF-BB and found that while PDGF-BB did not activate Smad2 phosphorylation, high glucose significantly stimulated Smad2 phosphorylation (Supplementary Fig. 13A–D). These effects were consistent with analogous findings in mesangial cells as described in our previous work. Moreover, we determined the expression of Myosin, α-SMA, Cyclin D1, and PCNA expression in response to DN in different genetic mice. We found that the expression of proliferation and VSMC markers remained unchanged in kidneys of Nlrc5+/+ diabetic mice compared with Nlrc5−/− diabetic mice (Supplementary Fig. 13E and F).

Given the structural homology of NLRC5 and the reported association between another NLR member class II transactivator (CIITA) and PPARγ, and also after excluding most of the traditional pathways that could potentially serve as downstream mediators of NLRC5, we then studied whether NLRC5 functioned through interaction with PPARγ. PDGF-BB stimulation (10 ng/ml) remarkably promoted the intrinsic interaction of NLRC5 with PPARγ in HASMCs within 6–12 h. These effects were consistent with analogous findings in mesangial cells as described in our previous work. Moreover, we determined the expression of proliferation and VSMC markers remained unchanged in kidneys of Nlrc5+/+ diabetic mice compared with Nlrc5−/− diabetic mice (Supplementary Fig. 13E and F).
transcriptional network, we examined the expression of well-known PPARγ target genes related to proliferation and migration, including CD36, AP2, and CITED2. In response to siRNA-mediated NLRC5 knockdown, the mRNA expressions of CD36, AP2, and CITED2 were significantly decreased (Fig. 4). Given that PPARγ/retnoid X receptors (RXRα) heterodimers bind to PPRE in the regulatory regions of target genes and activate gene transcription, we performed IP to determine whether the presence of NLRC5 regulated PPARγ–RXRα complex formation. However, absence of NLRC5 did not alter the PPARγ–RXRα interaction in HASMCs (Fig. 4). To dissect whether NLRC5 serves as a PPARγ ligand without interfering with the
PPARγ/RXRα complex, we generated a Flag-PPARγ LBD and determined the intrinsic interaction between NLRC5 and LBD of PPARγ (Fig. 4k). Co-immunoprecipitation revealed the interaction of NLRC5 with the LBD of PPARγ, suggesting that the LBD domain, which was responsible for the binding of PPARγ ligand, was required for the interaction with NLRC5 (Fig. 4l). These experiments indicate that NLRC5 predominantly interacts with PPARγ, acts as a PPARγ ligand and regulates PPRE-dependent gene transcription. Indeed, because of the residual levels of NLRC5 protein after knockdown of NLRC5, we could...
not entirely exclude the potential effect of NLRCS on PPARγ complexes in HASMCs.

Activation of PPARγ is known to restrict vascular hyperplasia in response to vascular injury. Similarly, our results depicting an interplay between NLRCS and PPARγ inspired us to determine whether NLRCS mitigated vascular hyperplasia through activation of PPARγ. We confirmed that the PPARγ agonist pioglitazone (10 nM) was able to moderately promote PPARγ expression in HASMCs and gradually enhanced PPRE activity as measured by luciferase assay in HEK293T cells (Supplementary Fig. 1A and C). Conversely, treatment of PPARγ antagonist T0070907 (100 nM) for 6 h inhibited PPARγ expression in HASMCs and repressed PPRE activity in HEK293T cells (Supplementary Fig. 1C and D). Second, we found that treatment of pioglitazone only partly rescued the excessive neointimal formation in Nlrc5−/− mice as compared to Nlrc5−/+ mice without pioglitazone treatment (Fig. 4m–o). Concomitantly, pioglitazone significantly alleviated the in vivo proliferation as quantified by PCNA staining in ligated carotid arteries from Nlrc5−/− mice (Fig. 4p, q). To examine whether NLRCS contributed to NLRCS-mediated alleviation of VSMC proliferation and dedifferentiation in vitro, HASMCs were treated with PPARγ agonist pioglitazone (10 nM) with and without NLRCS depletion. Pioglitazone treatment led to a significant reduction in PCNA and Cyclin D1 expression, accompanied with a recovery of α-SMA, Calponin, and Myosin expression, in the presence and absence of NLRCS (Fig. 4r and Supplementary Fig. 1A). Conversely, the enhancement of α-SMA, Calponin, and Myosin expression in HASMCs transduced with Ad-NLRCS was blocked in the presence of the T0070907, and accompanied by increases in PCNA and Cyclin D1 expression. These findings indicate that T0070907 counteracts the protective effect of NLRCS on VSMC proliferation and dedifferentiation (Supplementary Fig. 1B and C). Collectively, the aforementioned in vivo and in vitro data suggest that NLRCS, at least in part, alleviates vascular remodeling through activation of PPARγ in VSMCs.

