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TLR3 deficiency protects against collagen degradation and medial destruction in murine atherosclerotic plaques

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Abstract

Objective—Inflammatory cell activation plays a key role in atherosclerotic plaque growth and acute complications. While secretion of proteases and inflammatory cytokines are likely involved in the development of plaque instability, the precise mechanistic pathways are not well understood.

Methods and results—Based on our previous study, we crossed *Toll-like receptor 3* (*Tlr3*)$^{-/-}$ mice with a unique BALB-*Apoe*$^{-/-}$*Npc1*$^{-/-}$ plaque complication-susceptible mouse model, as well as the widely-used B6-*Ldlr*$^{-/-}$ atherosclerosis model, to test the role of TLR3 signaling in the development of plaque instability. TLR3-deficient mice showed no change in aortic root lesion area, but displayed a marked increase in collagen and smooth muscle cell (SMC) content of lesions. Notably, *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ mice exhibited a 50% reduction in the incidence of medial destruction, a precursor to aortic aneurysm formation. MMP-2 activity was markedly reduced in aortic extracts from *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ compared to controls, while both MMP-2 and -9 activities were reduced in *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ extracts. Consistent with the *in vivo* data, TLR3 deficiency suppressed MMP-2 activity induced by TNF-α or polyinosine–polycytidylic acid in macrophages from *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice.

Conclusions—TLR3 plays a critical role in regulating the degradation of extracellular matrix in lesions, in part by modulation of macrophage MMP-2 and -9 activities.

Keywords

Atherosclerosis; Metalloproteinase; Macrophage; Toll-like receptor

1. Introduction

While hypercholesterolemia is an important initiating factor in atherosclerotic plaque formation, plaque growth and acute complications involve the infiltration and activation of inflammatory cells including macrophages and lymphocytes [1,2]. Rupture-prone vulnerable plaques and aneurysm formation are thought to occur downstream of protease secretion from inflammatory cells, including matrix metalloproteinases (MMPs) and cathepsins [3–8]. Control of hypercholesterolemia by diet or lipid-lowering drugs reduces macrophage numbers, decreases expression and/or activity of MMP-1/2/9 and increases collagen content in a rabbit model of atherosclerosis [9]. However, the molecular events operating at the

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Appendix A. Supplementary data: Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.atherosclerosis.2013.03.035

interface between lipoprotein cholesterol uptake by macrophages, MMP expression and development of plaque instability and complications are not well understood.

A major limitation to the study of plaque complications is the fact that standard mouse models of atherosclerosis, including apolipoprotein E knockout (*Apoe*$^{-/-}$) and low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice, show only limited features of atherosclerotic plaque instability [10,11]. We recently reported spontaneous occurrence of plaque complications in *Apoe*$^{-/-}$ mice bearing a mutation in the *Niemann-Pick C1* (*Npc1*) gene [12], which regulates the exit of LDL-derived FC from late endosomes [13]. *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice develop large foam cell rich lesions with thin collagen caps and a significant incidence of medial destruction and athero-thrombosis. Mechanistically, late endosomal accumulation of FC in macrophages led to activation of various Toll-like receptor (TLR) signaling pathways, especially TLR3, and sustained activation of p38 mitogen-activated protein kinase (MAPK) signaling, leading to induction of various *Mmps* and *cathepsin K* [14]. To study the specific role of TLR3 in plaque complications, we crossed *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice with *Tlr3*$^{-/-}$ mice and discovered a role for TLR3 in determining the collagen content of lesions and integrity of the underlying *tunica media*, likely acting via *Mmp2* and possibly *Mmp9*.

2. Methods

Mice

BALB-*Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice have been previously described [12]. B6.129S1-Tlr3^{tm1Flv}/J (*Tlr3*$^{-/-}$) and B6.129S7-*Ldlr*^{tm1Her}/J (*Ldlr*$^{-/-}$) mice were obtained from The Jackson Laboratory. Following backcrossing of the *Tlr3* mutation into the BALB background, *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ littermates were fed standard chow diet for 12 weeks. *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ mice were weaned onto chow and switched to western-type diet (WTD) (TD88137; Harlan Teklad) at 5–6 weeks of age, for 10 weeks.

Additional methods can be found in the Online Supplement.

