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# NOD1 ligand FK565 promotes atherogenesis and accumulation of NOD1$^{+}$ smooth muscle cells in atherosclerotic lesions

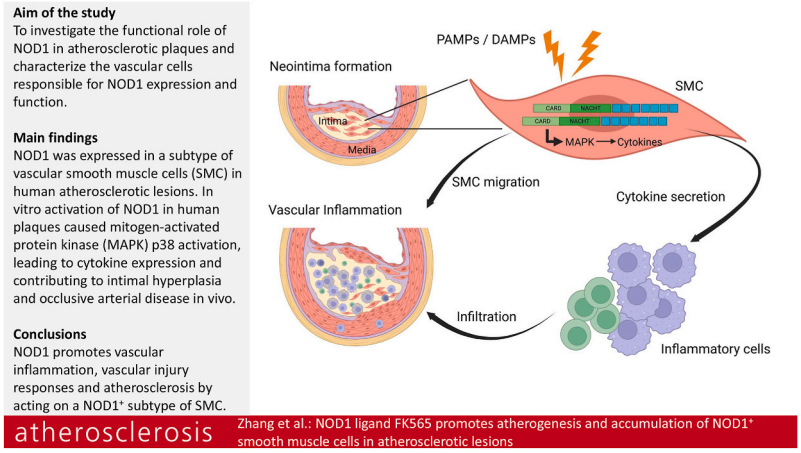
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## HIGHLIGHTS

- NOD1 stimulation activated MAPK p38 pathway and cytokine expression in human endarterectomy specimens.
- NOD1+ SMCs were found in *Ldlr-/-* mouse arteries, and NOD1 activation induced strong vascular inflammation and atherogenesis.
- In rats, mechanical injury increased NOD1+ SMC expansion and neointima formation.
- In vitro, NOD1+ neointimal SMCs showed enhanced migration, increased iNOS+ cells, and elevated CCL5 after NOD1 stimulation.
- NOD1 drives vascular inflammation, injury response, and atherosclerosis through its action on a specific SMC subtype.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Keywords:*
NOD1
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## ABSTRACT

*Background and aims:* Nucleotide-binding oligomerization domain-containing protein (NOD)1 is an intracellular pattern recognition receptor that initiates immune responses upon ligation of molecules such as bacterial peptidoglycan containing a D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) moiety. NOD1 ligation has been

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Innate immunity
Inflammation
Atherosclerosis

shown to promote vascular inflammation and atherosclerosis. In this study, we investigate the functional role of NOD1 in atherosclerotic plaques and characterize the vascular cells responsible for NOD1 expression and function.

*Methods and results:* NOD1 was mainly expressed in a subtype of vascular smooth muscle cells (SMC) in human atherosclerotic lesions. In *ex vivo* cultures, human endarterectomy specimens reacted to NOD1 ligand by activation of mitogen-activated protein kinase (MAPK) p38 pathway, leading to cytokine expression. Levels of NOD1 mRNA were higher in carotid endarterectomy specimens obtained from symptomatic patients compared to asymptomatic ones. $NOD1^+$ SMC were also found in arteries of atherosclerosis-prone $Ldlr^{-/-}$ mice. Challenging these mice with a NOD1 agonist resulted in transmural vascular inflammation, severe arterial damage, accelerated atherogenesis throughout the aorta, and evidence of occlusive coronary artery disease. In rats, mechanic injury to carotid arteries promoted $NOD1^+$ SMC expansion and neointima formation. *In vitro*, neointima derived $NOD1^+$ SMCs responded to NOD1 ligand exposure by enhanced migration, increased $iNOS^+$ cells and amplified CCL5 production.

*Conclusion:* Our findings show that NOD1 promotes vascular inflammation, vascular injury responses and atherosclerosis by acting on a $NOD1^+$ subtype of SMC.

## 1. Introduction

Several lines of evidence identify a pathogenetic role for innate immunity in atherosclerosis [1,2]. Innate immune responses are initiated by ligation of pattern recognition receptors such as Nucleotide-binding oligomerization domain (NOD) like receptors (NLR) and Toll-like receptors (TLR). NOD1 and its sister molecule, NOD2, act as intracellular innate immune sensors of microbial components [3]. Specifically, NOD1 recognizes γ-D-glutamyl-mesodiaminopimelic acid (iE-DAP) in peptidoglycan of Gram-negative bacteria and certain Gram-positive bacteria [4], whereas NOD2 recognizes a muramyl dipeptide (MDP) structure in peptidoglycan of virtually all Gram-positive and Gram-negative bacteria [5]. Upon ligation, NOD1 and NOD2 activate downstream nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK)s pathways via interaction with scaffolding kinase receptor-interacting protein 2 (RIP2) [6,7].

We have previously observed a macrophage-dependent pro-atherogenic effect of NOD2 by enhancing macrophage uptake of low-density lipoprotein (LDL) and diminishing macrophage efferocytosis of cholesterol. This, in turn, leads to expansion of the lipid-rich necrotic core of atherosclerotic lesions [8].

NOD1 is ubiquitously expressed in various cell types, including arterial endothelial cells and vascular smooth muscle cells (SMC) [9–11]. Endothelial cells respond to NOD1 ligation by expressing adhesion molecules that are instrumental to leukocyte adhesion, whereas SMC generate nitric oxide in response to NOD1 agonist [10]. Consistent with these *in vitro* observations, evidence from *in vivo* studies demonstrates that NOD1 agonist induces site-specific vascular inflammation [11] and promotes atherosclerosis in atherosclerosis-prone mice [12]. Despite these advances, little is known about the contribution of individual cells to NOD1-induced immunity in vascular inflammation and atherosclerosis.

By phenotyping of NOD1 expressing cells and functional assessment of NOD1 signaling in atherosclerotic plaques from patients and experimental animals, we have identified a distinct population of SMC with a high expression of NOD1, referred to as $NOD1^+$SMC, reacting to danger signals of both exogenous and endogenous origin. This response led to enhanced cell migration, cytokine and chemokine production *in vitro*, and contributed to intimal hyperplasia and occlusive arterial disease *in vivo*.

## 2. Materials and methods

Detailed methods can be found in the online data supplement.

## 3. Results

### 3.1. Human atherosclerotic lesion contains $NOD1^+$ SMC

Coronary atherosclerosis specimens obtained during autopsy were used to characterize NOD1 localization. Immunostaining analysis of specimens from five individuals was examined and revealed that NOD1 protein was preferentially co-localized with a population of α-actin-positive SMC in the subcapsular region of atherosclerotic plaques and in the tunica media (Fig. 1A). This $NOD1^+$SMC were also found in human carotid endarterectomy plaques with a similar expression pattern (Fig. S1). Co-localization analysis revealed that 33 ± 5% of all NOD1-positive cells were α-actin-positive SMC. In comparison, 40 ± 6% of all α-actin-positive SMC express NOD1. Further analysis of samples from the biobank of Karolinska Endarterectomy (BiKE) [13,14] revealed an association between *NOD1* levels and disease manifestations: Carotid plaque *NOD1* mRNA abundance was significantly higher in patients with a preceding stroke or transient ischemic attack compared to asymptomatic individuals (Fig. 1B).