We next performed an in silico search within the promoter of NLRCS gene for putative transcription factor-binding sites using binding profiles from JASPAR CORE database (jaspar.genereg.net). Interestingly, a predicted binding motif for PPARγ (JASPAR MA0066.1) was found in NLRCS promoter with a score of 16.4 (Fig. 5a). Thus, we hypothesized that NLRCS promoter sequences containing PPRE were a potential PPARγ-binding site (Fig. 5b). As an initial step, when HASMCs were treated with pioglitazone (10 nM), the mRNA and protein expression levels of NLRCS were gradually increased in a time-dependent manner (Fig. 5c, d). Notably, the intrinsic interaction between NLRCS and PPARγ existed without stimulation of the exogenous PPARγ ligand (Fig. 5e). However, since the increased combination of NLRCS and PPARγ paralleled the elevated expression of NLRCS induced by pioglitazone, we could not conclude whether exogenous PPARγ ligand, pioglitazone, enhanced or competed with NLRCS–PPARγ interaction. Moreover, transient transfection with PPARγ siRNA in HASMCs and co-immunoprecipitation followed by immunoblot analyses demonstrated that knockdown of PPARγ reduced NLRCS expression and abrogated the interaction between NLRCS and PPARγ as well (Fig. 5f). Furthermore, ChIP assay identified the PPARγ enrichment at the NLRCS promoter in HASMCs under native conditions (Fig. 5g). We then used anti-PPARγ antibodies to immunoprecipitate protein/DNA complexes from HASMCs pretreated with pioglitazone for 0 and 12 h and quantitative PCR (ChIP-qPCR) amplified the putative PPRE of the promoter. The ChIP-qPCR studies showed that the PPARγ–binding to the NLRCS promoter significantly increased in response to Pioglitazone (10 nM) (Fig. 5h), further supporting our hypothesis. We next generated a luciferase reporter construct carrying NLRCS promoter (pGL3–NLRCS–promoter–Luci) and measured the luciferase activity. We found that PPARγ overexpression and pioglitazone facilitated NLRCS–luc activities in HEK293T cells (Fig. 5i, j). Based on the above results, we proposed that NLRCS protected vascular remodeling through a positive feedback loop with PPARγ in VSMCs.

The NACHT domain of NLRCS interacted with PPARγ. To better understand the underlying molecular mechanism, we generated different plasmids expressing different NLRCS mutants (Fig. 6a) and evaluated their effects on PPARγ activity. NLRCS consists of a tripartite structure, including an N-terminus, a central NACHT domain, and a C-terminal leucine-rich repeat domain (LRR)[39]. Unlike most of the NLR family members, the N-terminus of NLRCS possesses an atypical CARD domain, also known as death-domain-like fold (DD). Based on the online structural database information (UniProtKB-Q86W13, NLRCS_HUMAN, https://www.uniprot.org/uniprot/q86w13#structure), we defined DD domain of NLRCS as amino acid (aa) 1–221 and the NACHT domain as aa 222–539. The mutants were individually co-transfected with the PPARγ vector in HEK293T cells, and cell lysates were then subjected to co-immunoprecipitation after 24 h transfection. We found that the DD mutant completely abolished NLRCS and PPARγ interaction, whereas the NACHT domain of NLRCS was essential to recognize and interact with PPARγ (Fig. 6b). To further validate the interaction of the NACHT domain with PPARγ, we applied confocal microscopy and detected that both myc-tagged DD + NACHT mutant and myc-tagged NACHT mutant were co-localized with Flag-tagged PPARγ in nuclei of...
HEK293T cells (Fig. 6c). Consistent with these observations, NACHT, NACTH + DD, and ISO3 led to a remarkable increase in PPARγ activity (Fig. 6d) among the four established mutants. Moreover, compatible with increased protein expression, induction of NACHT, NACTH + DD, and ISO3 remarkably enhanced PPARγ activity and reached their peaks at 2 or 3 μg concentrations, while DD mutant did not affect PPARγ activity (Fig. 6e–h).