3. Results

TLR3 deficiency inhibits medial destruction, increasing plaque collagen and SMC content, in plaque complication-susceptible BALB-***Apoe***$^{-/-}$***Npc1***$^{-/-}$**mice.** Although wild-type BALB/c and BALB-*Apoe*$^{-/-}$ mice are highly resistant to atherosclerotic lesion formation, NPC1 deficiency in this background leads to spontaneous lesion formation and acute complications [12]. Thus, we crossed the *Tlr3*$^{-/-}$ mutation into the BALB-*Apoe*$^{-/-}$*Npc1*$^{-/-}$ background to evaluate effects on medial destruction and athero-thrombosis. *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice showed no significant differences in body weight, plasma total cholesterol or triglyceride levels (Supplemental Table 1), lipoprotein profiles (Supplemental Fig. 1A), or atherosclerotic lesion area in the proximal aorta (Fig. 1B). However, there was a significant reduction of en face lesional staining area in the aortic arch in the latter group (Supplemental Fig. 1B). Notably, *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ mice exhibited a 50% reduction in the incidence of medial destruction with medial/adventitial infiltration of foam cells compared to *Apoe*$^{-/-}$*Npc1*$^{-/-}$controls (Fig. 1A, left panel, and C). This was accompanied by increased collagen content in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ lesions, as shown by both Masson's trichrome (Fig. 1A, middle panel, and 1D) and Picro Sirius red staining (Supplemental Fig. 1C). The incidence of athero-thrombosis was unchanged between the two groups (Fig. 1A right panel, and E). There was a 2-fold increase in intimal SMC, but not macrophage, area in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ compared to *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice (Fig. 1F, G). Together, the data indicate that TLR3 regulates medial degradation, collagen content, and SMC accumulation in atherosclerotic lesions in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice.

TLR3 deficiency increases collagen and SMC content, and cap thickness, of plaques in B6-*Ldlr*$^{-/-}$ mice

To determine whether TLR3 deficiency affects matrix composition in a more standard atherosclerosis model, we crossed the *Tlr3*$^{-/-}$ mutation into the B6-*Ldlr*$^{-/-}$ background. Following 10-week WTD feeding, there were no significant differences in body weight, plasma total cholesterol or triglyceride levels (Supplemental Table 2), lipoprotein profiles (Supplemental Fig. 2A), or lesion area in the aortic valve (Fig. 2A, B) or aortic arch (Supplemental Fig. 2B) between littermate *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ mice. However, a striking > 2-fold increase in collagen content and thickness of lesional caps was observed in lesions from *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ compared to *Ldlr*$^{-/-}$ mice (Fig. 2A, C, D and Supplemental Fig. 2C). This was accompanied by significantly increased intimal SMC, but not macrophage, area in *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ compared to *Ldlr*$^{-/-}$ mice (Fig. 2E, F). The results confirmed that TLR3 regulates collagen and SMC accumulation in hypercholesterolemia-induced atherosclerotic plaques.

TLR3 deficiency suppresses MMP-2 protease activity in atherosclerotic lesions of *BALB-Apoe*$^{-/-}$*/Npc1*$^{-/-}$ and B6-*Ldlr*$^{-/-}$ mice

In contrast to TLR4/Myd88 deficiency, and consistent with a recent report [15], TLR3 deficiency did not change inflammatory cytokine expression levels in aorta from *BALB-Apoe*$^{-/-}$*Npc1*$^{-/-}$ or B6-*Ldlr*$^{-/-}$ mice (Supplemental Fig. 3). Because MMPs and cathepsins promote collagen degradation and features associated with plaque instability [4,10], we analyzed aortic gene expression and activity levels. In the BALB-*Apoe*$^{-/-}$*Npc1*$^{-/-}$ model, *Mmp2* expression was induced in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and repressed in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ aorta (Fig. 3A). *Mmp9* was not induced in *Apoe*$^{-/-}$*Npc1*$^{-/-}$, but was repressed in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ aorta. *Mmp14* and *Ctss* were increased in both *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$ aorta, showing no difference with TLR3 deficiency. *Mmp12* was increased in *Apoe*$^{-/-}$*Npc1*$^{-/-}$, but not significantly repressed in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ aorta. In summary, TLR3 deficiency in the BALB-*Apoe*$^{-/-}$*Npc1*$^{-/-}$ background resulted in decreased expression of both *Mmp2* and *Mmp9*, encoding gelatinases known to cleave collagen type 4 fibrils [16].

Next, protease activity was assessed via collagen type 4 degradation and gelatin zymography assays. Enhanced collagenolytic activity was observed in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ aortic extracts, and this was suppressed in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ extracts (Fig. 3B). By zymography, MMP-2 activity was increased in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ aortic homogenates and reversed by concomitant TLR3 deficiency (Fig. 3C), while the activity of MMP-9 was too low for accurate quantification. The decrease in MMP-2 activity reflected an overall decrease in protein as the ratio of mature form to total MMP-2 protein did not differ between *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr*$^{-/-}$ aortic extracts (*Apoe*$^{-/-}$, 55.8 ± 9.9%; *Apoe*$^{-/-}$*Npc1*$^{-/-}$, 41.3 ± 3.0%; *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$, 41.2 ± 0.9%, mean ± SD).

In the B6-*Ldlr*$^{-/-}$ model, TLR3 deficiency repressed *Mmp2* expression, increased *Mmp14*. and had no effect on the expression of other *Mmps* in aorta (Fig. 4A). Decreased collagenolytic activity was observed in *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ protein extracts compared to *Ldlr*$^{-/-}$ controls (Fig. 4B). By zymography, both MMP-2 and -9 activities were substantially lower in *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ compared to *Ldlr*$^{-/-}$ aortic homogenates (Fig. 4C). Again, the ratio of the mature form to total MMP-2 was unchanged between groups (*Ldlr*$^{-/-}$, 35.4 ± 2.9%; *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$, 43.8 ± 4.0%, mean ± SD) indicating a decrease in total protein, and this was confirmed by Western blotting (Fig. 4D). Thus, decreased MMP-2 and -9 activities may contribute to the effect of TLR3 deficiency on lesional collagen content in *Ldlr*$^{-/-}$ mice.