To characterize NOD1 function in atherosclerosis, we used an *ex vivo* culture system with atheromatous tissue from patients undergoing carotid endarterectomy as previously described [8]. Western blot analysis of *ex vivo* cultured atheromatous tissue with and without stimulation of the NOD1 ligand C12-iE-DAP demonstrated activated p38 in all examined plaques. Extracellular signal-regulated kinases (ERK)1/2 and c-Jun N-terminal kinase (JNK) displayed a large variation in the activation level between the individual plaques. Although, NF-κB activation, as detected by phosphorylated IκBα, was found in three of four plaques, this did not reach significance (Fig. 1C). Moreover, *ex vivo* stimulation of atheromatous tissue with NOD1 ligand resulted in enhanced secretion of interleukin (IL)-1β, IL-6 and IL-8, however, we did not detect a difference in IL-10 secretion (Fig. 1D). IL-6 and IL-8 cytokine secretion was decreased by inhibition of p38 while ERK inhibition seemed to partly decrease IL-6 secretion, although, this did not reach statistical significance (Fig. 1D). Cytokine secretion appeared to be independent of JNK and NF-κB pathways.

### 3.2. NOD1 stimulation promotes experimental atherosclerosis with coronary occlusion

To examine the effect of NOD1 stimuli on atherogenesis, high-fat-diet-fed $Ldlr^{-/-}$ mice were challenged with drinking water containing the NOD1-specific agonistic ligand FK565. After ten weeks of intervention, gross examination of aorta revealed extensive atherosclerosis with outward vessel remodeling throughout the entire aorta in mice exposed to the NOD1 agonist, as compared with sham-treated animals (Fig. 2A). Quantitative analysis of atherosclerosis confirmed that NOD1 ligation was associated with a 3-fold enlarged lesion coverage in the aortic arch and a 2-fold increased lesion burden in the aortic root compared to the

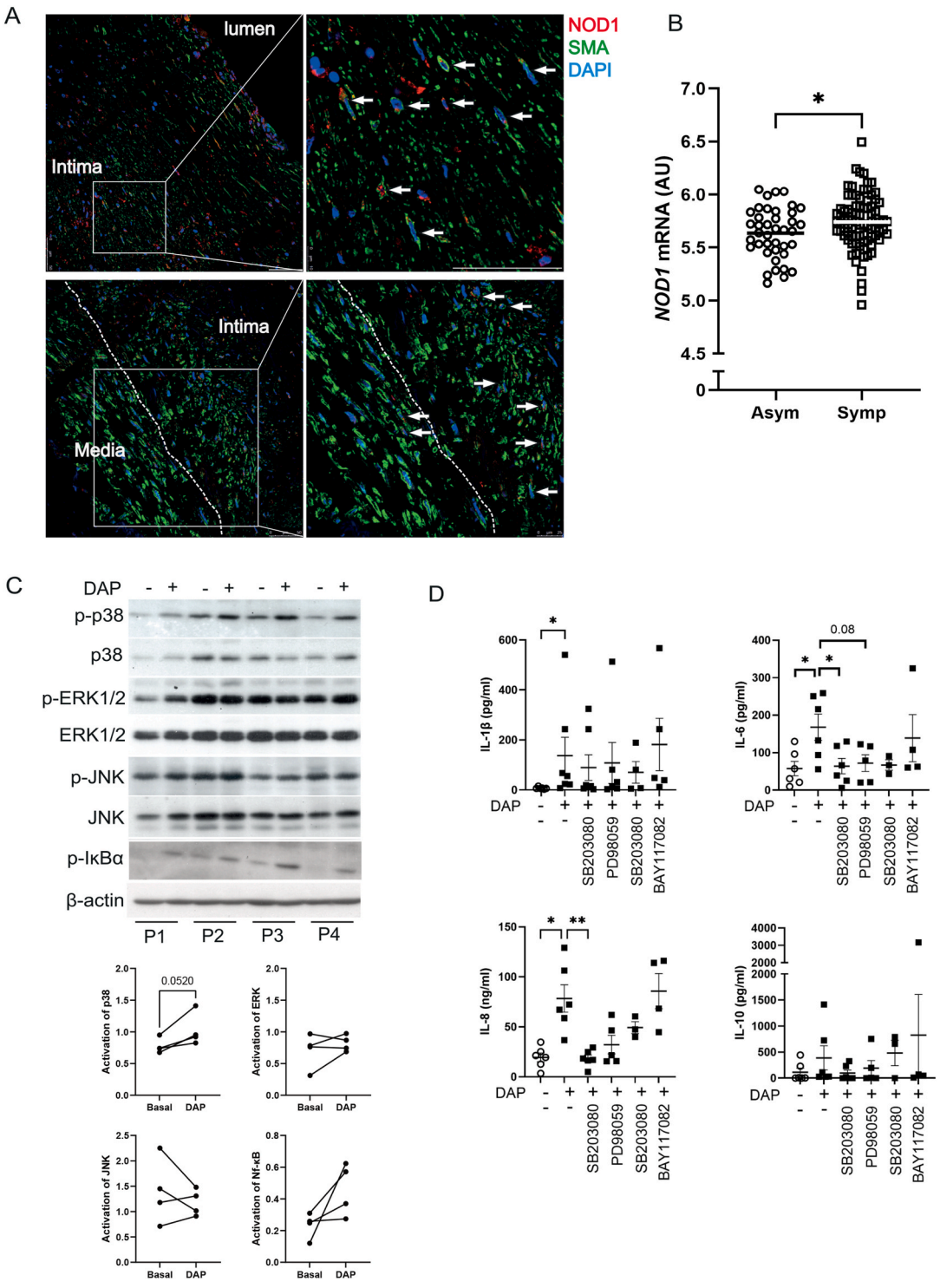



**Fig. 1.** NOD1 mediates innate immune inflammation in human atherosclerosis.
(A) Representative immunofluorescence micrographs of NOD1 expression in human coronary atherosclerosis lesion. Arrows indicate the cells that co-express smooth muscle α-actin (SMA, green) and NOD1 (red). Cell nuclei are labeled by 4, 6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 50 μm. (B) Levels of *NOD1* mRNA in carotid plaques from symptomatic (n = 85) and asymptomatic (n = 40). Mann-Whitney test, *P < 0.05, data presented as scatter plots where a line denotes mean values. AU, arbitrary units. (C) Top panel shows Western blot analysis of phosphorylated p38 (p-p38), p38, phosphorylated ERK (p-ERK), ERK, and phosphorylated IκBα (*p*p-IκBα) in four human carotid plaques (P1–P4) treated *ex vivo* for 15 min with or without C12-iE-DAP (DAP, 1 μg/ml). Lower panel shows degree of activation. β-actin was used as loading control. (D) NOD1-induced IL-1β, IL-6, IL-8 and IL-10 production by human carotid plaques pre-treated with or without (n = 6) p38 inhibitor (SB203080, n = 6-7), ERK inhibitor (PD98059, n = 5-6), JNK inhibitor (SP00125, n = 3-4), or NF-κB inhibitor (BAY117082, n = 4) 30 min in prior to C12-iE-DAP (DAP, n = 7). Kruska-Wallis test followed by Dunn's multiple comparisons test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

situation in mice fed high-fat diet alone (Fig. 2B and C). Mice subjected to the NOD1 agonist developed severe occlusive atherosclerosis in their coronary arteries (Fig. 2C and D). The coronary lesions were characterized by α-actin positive SMC and CD68-positive macrophages (Fig. 2D).

In addition to the accelerated atherogenesis, 6 of 10 mice treated with FK565 died unexpectedly during the course of experiment, with three of these deaths occurring within the final 24 h before sacrifice (Fig. 2E). Despite marked differences in severity and phenotype of atherosclerosis, plasma triglycerides and cholesterol levels did not differ between the two groups of mice. However, the mean body weight of FK565-treated mice was lower than that of the control mice ($p < 0.05$, Table S1).