We then administered adenovirus containing the NACHT domain of NLRC5 (Ad-NLRC5 NACHT) or empty adenovirus (Ad-Ctr) in HASMCs and assessed the effect of the NACHT domain on VSMC proliferation. The Edu incorporation assay showed that adenoviral overexpression of the NACHT domain substantially attenuated VSMC proliferation compared with Ad-Ctr groups (Fig. 6i, j). Furthermore, after adenoviral overexpression of the NACHT domain of NLRC5 (Ad-NLRC5 NACHT), the expression of the PPARγ target genes CD36, AP2, and CITED2 were markedly higher than those measured in Ad-Ctr-transduced HASMCs (Fig. 6k). To assess whether the suppressive role of the NACHT domain on VSMC proliferation in vitro similarly regulates neointimal formation in vivo, we ligated common carotid arteries in mice and performed local transduction of Ad-Control or Ad-Nlrc5 NACHT. Immunoblots for Flag tag were performed and the efficiency of localized delivery of adenovirus into carotid arteries was confirmed (Fig. 6l). In accordance with the in vitro observations, the cross-sectional areas of neointima (Ad-Control 1.29 ± 0.38 × 10^4 μm²...
vs. Ad-Nlrc5 NACHT 0.48 ± 0.17 × 10^4 μm^2, P < 0.01 by Student’s t-test) and intima/media ratios (Ad-Control 0.60 ± 0.11 vs. Ad-Nlrc5 NACHT 0.32 ± 0.07, P < 0.01 by Student’s t-test) in ligated carotid arteries were markedly decreased in Ad-Nlrc5 NACHT mice compared with Ad-Control mice (Fig. 6m–o). Immunohistochemistry analysis of PCNA-positive cells within ligated carotid arteries indicated that VSMC proliferation of Ad-Control mice were more severe than that of Ad-Nlrc5 NACHT mice (Fig. 6p, q). Taken together, these results strongly suggested that the NACHT domain of NLRC5 was responsible for NLRC5 binding to PPARγ and positively regulating PPARγ activity.
Vascular NLRC5 was required for neointimal formation. We further examined the possible contribution of vascular or hematopoietic Nlrc5 in neointimal formation using mismatched bone marrow transplantation (BMT). Nlrc5<sup>−/−</sup> and Nlrc5<sup>+/+</sup> mice were sufficiently irradiated and reconstituted with either Nlrc5<sup>+/+</sup> or Nlrc5<sup>−/−</sup> BM (Fig. 7a). Firstly, BM cells from GFPexpressing mice were transplanted into Nlrc5<sup>−/−</sup> and Nlrc5<sup>+/+</sup> mice after irradiation. Using flow cytometry, the ratios of GFP-positive cells in BM, spleen, and peripheral blood from recipients exceeded 80%, which verified the success of BMT (Supplementary Fig. 15). In addition, we found few and comparable GFP-positive cells from BM infiltrating into neo-intima and media in carotids between Nlrc5<sup>−/−</sup> and Nlrc5<sup>+/+</sup> mice (Fig. 7b). By genotyping and quantitative PCR in BM cells, splenocytes, and peripheral blood cells from Nlrc5<sup>+/−</sup> recipients, we further confirmed the high efficiency of BMT regardless of donor genotypes (Supplementary Fig. 17A–D). Nlrc5<sup>−/−</sup> background mice had equally increased intima areas and intima/media ratios when receiving Nlrc5<sup>+/+</sup> or Nlrc5<sup>−/−</sup> BM cells. Similarly, there was no difference in intima areas and intima/media ratios between Nlrc5<sup>+/+</sup> background mice receiving Nlrc5<sup>+/+</sup> or Nlrc5<sup>−/−</sup> BM cells (Fig. 7a–e). Accordingly, while depletion of Nlrc5 in carotid parenchymal cells markedly aggravated VSMC proliferation indicated by PCNA-positive staining, depletion of Nlrc5 in BM-derived cells did not efficiently affect vascular hyperplasia (Fig. 7f, g). Collectively, this mismatched BMT experiment excluded the contribution of hematopoietic Nlrc5 in neointimal formation after carotid ligation, indicating an essential role for non-hematopoietic Nlrc5 in vascular remodeling.