TLR3 deficiency represses macrophage pro-MMP-2 activity induced by inflammatory cytokines present in atherosclerotic plaques. As observed in aortic homogenates (Fig. 3B), collagen degradation was increased in media from *Apoe*$^{-/-}$*Npc1*$^{-/-}$ macrophages, and this effect was reversed using media from *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ cells (Fig. 5A). Next, we assessed cytokine-induced MMP2/9 activity in BM-derived macrophages using gelatin zymography (Fig. 5B). Treatment with TLR3 ligand (polyinosine–polycytidylic acid [Poly(I:C)]), phorbol-12-myristate-13-acetate (PMA) and tumor necrosis factor (TNF)-α increased pro-MMP-2 activity in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ macrophages, but not in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ cells. Pro-MMP-9 activity was induced by TNF- α stimulation in *Apoe*$^{-/-}$*Npc1*$^{-/-}$macrophages and modestly reduced in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ macrophages. Interferon (IFN)-γ reduced both pro-MMP-2 and -9 activities in a non-genotypic fashion, consistent with a previous report [5]. Since these stimuli are likely relevant to the inflammatory milieu of atherosclerotic plaques [2], these data suggest that the effect of TLR3 deficiency on collagenolytic activity in aortic homogenates is mediated, at least partly, by decreased activity of MMP-2 and possibly MMP-9 in macrophages.

Next, we analyzed *Mmp2* and *Mmp9* expression http://circ.ahajournals.org/cgi/content/full/117/7/931 - R33-188785in basal and TNF-α-stimulated macrophages (Fig. 5C). In TNF-α-stimulated macrophages, NPC1 deficiency enhanced *Mmp2* and *Mmp9* expression and these were inhibited by TLR3 deficiency. Genotypic regulation of macrophage *Mmp2* expression paralleled differences in MMP-2 activity under similar conditions (Fig. 5B), suggesting that altered gene expression regulates MMP-2 activity in media of cultured macrophages and aortic homogenates.

MMP-2 is also secreted by SMCs and endothelial cells [17,18]. *In vitro*, pro-MMP-2 activity was readily detected in basal SMCs (Supplemental Fig. 4A). However, pro-MMP-2 activity was similar in all genotypes, and was not affected by inflammatory mediators. TNF-α stimulation induced pro-MMP-9 activity, but to a similar level in all genotypes. Moreover, intimal MMP-2-positive immunostaining overlapped F4/80-positive macrophages in BALB-*Apoe*$^{-/-}$*Npc1*$^{-/-}$ and B6-*Ldlr*$^{-/-}$ mice (Supplemental Fig. 4B). It has been proposed that macrophages activate SMC-derived pro-MMP-2 in response to local cytokine activation of macrophage MMP-14 [19]. In our model, macrophage MMP-14 protein level was unchanged by TLR3 deficiency (Supplemental Fig. 4C) and there was no effect of macrophage genotype on MMP-2 activity in media derived from SMCs (Supplemental Fig. 4D). These data are consistent with an effect of TLR3 on pro-MMP2 activity in macrophages but SMCs.

Macrophage MMP-2 activity induced by TNF-α is suppressed by p38 MAPK and NF-κB inhibitors

TLR3 signals through the adaptor protein, TRIF, leading to activation of MAPKs and nuclear factor (NF)-κB [20]. We previously reported increased basal and stimulated p38 MAPK phosphorylation in *Npc1*$^{-/-}$ macrophages compared to wild-type [14]. Herein, we assessed the role of MAPKs and NF-κB in regulating macrophage MMP-2 activity. Inhibitors of both p38 MAPK (SB202190) and NF-κB (IκBα phosphorylation inhibitor, BAY11-7085) reduced TNF-α–stimulated MMP-2 activity to a similar low level in all genotypes (Fig. 5D), consistent with a recent report [21]. This suggests that activation of p38 MAPK and NF-κB by endosomal cholesterol loading is responsible for induction of *Mmp2* expression.

TLR3 deficiency enhances SMC proliferation and collagen secretion, but not migration

To understand the mechanism of increased SMC content in TLR3-deficient lesions, we performed a series of studies in SMCs. Like macrophages, *Apoe*$^{-/-}$*Npc1*$^{-/-}$-derived SMCs