### 3.3. NOD1 stimulation enhances vascular inflammation and impairs vessel wall integrity

To investigate how NOD1 ligand treatment promotes atherosclerosis, we analyzed factors known to promote vascular inflammation in atherosclerotic lesions of FK565-treated mice. FK565-treatment increased both the lesional macrophages (CD68) and and $CD4^+$ T cells, however, when normalized to lesional area, there was no

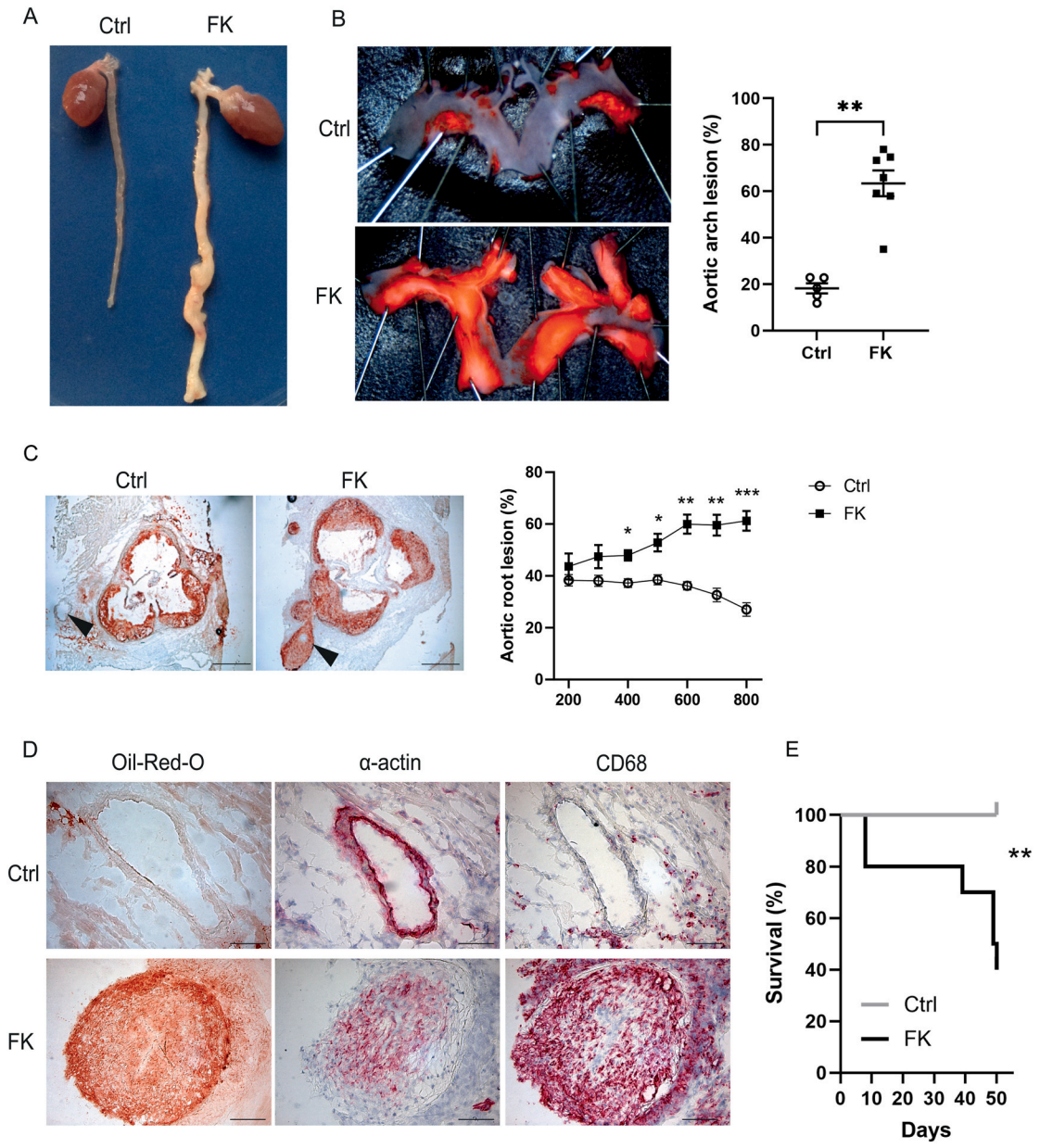



**Fig. 2.** Exposure to NOD1-ligand in drinking water exacerbates development of atherosclerosis and results in coronary and brachiocephalic artery occlusion in hyperlipidemic mice. High-fat diet-fed *Ldlr*$^{-/-}$ mice were treated for 10 weeks with NOD1 ligand FK565 (FK, 21 μg/ml) or without the ligand (Controls, Ctrl) in drinking water. (A) Representative gross specimens of heart and aorta in FK and Ctrl group after 10 weeks treatment. (B and C) Representative images of atherosclerotic lesion in (B) aortic arch stained with Sudan IV and in (C) aortic root stained with Oil Red O. Arrowheads indicate coronary arteries. Scale bar, 400 μm. Quantifications of atherosclerotic lesion area in (B) aortic arch (Ctrl n = 5, FK n = 7) and in (C) aortic root 200 and 800 μm from the aortic root (C) are shown at the right of images (Ctrl n = 10, FK n = 7). Data are presented as mean ± SEM. (B) Mann-Whitney test or (C) Mixed-effects analysis ($p < 0.0001$) followed by Sidak's multiple comparisons test. (D) Representative histological analysis of coronary artery stained with Oil Red O, smooth muscle a-actin (SMA) and CD68. Scale bar, 100 μm. (E) Survival curve in Ctrl and FK treated mice, Logrank (Mantel-Cox) test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significant difference between the experimental groups (Fig. 3A and C). FK565-treatment also caused an upregulation of mRNA encoding *Cd68*, nitric oxide synthase-(*Nos)2, Tnf*, *Il1b, Il12* (Fig. 3B–E) and T cells markers *Cd3*, *Cd4* and *Cd8* with an concomitantly elevated *Ifng* and *Il17a* mRNA levels (Fig. 3C–E). Transcripts of key chemokines, including the ones encoding C–C motif ligand (*Ccl)2*, *Ccl5*, and C-X-C motif chemokine ligand (*Cxcl)10*, were increased approximately 40-fold in diseased arteries from the mice treated with NOD1 ligand as compared with sham treated mice (Fig. 3F).

Infiltration of $CD68^+$ macrophages, $Ly6G^+$ neutrophils and $CD3^+$ T lymphocytes into the media was also significantly increased in FK565-treated animals (Fig. 4A). Analysis of Verhoeff-stained aorta sections revealed extensive destruction of elastic lamellae in the media of NOD1 ligand-treated mice. In total, 10 of 15 aortic valves in the FK565-treated mice had impaired medial integrity compared to one of 28 valves in the control group (Fig. 4B). Immunohistochemical analysis showed a significant reduction of smooth muscle α-actin (α-SMA) protein in the media and increased α-actin in the lesion, together with changes in medial SMC morphology in the brachiocephalic artery of FK565-treated mice (Fig. 4C). Despite we did not find any difference in collagen content in the aortic root lesions (Fig. 4D), the relative levels of transcripts encoding matrix metalloproteinase (*Mmp)9, Mmp10,* and *Mmp12* were increased in FK565-treated animals (Fig. 4E).