Since the discovery of NOD-like family member Nlrc5, it has been studied primarily in the immune system and under inflammatory conditions. The innate immune system, such as TLR family members, have been involved in vascular injury and remodeling through regulating VSMC function. Moreover, NLRC5 contributes to both innate immunity and inflammation. Recent studies also revealed its role in chronic disease such as cancer. Our recently published study showed that deficiency in NLRC5 ameliorated DN, a disease exemplified by inflammation, immune response, and vascular remodeling. In response to DN, NLRC5 regulated cellular effects by inhibiting high glucose-related NF-kB and TGF-β-signaling pathways. We not only found enhanced NLRC5 expression in remodeled arteries, but also identified that NLRC5 was specifically located in the newly formed intima, co-localizing well with VSMC markers, rather than in adventitia or medial layers. Gain-of-function and loss-of-function studies revealed a distinct role for NLRC5 in vascular remodeling. Our in vitro data demonstrated that NLRC5 deficiency triggered VSMC dedifferentiation, proliferation, and migration, supporting the role of NLRC5 in regulating VSMC phenotypic switching. Because NLRC5 deficiency also affects the immune system, such as T cell subtype distribution as described by our group and others, we could not exclude the potential contribution of NLRC5 from immune cells on neointima formation. In addition, the neointima itself harbors macrophages and T cells, suggesting the potential role of innate and/or adaptive immunity in vascular remodeling. Therefore, we generated chimeric mice with mismatched bone marrow to investigate the possible contribution of hematopoietic cells to NLRC5-related vascular remodeling.

Discussion

The findings in the present study bear therapeutic relevance based on: (1) accumulating data pointing to the essential role of immunity in the initiation and development of cardiovascular disease through vascular remodeling; (2) the reported function of NLRC5 in the immune response; and (3) our previous report of NLRC5 in facilitating DN, a disease where vascular remodeling is highly involved. The current report extends these findings and establishes a direct role of NLRC5 in vascular remodeling. Nlrc5<sup>−/−</sup> mice exhibit more severe intimal hyperplasia as compared with Nlrc5<sup>+/+</sup> mice. Mechanistically, we identify that NLRC5 forms a positive feedback loop with PPARy in VSMCs. Notably, NACTH domain is the essential domain of NLRC5 mediating PPARy interaction. These findings demonstrating a key role of NLRC5 in orchestrating a complex process of vascular remodeling are summarized in Fig. 8.
Fig. 7 NLRC5 deficiency in BM-derived hematopoietic cells does not affect neointimal formation. 

The second significance of this study is that we characterize PPARγ as the downstream mediator of NLRC5 signaling in VSMCs. The cytoplasmic NLRC5 protein is highly involved in regulating NF-κB activities and modified by different deubiquitinases, while nuclear NLRC5 is thought to transactivate MHC I and related genes through its interaction with RFX. In our previous study in DN, we found that NLRC5 deficiency inhibited high glucose-related NF-κB activity in peritoneal macrophages and ameliorated inflammation. Interestingly, our current study shows that NLRC5 protein is highly expressed in the nucleus of stimulated VSMCs. This is consistent with the lack of influence of NLRC5 on NF-κB activation in stimulated VSMCs, since the latter is believed to function through NLRC5 in the cytoplasm. These data inform a shift in the mechanisms by which NLRC5 functions—

BM: Nlrc5+/+ Nlrc5+/+ Nlrc5+/+ Nlrc5–/–
BM: Nlrc5–/– Nlrc5–/–

Nlrc5+/+ Nlrc5+/+ Nlrc5–/–

GFP GFP GFP GFP

WT KO WT KO

WT KO WT KO

WT KO WT KO

WT KO WT KO

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transcription factor RFX5. However, RFX5 expression could not be induced in VSMCs upon PDGF-BB stimulation, thereby explaining in part the similar MHC I expression levels. The above results imply that the effects of NLRC5 in VSMCs are beyond inflammation or immunity. It is known that PPARγ functions with the obligate heterodimer RXRα. PPARγ/RXRα heterodimers binds to PPRE in the regulatory regions of target genes and activate gene transcription. Apart from this canonical PPARγ receptor, Zhang et al. revealed that NF-κB p65 subunit and PPARγ formed an active transcription factor complex and cooperatively inhibited atherosclerotic progression. Nevertheless, our results show that absence of NLRC5 does not markedly disrupt PPARγ/RXRα heterodimer formation, but suppresses PPRE transcriptional activity. Furthermore, the observations that the LBD domain of PPARγ directly interacted with NLRC5, together with the in vivo and in vitro rescue experiments using PPARγ agonist pioglitazone, suggest that NLRC5 may serve as an endogenous ligand for PPARγ and provides a protective shield against vascular injury via activation of PPARγ.