displayed increased cellular FC compared to *Apoe*$^{-/-}$ controls, and this was not affected by TLR3 deficiency (Fig. 6A). Cell migration was similar in *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ SMCs (Fig. 6B). However, cell proliferation was decreased in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ SMCs, while both proliferation and collagen secretion were increased in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ SMCs (Fig. 6C and D). Many cytokines secreted from macrophages regulate SMC proliferation, migration and collagen secretion [22]. Although media from macrophages reduced SMC proliferation in all groups, the pattern of SMC proliferation did not change between genotypes (Fig. 6C), suggesting a cell autonomous effect of TLR3 deficiency. Thus, we looked at SMC gene expression by quantitative real-time PCR. *Mmp* gene expression was similar between *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ SMCs (Fig. 6E). Pro-collagen *Col1a1* gene expression was significantly reduced in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ SMCs, while *Col4a3* gene expression was significantly increased in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ SMCs. PDGF–B is known to increase SMC proliferation in atherosclerotic lesions [22], and *Pdgfb* gene expression is down-regulated by TLR3 activation in macrophages [23]. Accordingly, *Pdgfb* gene expression was decreased in *Apoe*$^{-/-}$*Npc1*$^{-/-}$ SMCs, and increased in *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ SMCs (Fig. 6E). These results suggested that induction of *Pdgfb* by TLR3 deficiency facilitates cell proliferation and collagen secretion in SMCs, contributing to increased collagen accumulation and SMC number in lesions of *Tlr3*$^{-/-}$ mice.

4. Discussion

We have used a unique plaque complication-susceptible mouse model, involving NPC1 - related endosomal accumulation of cholesterol, to reveal an effect of TLR3 signaling on medial destruction, collagen degradation, and SMC cell content in atherosclerotic lesions. In humans, collagen degradation is associated with plaque vulnerability, and medial destruction with loss of SMCs is a precursor for aortic aneurysms. The effects of TLR3 deficiency on collagen and SMC content were also observed in the more commonly used B6-*Ldlr*$^{-/-}$ model. The effects were associated with decreased macrophage *Mmp2*, and possibly *Mmp9*, expression. Together with earlier studies, these findings suggest that cholesterol accumulation in the macrophage endosomal system leads to TLR3 signaling via p38 MAPK and NF-κB, and increased *Mmp2* and *Mmp9* expression. We also observed SMC-mediated effects of *Pdgf* expression on cell proliferation, collagen secretion and collagen gene expression.

TLRs play a pivotal role in the sterile inflammation associated with atherogenesis. Deletions of *Tlr2, Tlr4*, and the shared TLR-signaling adaptor protein, *Myd88*, have been shown to significantly reduce atherosclerotic lesion area in *Apoe*-deficient mice by decreasing macrophage content and expression of inflammatory and chemokine genes [24–26]. Consistent with a recent report [15], we observed no effect of TLR3 deficiency on aortic cytokine/chemokine expression (Supplemental Fig. 3). Other studies have implicated combinatorial signaling of CD36/TLR4/6 in macrophage inflammatory effects of oxidized LDL [27], as well as inflammasome activation by small cholesterol crystals in the endosomal system [28]. Although we cannot rule out an effect of inflammasome activation in the *Apoe*$^{-/-}$*Npc1*$^{-/-}$ background, our findings suggest that TLR3 may also be an important player at the lipoprotein/macrophage interface, sensing signals derived from endosomal cholesterol deposition and regulating MMP activity, lesional collagen and SMC content, as well as medial degradation. Moreover, TLR3 ligand inhibits cholesterol efflux from macrophages by inhibiting liver × receptor-dependent gene expression, including *Abca1* and *Abcg1* [29].

Medial destruction, with loss of SMCs and macrophage infiltration into the adventitia, is a hallmark of atherosclerotic aortic aneurysms [30,31]. *Mmp9* and *Mmp2* have been

previously implicated in the destruction of medial elastic fibers. Increased expression of both genes has been observed in aneurysm walls [32–34] and targeted disruptions of both genes suppress the development of experimental abdominal aneurysms [35,36]. In addition, targeted *Mmp9* disruption suppressed atherosclerosis-related medial destruction in one study [37] but not another [38]. The disparate findings may have been due to differences in experimental design such as diet and site of plaque characterization. Although we observed a strong protective effect of TLR3 deficiency on atherosclerotic medial destruction in the BALB-$Apoe^{-/-}Npc1^{-/-}$ model, Cole et al. reported [39] the opposite effect on elastic lamina damage following carotid collar-induced injury. However, injuryinduced neointima formation primarily involves SMC proliferation and the underlying mechanism may be different. In our model, the effect of TLR3 deficiency on pro-MMP2 activity was observed in macrophages but not SMCs. It is notable that aortic mRNA expression of *Mmp 14* and *Timp2*, known mediators of MMP2 activation in other models, was not affected by TLR3 deficiency.

The impact of TLR3 signaling on collagen type 4 degradation observed in our study may have clinical relevance in humans. Collagen type 4 is the major structural component of basement membranes. Genome-wide association studies have reported associations of common polymorphisms of *COL4a1* and *COL4a2* structural genes with arterial stiffness [40], coronary artery calcification/myocardial infarction [41], CAD [42], and intracranial aneurysms [43]. Three other genes involved in ECM integrity – *ADAMTS7* and *HSPG2/CSPG2* – have been associated with CAD [42] and intracranial aneurysms [43], respectively, further supporting the importance of the ECM in atherogenesis and vascular complications. Although collagen type I is an important structural component of plaque caps, this protein was not studied herein and we cannot rule out a potential role of TLR3-deficiency in collagen type I degradation.