### 3.4. Myeloid NOD1 alone is insufficient to promote atherosclerosis

To investigate whether the presence of myeloid cell NOD1 by itself aggravates lesion size in atherosclerosis, lethally irradiated $Ldlr^{-/-}$ mice were reconstituted with bone marrow from $Nod1^{-/-}$ mice. After 13 weeks of high-fat diet feeding, atherosclerotic lesions were quantified in the aortic root and the aortic arch. There were no significant differences in aortic root or aortic arch lesion size (Fig. 5A and B) or any differences in $CD68^+$ macrophages, $CD3^+$ T cells, necrotic core or collagen content in the lesions (Fig. 5C). mRNA levels of *Cd68*, *Cd4*, and *Tnf* in the aorta remained unaltered (Fig. 5D). This implies that myeloid NOD1 is insufficient and hence vascular NOD1 is required to promote atherosclerosis.

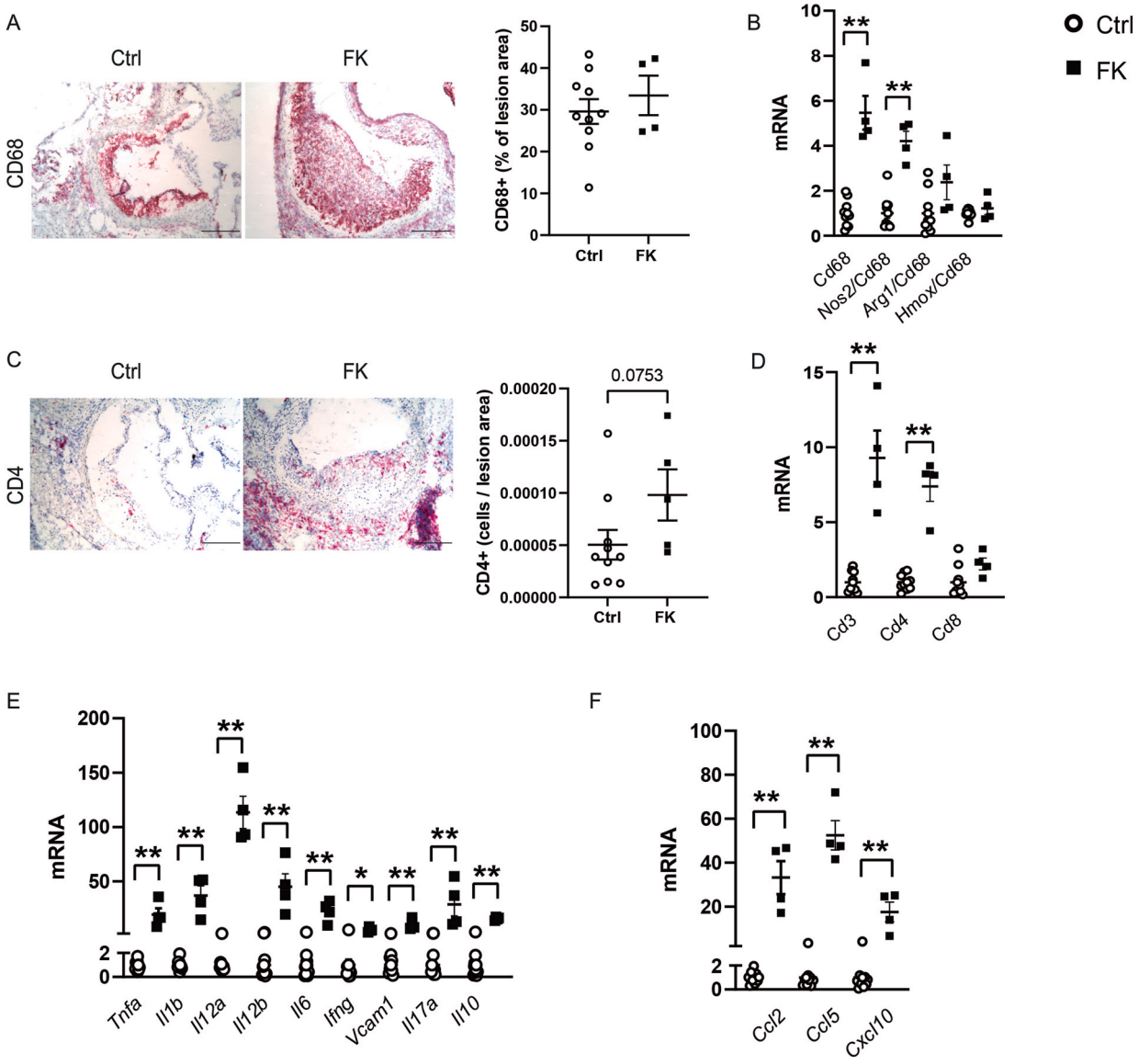



**Fig. 3. Stimulation of NOD1 enhances vascular inflammation in hyperlipidemic mice.** (A and C) Representative micrographs and quantification of $CD68^+$ macrophage and $CD4^+$ T lymphocyte in aortic root sections from mice as in Fig. 1. Scale bar, 200 μm. (B, D, E and F) qPCR analysis of (B) macrophage marker *Cd68* and phenotype-related genes related to *Cd68.* (D) qPCR analysis of T-cell markers, (E) cytokines and (F) chemokine transcripts in aortas. Data are presented as mean ± SEM. Mann-Whitney test, *p < 0.05, **p < 0.01. n = 4-10/group.