Finally, our data substantiate that the NACHT domain is a key domain of the NLRC5 interaction with PPARγ, which potentially provides a promising therapeutic target for vascular remodeling-related diseases. NLRC5 is composed of DD domain, NACHT domain and LRR domain, and exhibits structural homology to CIITA. Previous studies revealed that the DD domain conferred transcriptional activity and regulated MHC-I/HLA transcription. The NACHT domain is known as the nucleotide-binding domain thought to be critical for nuclear import and transactivation activity, whereas the LRR domain is responsible for ligand binding. Besides finding the NACHT domain as an essential domain for the interaction between NLRC5 and PPARγ, we also identify that treatment with the PPARγ agonist pioglitazone significantly reduces VSMC proliferation and migration in HASMCs transfected with NLRC5 siRNA. Therefore, our study not only implicates NLRC5 as a therapeutic target for human disease characterized by vascular remodeling, but it also provides a precise domain that may serve as the foundation for future rational drug design. While PPARγ agonists and most of PPARγ target genes are proven protection in type 2 diabetes and cardiovascular diseases, a minority of PPARγ target genes, such as CD36, are reported to promote VSMC proliferation and tumor metastasis. Given the side effect issues associated with PPARγ agonists, including tumor formation, bone fractures, weight gain, and fluid retention, targeting NLRC5 may provide a valuable means to modulate PPARγ signaling and potentially overcome these issues.

Collectively, this study demonstrates an essential role of NLRC5 in vascular intimal hyperplasia and establishes NLRC5 as a key transcriptional regulator in VSMCs uniquely through interaction with PPARγ.
Methods

Human aorta sample collection. Healthy coronary arteries were obtained from three patients undergoing trauma surgery without coronary plaques. Human coronary plaques were obtained from three patients undergoing coronary artery bypass grafting. The baseline characteristics of the patients are listed in Supplementary Table 1. The patient with Kawasaki disease was diagnosed according to the diagnostic criteria of the American Heart Association31. The study received approval by the Ethics Committee of Shanghai Ten People’s Hospital and experiments were conducted in compliance with all relevant ethical regulations. The written informed consent was collected from each patient and/or their relatives.

Mice and complete carotid artery ligation. Nlrc5 knockout (KO, Nlrc5+/−) mice (on C57BL/6 Background) were generated by Shanghai Biomed Model Organism. Brieﬂy, Nlrc5−/− mice (C57BL/6 background) were created by replacing exons 1–4 of the Nlrc5 gene with a neomycin-resistance gene24. The littermate wild type (WT, Nlrc5+/+) mice were used as control mice. Genotyping was conducted using the following primers: Nlrc5-forward 5’TGGGCCAGGAATTATGCTA-3’, Nlrc5-WT-reverse 5’TTCCTGCTGCTGCTCAGT-3’ and Nlrc5-KO-reverse 5’AATGTCGGCAGACCCAGA-3’. Ten-week-old male Nlrc5−/− and Nlrc5+/+ mice fed with normal chow diet were subjected to complete carotid ligation. In brief, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was completely ligated briefly.

Immunofluorescence staining. Cells were seeded at 1 × 105 cells/well on glass-bottomed culture dishes and then fixed with freshly prepared 4% paraformaldehyde for 10 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min. Human artery and mouse common carotid artery tissues were harvested and processed in optimal cutting temperature compound and sliced into 5 μm-thick sections. Paraformaldehyde-fixed sections, cryosections, and cells were incubated with anti-NLRCS (ab105411, Abcam), anti-u-smooth muscle actin (a-SMA, ab7817, Abcam, 1:200), anti-PPAR (sc-7196, Santa Cruz, 1:100), and anti-CD31 (557355, BD Bioscience, USA, 1:100), anti-FLAG (ab49763, Santa Cruz, 1:100), and anti-myc (2276, Cell Signaling Technology, 1:100) overnight at 4 °C. Normal isotype IgG (sc2027, Santa Cruz) was used as negative control. After washing with phosphate-buffered saline (PBS), secondary antibodies (Alexa Fluor 647-conjugated goat anti-rabbit and Alexa Fluor 488-conjugated goat anti-mouse; Thermo Fisher Scientific, 1:200) were incubated for 1 h at 37 °C in the dark. Nuclei were labeled with DAPI (Vector Laboratories), and cells were visualized using an LSM710 laser confocal microscope (Carl Zeiss, Germany).