The emerging role of inflammation on plaque growth and complications suggests that anti-inflammatory therapies could effect plaque stabilization. Our study suggests that decreasing TLR3/TRIF signaling in macrophages might lead to an increase in plaque collagen content and cap thickness, and a decrease in medial destruction and aneurysm formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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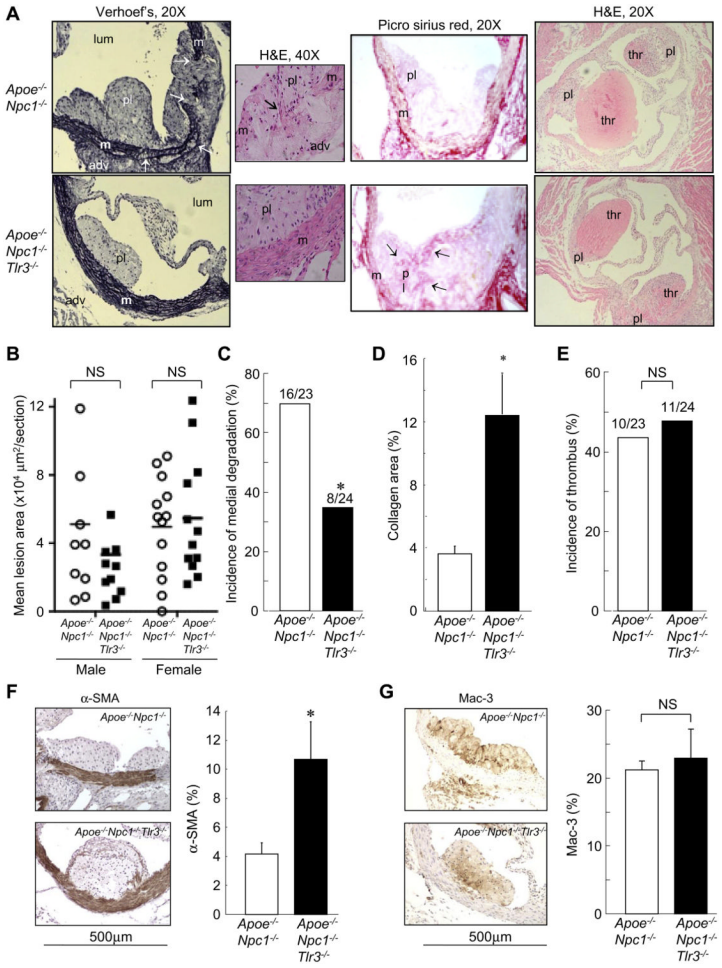

Fig. 1.

TLR3 deficiency results in dramatic inhibition of medial degradation, with increased collagen and SMC content of atherosclerotic plaques in BALB-***Apoe*$^{-/-}$***Npc1*$^{-/-}$**mice**. (A) Representative sections from the proximal aorta of twelve-week-old, chow-fed, *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ mice. Left panel, Verhoef's staining for elastin (black). Arrows indicate medial destruction. Small insert, high-power field of medial area. Arrow indicates foam cells in the region of destructed media. Middle panel, Picro Sirius red staining for collagen (red). Arrows indicate intimal collagen accumulation. Right panel, H&E staining shows thrombi associated with atherosclerotic plaques. pl, plaque; lum, lumen; m, media; thr, thrombus. (B) Quantification of lesion area by morphometric analysis (n = 23−24 mice/genotypic group). Horizontal bars represent mean values. (C, E) Incidence of medial degradation (C) and thrombus formation (E) associated with atherosclerosis in the proximal aorta. (D) Quantification of collagen-positive area relative to total lesion area (n = 12 mice/group). (F, G) Immunostaining for SMCs (α-SMA, F) and macrophages (Mac-3, G) and quantification of positively-stained areas (brown) relative to total intimal lesion area (n = 8 mice/group). NS, not significant. *$p < 0.05$ *vs.* *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice. Data are mean ± SD.