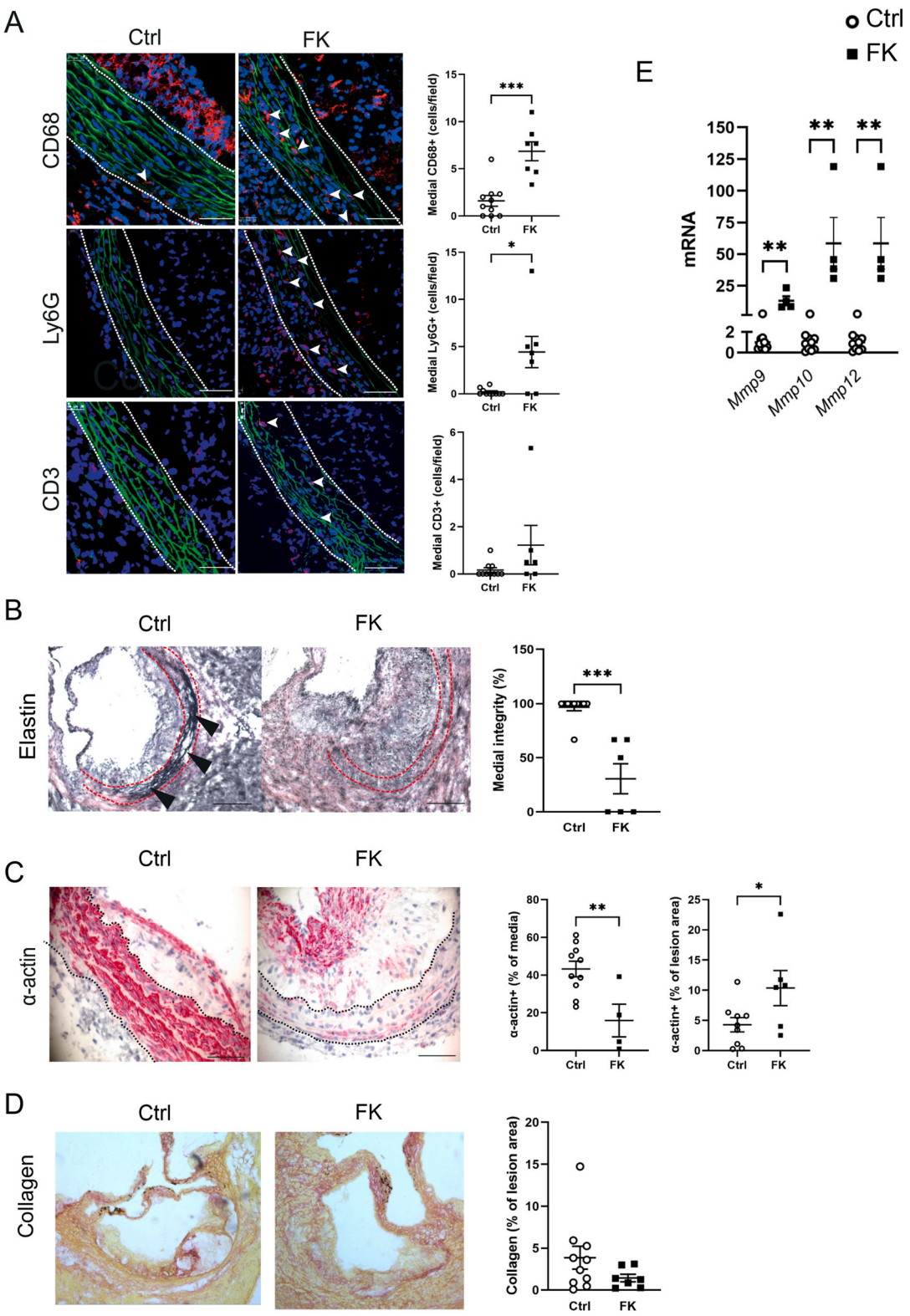



**Fig. 4. Immune cell infiltration into the arterial media layer and impaired vascular wall integrity.** (A) Immunofluorescence micrographs of macrophage (CD68), neutrophil (Ly6G), and T lymphocytes (CD3) in aortic root of *Ldlr*$^{-/-}$ mice. Dotted lines mark the media layer of aorta. Scale bars, 50 μm. Scatter plots show quantification of inflammatory cells in the media. (B) Representative micrographs show Verhoeff-Van Gieson stained elastic fibers in aortic root from the mice as in Fig. 1. Scale bar, 200 μm. Scatter plots show quantifications of elastic fibers in the aortic roots. (C) Representative micrographs showing immunostaining of smooth muscle α-actin (α-actin) in brachiocephalic artery sections. Scale bar, 200 μm. Scatter plot shows the percentage of positive area for SMA in the medial layer (left) and in the lesion (right). (D) Representative micrographs shows picrisirius red staining in tha aortic root and scatter plot shows percentage collagen content in the lesions. (E) qPCR analysis of mRNA encoding matrix metalloproteinase (MMP) in aorta. Mann-Whitney test, Control, n = 9-10, FK, n = 4-7. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

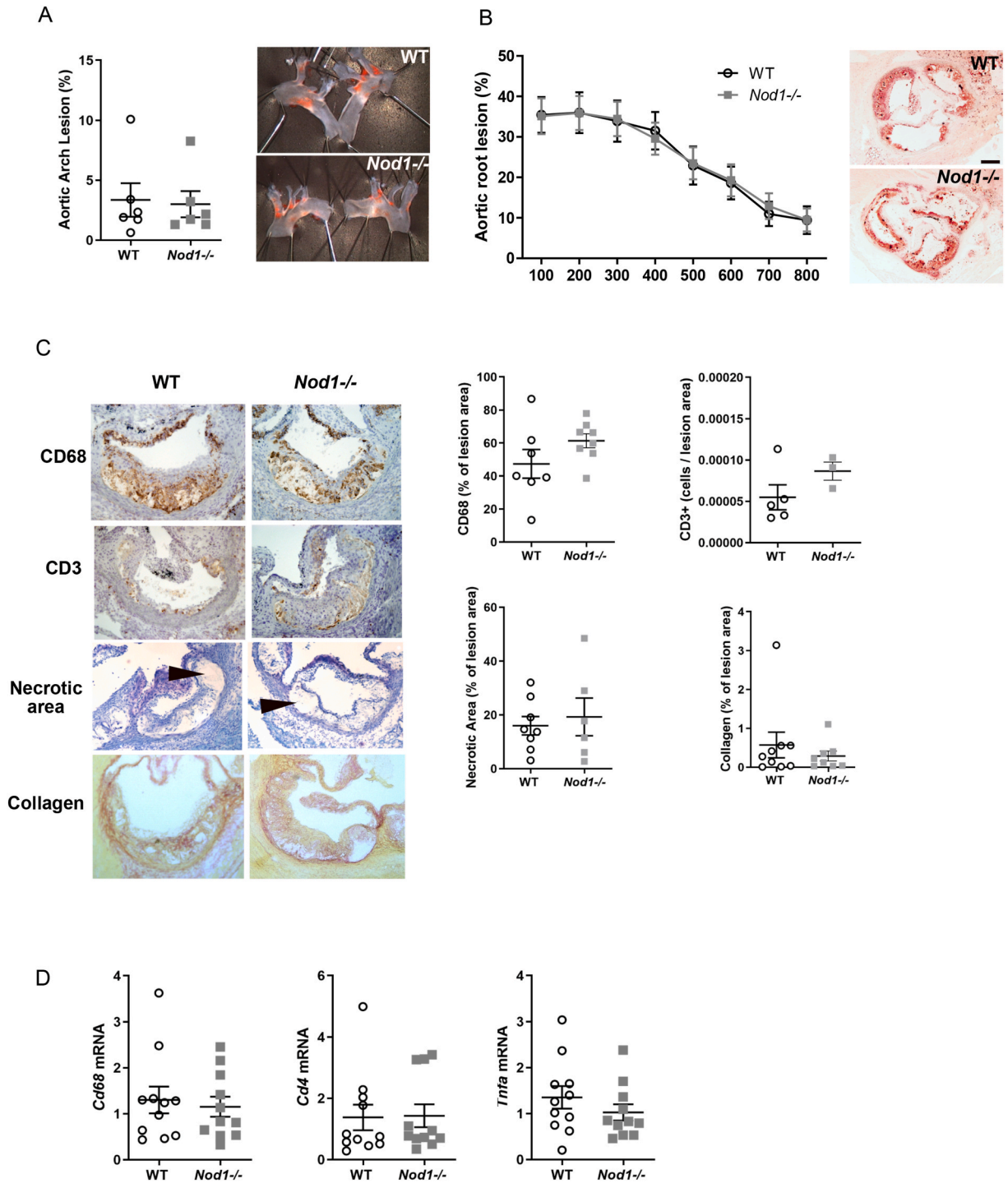



**Fig. 5. *Ldlr*$^{-/-}$ mice transplanted with *Nod1*$^{-/-}$ bone marrow** (A) Quantifications of atherosclerotic lesion area in (A) aortic arch stained with Sudan IV, representative images of aortic arch from WT chimeras (top panel) and *Nod1*$^{-/-}$ chimeras (lower panel, WT, n = 6, *Nod1*$^{-/-}$ n = 6) and (B) aortic root stained with Oil Red O from 100 to 800 μm from the aortic root, representative micrographs of (WT n = 10, *Nod1*$^{-/-}$ n = 10). Scalebar indicates 200 μm. (C) Representative stainings of macrophage marker CD68, T cell marker CD3, necrotic area visulised by toulidine blue staining and collagen content visualized with picrosirius red staining. Arrow heads indicate areas defined as necrotic areas. Scatter plots show CD68$^{+}$, necrotic area and collagen as percentage to lesion area. percentage CD68$^{+}$ areas, number of CD3$^{+}$ cells per lesion area. CD3$^{+}$ is expressed as number of CD3+cells per lesion area. (D) Gene expression of macrophage marker (*Cd68*), T cell marker (*Cd4*) and the cytokine Tnf-α (*Tnfa*) in aorta quantified by qPCR (WT n = 10, *Nod1*$^{-/-}$ n = 10). Data are presented as mean ± SEM. Mann-Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 3.5. Activation of NF-κB in NOD1$^{+}$ SMC