Plasmid construction. Mutant NLRCS domain constructs and mutant PPARy constructs were purchased from Shanghai Genechem Co., Ltd. All the following plasmids were generated from pGV219 vector with myc tag or pGV141 vector with Flag tag. T0070907 directing myc-NLRC5 ISD Full length Flag-NLRC5 ISD NACHT, myc-NLRCS DD→NACHT, Flag-PPARy, and Flag-PPARy ligand-binding domain (LBD). Myc-NLRC5 ISD3 vector with deletion of partial LRR fragments was purchased from Addgene Incorporation and generated by Neerincx et al36.

Real-time quantitative RT-PCR. Total RNA was isolated from tissues or cells with Trizol reagent (Thermo Fisher, USA). Purified RNA (500 ng) was reverse-transcribed using PrimerScript RT Reagent Kit (Takara, Japan) and quantitative PCR was performed on an ECO real-time PCR machine using FastStart Universal SYBR Green Master (Roche, USA) on a Roche Lightcycler. Information of primers is presented in Supplementary Table 3.
Protein extraction and western blot. Carotid artery tissue lystate or whole cells from in vitro experiments were prepared by 1 x cell lysis buffer (Cell Signaling Technologies, USA) containing protease inhibitors (Cat. 04693159001; Roche Molecular Biochemicals, USA). Given the low abundance of protein collected from mouse carotid artery, we mixed carotid arteries from two individuals under equal condition for the protein extraction. Lysates were cleared by centrifugation. Nuclear and cytoplasmic preparations were separated by NE-PER Nuclear and Cytoplasmic Extractions Reagents according to manufacturer’s instructions (Cat. 78833; Thermo Fisher, USA). Briefly, HASMCs were detached using trypsin and washed with PBS. The cell lysis named CER I was added to cell extractions. Cytoplasmic fractions were collected via vortex, incubation with CER II and centrifugation. The insoluble pellets were then extracted with cold NER. Nuclear fractions were collected via vortex and centrifugation without incubation with CER II. Protein concentrations were determined using bicinchoninic acid protein assay. Because the total protein amount in mice carotid tissues is very low, each lane comprised protein extracts of at least two carotids from mice in the same group when processing SDS–PAGE.

Proteins were separated by SDS–PAGE, transferred to polyvinylidene fluoride (PVDF) membranes and incubated overnight at 4°C with primary antibodies including anti-PPARγ (sc-7196, Santa Cruz, USA, 1:1000), anti-IRXα (3085, Cell Signaling Technology, USA, 1:1000), anti-PCNA (ab29, Abcam, USA, 1:1000), anti-Cyclin D1 (2978, Cell Signaling Technology, USA, 1:1000), anti-α-SMA (ab5094, Abcam, USA, 1:1000), anti-Calponin (ab6794, Abcam, USA, 1:1000), anti-Myc (ab353219, Abcam, USA, 1:1000), anti-NLRCS (ab17624, Abcam, USA, 1:1000), anti-GAPDH (60004-1-lg, Proteintech, USA, 1:1000), anti-Lamin B1 (66095-1-lg, Proteintech, USA, 1:2000), anti-α-tubulin (ab52866, Abcam, USA, 1:1000), anti-β-actin (60008-1-lg, Proteintech, USA, 1:5000), anti-vinculin (sc73614, Santa Cruz, USA, 1:2000), anti-FLAG (ab162, Abcam, USA, 1:2000), and anti-myc (2276, Cell Signaling Technology, USA, 1:2000). Primary antibodies were then incubated with secondary antibody for one hour and bends were visualized using chemiluminescence (ECL, TANON, China) and viewed under Amersham Imager 600 system (GE Health Care, USA). Uncropped scans of the most important immunoblots are supplied in the Source Data file.