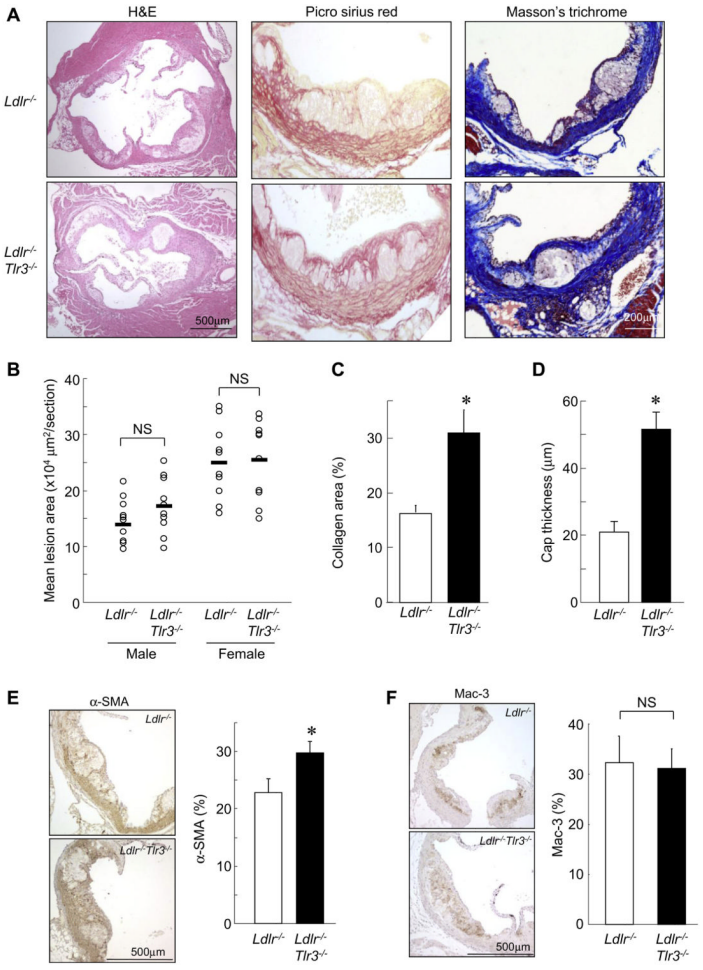

Fig. 2. TLR3 deficiency increases collagen and SMC content of plaques in B6-*Ldlr*$^{-/-}$mice

(A) H&E, Picro Sirius red, and Masson's trichrome staining of sections from the proximal aorta of 10wk WTD-fed *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Tlr3*$^{-/-}$ mice. (B) Quantification of lesion area by morphometric analysis (n = 10 mice/group). Horizontal bars represent mean values. (C, D) Quantification of collagen-positive area (red staining) relative to total lesion area (n = 8 mice/group) and fibrous cap thickness (n = 10 mice/group). (E, F) Immunostaining for SMCs (α-SMA, E) and macrophages (Mac-3, F) and quantification of positively-stained areas (brown) relative to total intimal lesion area (n = 8 mice/group). NS, not significant. *p < 0.05 vs. *Ldlr*$^{-/-}$ mice. Data are mean ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

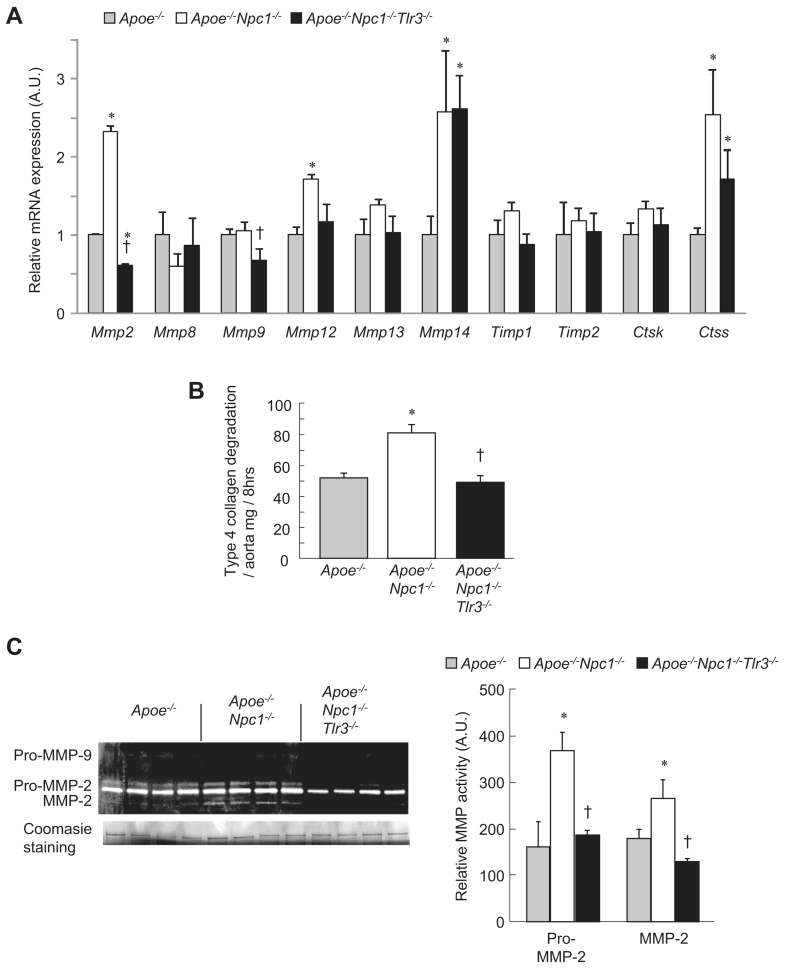

Fig. 3. Suppression of aortic collagen degradation and MMP-2 activity in TLR3-deficient *Apoe*$^{-/-}$*Npc1*$^{-/-}$ mice

(A) Analysis of gene expression by real-time PCR in aorta from twelve-week-old, chow-fed, *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Npc1*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ mice ($n = 4$ mice/group). (B) Collagen degradation in aortic extracts incubated with FITC-labeled native type IV collagen and measured as the release of soluble fluorescent material after the indicated time. (C) Relative MMP-2 activity measured by densitometric analysis of gelatin zymographs. Coomasie blue staining is for standard control. *$p < 0.01$ *vs.* *Apoe*$^{-/-}$ group. †$p < 0.01$ *vs.* *Apoe*$^{-/-}$*Npc1*$^{-/-}$ group. AU, arbitrary unit Data are mean ± SEM of three individual experiments.