Since hematopoietic NOD1 did not affect atherogenesis in *Ldlr*$^{-/-}$ mice, we proceeded to investigate cellular NOD1 expression in atherosclerotic lesions. Notably, immunostaining revealed a number of α-actin-positive SMC in sections of the brachiocephalic artery samples that were distinguished from other α-actin-positive SMC by expressing high levels of NOD1 (Fig. 6A). These NOD1$^{+}$SMC were an abundant cell type in atherosclerotic lesions of mice treated with NOD1 ligand, whereas, in control mice, NOD1$^{+}$SMCs were predominantly present in arterial

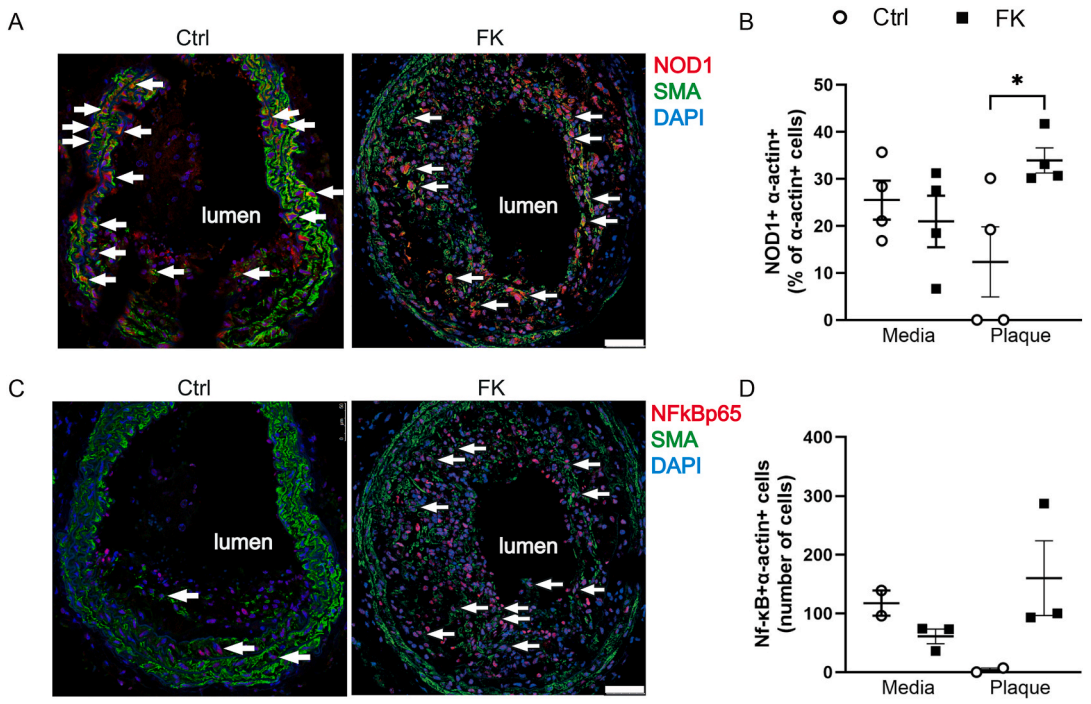



**Fig. 6. Involvement of NOD1$^+$ SMC in atherosclerosis in *Ldlr*$^{-/-}$ mice.** Immunofluorescence micrograph of (A) NOD1 and (B) NF-κB p65 in the brachiocephalic artery of high-fat-diet-fed *Ldlr*$^{-/-}$ mice treated with NOD1 ligand FK565 (FK, 21 μg/ml) or without the ligand (Ctrl) in drinking water. Arrowheads denote NOD1 or NF-κB in smooth muscle α-actin (SMA, green) positive SMC. Scatter plots show (A) percentage of NOD1$^+$SMA$^+$ cells of all SMA + cells and (B) number of NOD1$^+$ NF-κB p65$^+$ cells in media and in the atherosclerotic plaque. Scale bar, 50 μm. (C) Ctrl, n = 4 FK, n = 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

media. In fact, NOD1 expression was seen in the media in both control and FK565-treated mice, however, the percentage of NOD1$^+$ SMC$^+$ in the plaques was significantly increased in FK565-treated mice (Fig. 6B). Immunostaining for phosphorylated NF-κB p65, part of the canonical downstream signaling pathway of the NOD1 receptor, further showed that NF-κB activation was minimal in SMC in the brachiocephalic artery of *Ldlr*$^{-/-}$ control mice. However, in mice treated with NOD1 ligand, ample NF-κB activation was observed in atherosclerotic lesions and the staining was associated with regions rich in NOD1$^+$ SMC (Fig. 6C and D). These observations indicate that FK565-treatment promotes accumulation of NOD1$^+$ SMC in atherosclerotic lesions.

### 3.6. Unique response of NOD1$^+$ SMC to vascular injury

Neointimal development in response to vascular injury resembles many aspects of accelerated atherogenesis and restenosis. To corroborate NOD1 function in vascular injury, alteration in *Nod1* mRNA expression during the course of neointima development was assessed in a rat model of vascular injury. Immunostaining for NOD1 14 days after injury showed a massive NOD1 staining in the neointima (Fig. 7A). Analysis of isolated SMC populations confirmed that constitutive *Nod1* mRNA was threefold higher in neointima-derived SMC versus medial SMC (Fig. 7B). In culture, *Nod1* mRNA levels remained stable in neointima-derived SMC between three and seven passages (data not shown). Thus, imprinted *Nod1* expression seems to be a hallmark of neointimal SMC.

### 3.7. Medial vs intimal SMC

*In vitro* studies of neointima-derived NOD1$^+$ SMC revealed that these SMC responded strongly to tri-DAP, the cognate NOD1 agonist discovered in peptidoglycan [4]. Tri-DAP stimulation increased both *Ccl2 and Ccl5* mRNA levels (Fig. 7C), two of the chemokines induced *in vivo* in response to FK565. Another feature of NOD1$^+$ SMC was their spontaneous release of CCL5 in the absence of exogenous stimulation (Fig. 7D). Furthermore, when using a siRNA approach to knock down the NOD1 expression, this significantly decreased the DAP-mediated CCL5 secretion (Fig. 7E).

Interestingly, in an *in vitro* wound healing assay, NOD1 ligand tri-DAP promoted NOD1$^+$ SMC migration, whereas NOD1 inhibitor ML130 decreased migration (Fig. 7F). However, migration was not dependent on small ras homologous (Rho) nucleotide guanosine triphosphate-(GTP)ase ras-related C3 botulinum toxin substrate (Rac)1 given that blockade with Rac1 inhibitors did not significantly influence migration. Moreover, exposure to NOD1 ligand induced expression of matrix metalloproteinase 9 (*Mmp9*) and synthethic SMC marker *Klf4*, markers of synthethic SMCs, whereas mRNA expression of contractile SMC markers, calponin (*Cnn1*) and smooth muscle α-actin (*Acta2*) was attenuated in NOD1$^+$ SMC (Fig. 7G). Also the synthethic marker iNOS (*Nos2*) was significanly upregulated by NOD1 stimulation, both on mRNA levels (Fig. 7H), as well as, on protein level, showing a time-dependent increase in iNOS levels with NOD1 agonist (Fig. 7I and J).