Co-immunoprecipitation. HASMCs or HEK293T cells were lysed in 1 x cell lysis buffer (Cell Signaling Technologies, USA) containing protease inhibitors (Cat. 04693159001; Roche Molecular Biochemicals, USA). After centrifugation, 500 μg of cell lysate was incubated with 5 μg of the indicated primary antibodies at 4°C overnight. The lysate immunoprecipitated with anti-IgG serves as negative control. The immune complexes were then purified by 20 μl of protein A/G agarose (sc-2003, Santa Cruz, USA, 1:50) for 3 h, centrifuged and washed by ice-cold cell lysis buffer. The immunoprecipitated protein was further analyzed by immunoblot.

Dual-Luciferase assay. HEK293T cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycinin, then seeded in 12-well plates co-transfected with 1 μg of PPARα response element (PPRE) Reporter mixture (PPRE Luciferase Reporter; Renilla construct – 401, Iagen, Germany) and 1 μg of NLRCS or mutant NLRCS domain constructs using Lipofectamine 2000 (Thermo Fisher, USA). The amplified fragments of NLRCS promoter (Gene accession NM_001330552, pGL-NLRCS promoter) were inserted into pGL3-basic vector (Promega, USA) and were sequenced. Cellular lysates were collected 24 h after transfection using passive lysis buffer. PPRE and NLRCS promoter Luciferase activity was measured using Dual Luciferase Reporter Assay System (Catalog #E1910, Promega, USA) by SpectraMaxi reader.

Bone marrow transplantation. Recipient mice were irradiated with 9 Gy of radiation at least 6 h prior to injection. On the day of BMT, donors were sacrificed and diseminated. Femur and tibia were collected in sterilized PBS on ice, and muscle tissue was thoroughly cleaned. Both ends of the bones were cut off and bone marrow cells were flushed out using 1 ml syringe needle filled with RPMI medium. Bone marrow was filtered through 0.45 μm strainer. Cells were centrifuged and then suspended with 1 ml RPMI 1640. After being counted, cells were transplanted to recipients through tail vain injection. Each recipient was injected with 1 x 10^7 cells with an injection volume of 400 μl.

Cytokine profiling array. Thirty-six different inflammatory markers of HASMCs at the proteins level were examined using a Human Cytokine Array (R&D System ARY005B) according to the manufacturer’s instructions. Briefly, membranes were incubated with 100 μg of total protein lysate and a cocktail of biotinylated anti-bodies overnight at 4°C. Following three washes, membranes were incubated in the presence of 20 μl (1 μg) of streptavidin–horseradish peroxidase (HRP) for 30 min at room temperature, and the presence of immunocomplexes was detected by staining with 3,3’-diaminobenzidine (DAB) chromogen. Arrays were scanned and pixel density was quantified using Imagej software (V1.49, NIH).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using a SimpleChIP Enzymatic Chromatin IP kit (cat No. 9003; Cell Signaling Technologies). HASMCs were treated with 20 μg/ml PDGF-aa for 12 h at 37°C and then cross-linked with 37% formaldehyde at a final concentration of 1% at room temperature for 10 min. Fragmented chromatin was treated with nuclease and subjected to sonication.

ChIP was performed with rabbit anti-PPARγ antibody (ab5036, Abcam, 1:100), rabbit anti-histone H3 (a technical positive control; 1:50) (6626, Cell Signaling Technology) and related gastric cancer: a case-control study and gene expression analyses. PLoS ONE 9, e98899 (2014).

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Author contributions

P.L. performed the animal experiments and flow cytometry, conducted co-immunoprecipitation assays, and immunoblotting, analyzed data and interpreted the results. W.J. designed and performed experiments, analyzed data, and interpreted results. X.X. conducted the immunofluorescence staining of human artery samples, and interpreted results. W.K. performed the histology of mouse artery samples. Q.Y. performed immunoblotting and quantitative PCR, and analyzed the data. H.H. and D.L. conducted bone marrow transplantations. W.W. helped to design and interpret the experiments. M.W.F. helped interpret the data and wrote the manuscript. J.Z. designed experiments, performed the animal experiments, conducted luciferase and CHIP assays, interpreted results and wrote the manuscript. T.Y. supported the initiation of the study and helped design the study; W.P. conceived the project, designed experiments, analyzed data, interpreted results, and wrote the manuscript.

Additional information

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