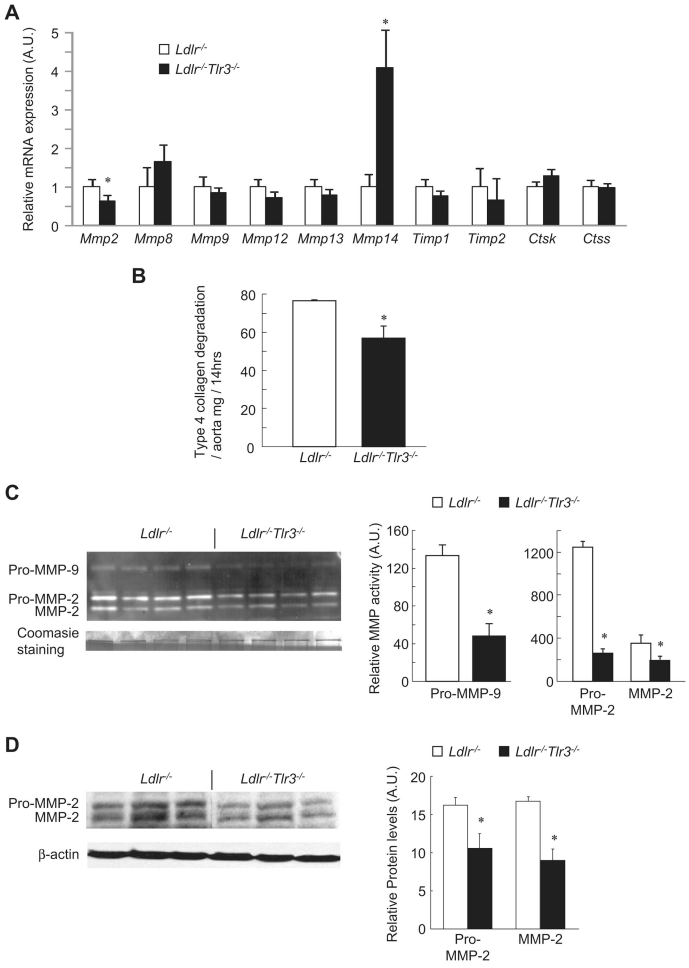

Fig. 4. Suppression of aortic collagen degradation and MMP-2 activity in TLR3-deficient *Ldlr*$^{-/-}$ mice

(A) Analysis of gene expression by real-time PCR in aorta from 10wk WTD-fed *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Tlr3*$^{-/-}$ mice (n = 4 mice/group). (B) Collagen degradation in aortic extracts incubated with FITC-labeled native type IV collagen and measured as the release of soluble fluorescent material after the indicated time. (C) Relative MMP-2 and -9 activities measured by densitometric analysis of gelatin zymographs. Coomasie blue staining is for standard control. (D) Western blot analysis of aortic MMP-2 (n = 3 mice/group). *p < 0.01 *vs.* *Ldlr*$^{-/-}$. AU, arbitrary unit. Data mean ± SEM of three individual experiments.

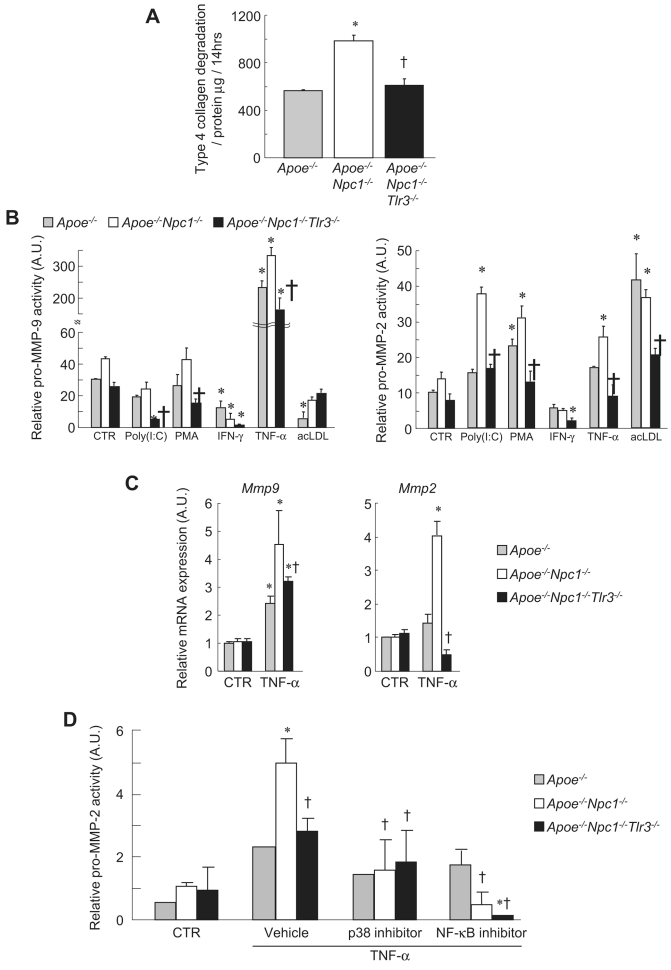

Fig. 5.