## 4. Discussion

NOD1 ligation can enhance vascular inflammation and atherosclerosis in experimental animals [10,11] but the cellular targets and responses mediating these responses have remained unclear. Here we report that a population of NOD1$^+$ SMC is present in human and murine atherosclerotic lesions. NOD1 ligation of such cells triggered a set of proinflammatory responses involving MAPK and NF-kB activation, adhesion molecule expression, and production of proinflammatory cytokines and chemokines. *In vivo*, NOD1 ligands instigated transvascular inflammation, provoked intimal hyperplasia, accelerated atherogenesis and promoted coronary artery obstruction in experimental animals. The proatherogenic effect of NOD1 ligation was unaffected by NOD1 ablation of myeloid cells, implying that it requires vascular NOD1 activation. Together, these findings identify an important, proinflammatory and

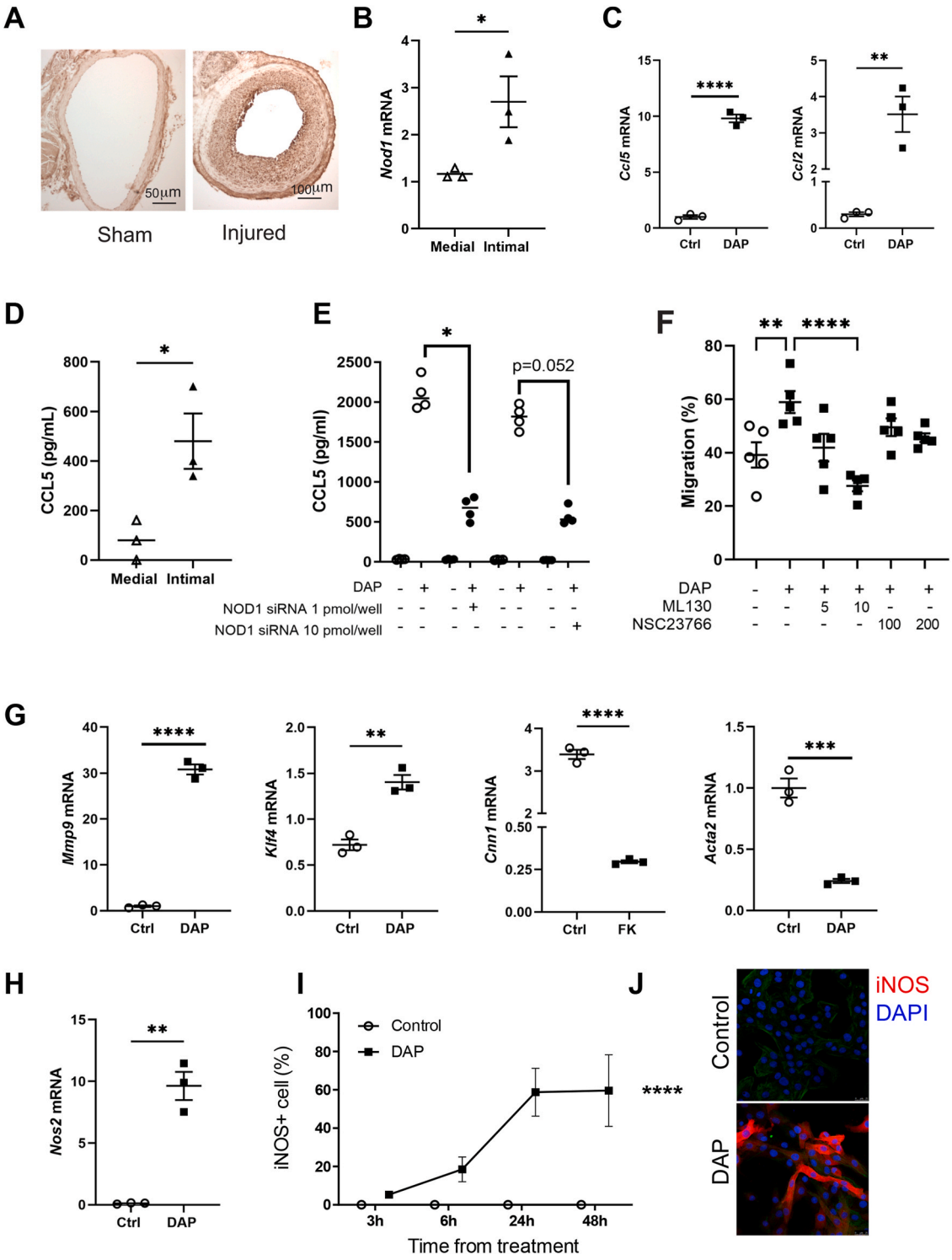



**Fig. 7. Intimal SMC function as NOD1 effector cells.** (A) Representative micrographs showing immunostaining of NOD1 in rat carotid arteries 14 days after balloon injury. (B) qPCR analysis of *Nod1* levels in rat primary SMC isolated from media of uninjured carotid artery (Medial) or from neointima 14 days after balloon injury (Intimal). (C) qPCR analysis of *Ccl5* and *Ccl2* in rat intimal SMC treated with or without C12-iE-DAP (DAP, 1 μg/ml) for 24 h. (D) ELISA assessment of CCL5 production by the SMCs as in (B) in the absence of exogenous stimulation for 24 h. (E) silencing RNA (siRNA) towards NOD1 receptor (F) Wound healing assay of rat intimal SMC migration in response to C12-iE-DAP (DAP, 1 μg/ml) and in the presence or absence of NOD1 inhibitor ML130 (5 μM or 10 μM) or small Rho GTPase Rac1 inhibitor NSC23766 (100 μM or 200 μM). One-way ANOVA followed by Sidak's multiple comparisons test. qPCR analysis of (G) matrix metalloproteinase-9 (*Mmp9*), Krüppel-like factor 4 (*Klf4*), calponin 1 (*Cnn1*), α-actin (*Acta2*) and (H) inducible nitric oxide synthase (iNOS, *Nos2*) mRNA levels in rat intimal SMC treated with or without C12-iE-DAP (DAP, 1 μg/ml) for 24 h. (I) time curve of iNOS expressing intimal SMC treated with or without DAP. (J) representative micrographs showing iNOS immunofluorescence staining (red) in cells from (I) at 24 h. Data are presented as mean ± SEM. Unpaired *t*-test was performed, n = 3-5/group, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

proatherosclerotic role of SMC responding to innate immune signals.

A few studies have investigated the expression and function of NOD1 in human atherosclerosis. NOD1 expression was recently reported in human coronary atherosclerotic lesions [15,16] yet, its functional role in human atherosclerosis remains unclear. We confirm the expression of NOD1 in human coronary and carotid plaques and report an increased expression of NOD1 in patients with symptomatic carotid plaques. Interestingly, NOD1 expression is mainly co-localized with α-actin positive SMCs in both the atherosclerotic lesions and the media layer of the vessel wall. Furthermore, using an *ex vivo* tissue culture system [8,17] we demonstrated that NOD1 stimulation activates p38, resulting in increased cytokine production. Thus, NOD1 activation could contribute to the increased inflammatory milieu in human atherosclerotic plaques, and possibly also contribute to a less stable plaque phenotype.

The present study demonstrates that NOD1 agonist, orally administered at high concentration, has potent effects on both innate and adaptive immunity. This is consistent with previous studies demonstrating the proatherogenic effect of NOD1 stimulation [12]. In addition to signs of macrophage and Th1-cell activation, NOD1 stimulation enhanced vascular Il-17 mRNA levels. IL-17 could contribute to several key mechanisms in NOD1-mediated vascular inflammation and atherogenesis, such as neutrophil infiltration [18], SMC migration, and collagen production [19,20].

Another prominent property of NOD1 stimulation was the induction of chemokines. FK565 treatment led to a 40-fold increase of *Ccl2*, *Ccl5*, and *Cxcl10* mRNA in arteries of mice. This coincided with signs of increased leukocyte infiltration, production of inflammatory cytokines and MMPs as well as impaired vessel wall integrity. The increased chemokine production is a feature of NOD1 stimulation also seen by others [11,12]. These observations support a concept where NOD1 ligand elicits an inflammatory cascade, initiated by local upregulation of chemokines and subsequent transvascular recruitment and activation of $Ly6G^+$ neutrophils, $CD68^+$ macrophages and $CD3^+$ T lymphocytes, contributing to vascular injury. The NOD1-induced increase in expression of several MMPs has been reported previously [11] and we can here demonstrate that MMP activation also coincides with a decreased α-actin expression and elastic lamellae destruction, all hallmarks of vascular wall remodeling [21] and plaque vulnerability [22].

From the bone-marrow transplantation experiment, we can conclude that myeloid NOD1 does not promote atherosclerosis under unstimulated conditions, thus, the major effector cell for NOD1 stimulation is not of hematopoietic origin, thereby confirming previous reports [12,16]. However, the findings in our NOD1-treated mice mirror the findings in *Apoe*$^{-/-}$ mice lacking NOD1, ie., *Apoe*$^{-/-}$*Nod1*$^{-/-}$ mice display decreased atherosclerosis, decreased immune cell infiltration and increased α-$actin^+$ SMC in the lesions [15,16]. Although there was no difference in proliferation in the atherosclerotic lesions, the decreased apoptotic activity in *Apoe*$^{-/-}$ mice lacking NOD1 further supports a role for NOD1 in regulating plaque stability [15,16].

Previous studies have reported an important role of NOD1 expression in endothelial cells, mediating an up-regulation of adhesion molecule VCAM-1 [16]. Although NOD1 is detectable in endothelial cells, expression was substantially higher in SMCs. The findings emphasize a SMC capacity to respond to NOD1 ligands, particularly for the subset with high NOD1 expression found both in mice and human arterial biopsies. Most $NOD1^+$ SMC express vascular smooth muscle cell conserved filament protein α-actin but are distinguished from conventional medial SMC by constitutive two-fold higher mRNA level of NOD1.

$NOD1^+$ SMC were highly responsive to exogenous NOD1 agonist and activated NF-κB was associated with $NOD1^+$ SMC in atherosclerotic lesion of *Ldlr*$^{-/-}$ mice treated with NOD1 ligand. In addition to bacterial peptidoglycan derived NOD1 ligand, $NOD1^+$ SMC also respond to endogenous danger associate molecular patterns (DAMP) derived from vascular injury. Indeed, our previous study in the rat model of vascular injury showed that activated NF-κB signal was present in approximately 80% of intimal SMC. Moreover, targeting IκB kinase β (IKKβ) in the injured artery could attenuate vascular inflammatory responses and neointima development [23]. The present study shows that a majority of cells in the injury-induced intimal lesion are $NOD1^+$ SMC. Although, we did not performe doublestainings for NF-κB and NOD1, given these similarities, the NF-κB positive intimal SMC and injury responsive $NOD1^+$ SMC are very likely overlapping populations. Nevertheless, exact co-localization needs to be addressed in future studies. Emerging evidence shows that NOD1 is involved in transduction of danger signal of endoplasmic reticulum (ER) stress via activation of inositol-requiring enzyme 1α-TNF receptor-associated factor 2 (IRE1α/TRAF2) pathway [24], implicating $NOD1^+$ SMC function in several inflammatory situations. Besides ER stress, high mobility group box 1 protein (HMGB1) released, as a consequence of cell death or actively secreted by living cells under stress, is another known danger signal that correlates closely with severity of injuries. Furthermore, generation of other danger signals such as heat shock proteins has been reported in the context of vascular injury. In addition, insulin and oxidized low-density lipoprotein (oxLDL) have also been demonstrated to activate NOD1 *in vitro* [15,25]. The exact danger signals involved in vascular injury that are recognized by $NOD1^+$ SMC remain to be elucidated in future studies.

$NOD1^+$ SMC have strong inflammatory activity, as demonstrated by the release of CCL5 in the absence of extra stimulation. In a study on *Apoe*$^{-/-}$ mice, Kanno et al. showed that CCL5 protein was present in the intima layer of arteries [12]. Thus, both *in vitro* and *in vivo* observations point to $NOD1^+$ SMC as a producer of CCL5 [12]. In a previous study, we also found that intima-derived SMC are characterized by high expression of a spectrum of chemokines including those highly expressed in the vessels of NOD1-ligand-treated mice from the current study [26]. These findings in combination suggest that NOD + SMC have considerable capability in producing chemokines, thereby contributing to NOD1-induced transvascular inflammation and accelerated atherosclerosis.

Interestingly, $NOD1^+$ SMC was the predominant cellular component of the intimal lesions found in both the stenotic atherosclerotic lesions in the mouse study, as well as, in the rat balloon injury model, a model of intimal hyperplasia. Given that NOD1 stimulation can induce a proliferative phenotype in vascular SMC [25], this suggests that $NOD1^+$ SMC drive intimal thickening, arterial narrowing and progressive occlusion in response to inflammatory or mechanical injury. Exploring the mechanisms behind these effects, we found that NOD1 ligation *per se* could promote SMC migration, since this effect was abolished when using NOD1 inhibitor. Futher characterizartion of synthethic and contractile markers [27] showed NOD1 stimulation to decrease expression of contractile markers SMC α-actin and calponin, whereas synthethic markers MMP9, Krüppel-like factor 4 and iNOS increased. Thus, $NOD1^+$ SMC are prominently contributing to vascular wall remodeling.

Notably, NOD1 agonist FK565 caused a 60% mortality. Oral administration of NOD1 agonist have previously been reported to cause septic shock and organ dysfunction [9] and this cannot be ruled out in the current study. However, no apparent pathological changes in livers or kidneys were identified in the necropsies by gross examination.

An important question for future studies is the origin of $NOD1^+$ SMC. Elegant lineage tracking studies in mouse arteries in scenarios of atherosclerosis, injury, and inflammation have established a consensus that the majority of intimal SMC are derived from tunica media [28,29]. Consistent with this view, tracking NOD1-expressing SMC we found a small number of NOD $^+$ SMC residing in the tunica media of arteries in *Ldlr*$^{-/-}$ mice.

In summary, these findings suggest that a subpopulation of vascular $NOD1^+$ SMC in humans and rodents can initiate transvascular inflammation and lesion development in response to bacterial motifs and/or vascular injury. Understanding where, when, and how the $NOD1^+$ SMC are activated may open up possibilities for new therapeutic interventions.

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## Conflict of interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2026.120729.

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