Macrophage MMP2 activity and gene expression is induced by NPC1 deficiency and suppressed by TLR3 deficiency. (A) Collagen degradation in macrophage media incubated with FITC-labeled native type IV collagen and measured as the release of soluble fluorescent material after 14 h (n = 3/group). (B) Relative activities of pro-MMP-2 and -9 quantified by densitometric analysis of gelatin zymographs. BM-derived macrophages stimulated by indicated cytokines, Poly(I:C) (5 μg/ml), PMA (40 ng/ml), IFN-γ (10 ng/ml), TNF-α (50 ng/ml), or acLDL (50 μg/ml). (C) *Mmp2* and *Mmp9* expression by real-time PCR in $Apoe^{-/-}$, $Apoe^{-/-}Npc1^{-/-}$ and $Apoe^{-/-}Npc1^{-/-}Tlr3^{-/-}$ macrophages incubated in TNF-α (50 ng/ml) for 4h. (D) Relative activities of pro-MMP-2 quantified by densitometric analysis of gelatin zymographs. BM-derived macrophages stimulated in serum-free medium containing TNF-α (50 ng/ml) and indicated inhibitor (10 μmol/L) for 24 h p38 inhibitor, SB202190; NF-κB inhibitor, BAY11-7085. *$p < 0.05$ *vs.* $Apoe^{-/-}$ CTR. †$p < 0.05$ *vs.* $Apoe^{-/-}Npc1^{-/-}$. AU, arbitrary unit. Data are mean ± SEM of three individual experiments.

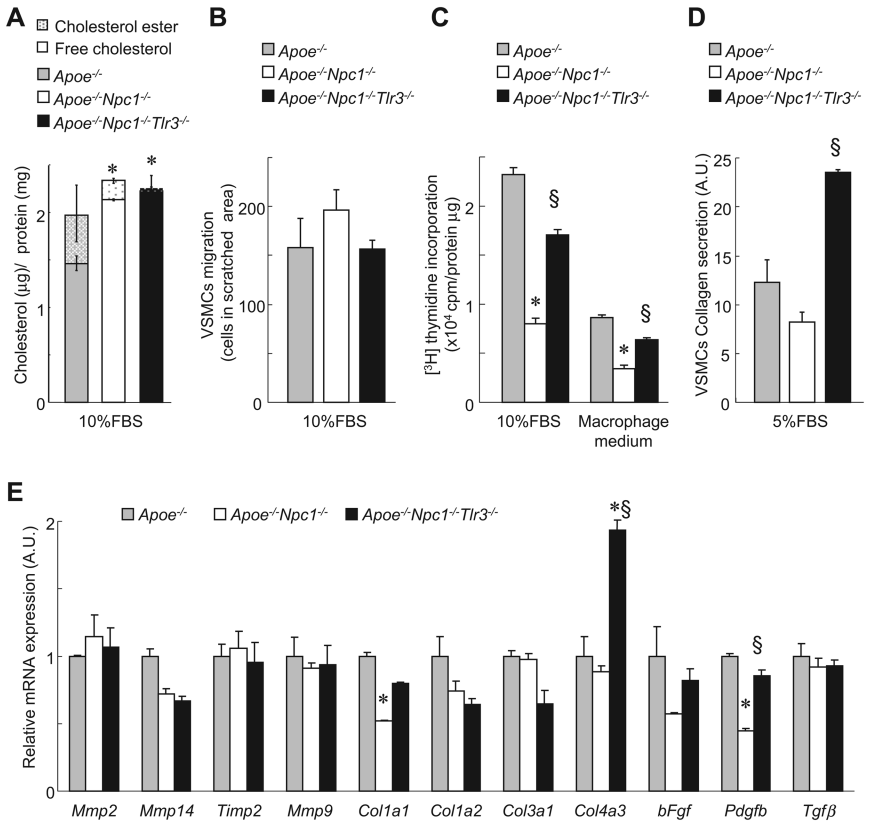

Fig. 6. SMC proliferation and collagen secretion are induced by TLR3 deficiency

(A) Cellular cholesterol content of SMCs derived from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ incubated in SMC medium. (B) SMC migration measured by wound healing assay after incubation in 10% FBS for 24 h (C) SMC proliferation measured by thymidine incorporation assay in 10% FBS or macrophage medium. (D) SMC collagen secretion measured by Sircol collagen assay. (E) mRNA expression by real-time PCR in SMCs derived from *Apoe*$^{-/-}$, *Apoe*$^{-/-}$*Npc1*$^{-/-}$, and *Apoe*$^{-/-}$*Npc1*$^{-/-}$*Tlr3*$^{-/-}$ mice and incubated in medium for 24 h *$p < 0.05$ *vs. Apoe*$^{-/-}$. f$p<0.05$ *vs. Apoe*$^{-/-}$*Npc1*$^{-/-}$. Results are mean ± SEM of triplicate experiments.