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ORIGINAL ARTICLE

# Toll-like receptor 4 signaling has a critical role in *Porphyromonas gingivalis*-accelerated neointimal formation after arterial injury in mice

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**Recently, we reported that a periodontopathic pathogen, *Porphyromonas gingivalis (P. gingivalis)*, infection induced neointimal hyperplasia with enhanced expression of monocyte chemoattractant protein (MCP)-1 after arterial injury in wild-type mice. Toll-like receptor (TLR) 4 is known to be a key receptor for virulence factors of *P. gingivalis*. The aim of this study is to assess the hypothesis that TLR4 has a critical role in periodontopathic bacteria-induced neointimal formation after an arterial injury. Wild-type and TLR4-deficient mice were used in this study. The femoral arteries were injured, and *P. gingivalis* or vehicle was injected subcutaneously once per week. Fourteen days after arterial injury, murine femoral arteries were obtained for histopathological and immunohistochemical analyses. The anti-*P. gingivalis* IgG levels in *P. gingivalis*-infected groups were significantly increased compared with the anti-*P. gingivalis* IgG levels of the corresponding non-infected groups in both wild-type and TLR4-deficient mice. TLR4 deficiency negated *P. gingivalis*-induced neointimal formation compared with that observed in wild-type mice and reduced the number of MCP-1 positive cells in the neointimal area. We conclude that *P. gingivalis* infection may promote neointimal formation after an arterial injury through TLR4 signaling.**
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**Keywords:** arterial injury; periodontal disease; *Porphyromonas gingivalis*; toll-like receptor

## INTRODUCTION

Percutaneous coronary intervention is a well-established therapy in coronary artery disease; however, the resulting mechanical damage to the vascular wall causes neointimal hyperplasia and blood vessel remodeling.[1,2] Upregulation of monocyte chemoattractant protein (MCP)-1 gene expression after coronary angioplasty induces neointimal hyperplasia and recruits monocyte and tissue macrophages to the arterial wall.[3] Medical treatment with neutralizing MCP-1 antibody has resulted in a remarkable reduction of neointimal formation in a rat model of carotid injury.[4]

Periodontitis is an inflammatory disease that leads to the destruction of tooth-supporting tissue. The pathogen *Porphyromonas gingivalis* (*P. gingivalis*) is a major cause of human periodontitis.[5] Toll-like receptors (TLRs) are a group of pattern recognition receptors that mediate the innate host response to microbial pathogens.[6,7] It is well known that *P. gingivalis* is recognized by TLR4; *P. gingivalis* lipopolysaccharides (LPS) treatment of gingival fibroblasts upregulates the expression of MCP-1 and nuclear factor-κB (NF-κB) via TLR4.[8] Recent studies have shown that periodontal pathogens contribute to the pathogenesis of arterial diseases.[9,10] Periodontal pathogens may have a role in the development and progression of atherosclerosis, leading to cardiovascular disease.[11,12] We previously showed that *P. gingivalis* infection induced neointimal hyperplasia after arterial injury[13] and that TLR2 deficiency suppressed the progression of the neointimal formation.[14] However, the role of TLR4 blockade in neointimal formation accelerated by *P. gingivalis* infection after wire injury remains to be elucidated.

TLR4 is known to have a pivotal role in the progression of vascular remodeling.[15] Bai *et al.* also showed that the expression of TLR4 protein increased in balloon-injured arteries compared with uninjured arteries. The inhibition of IL-1 receptor-associated kinase (IRAK) 1 and IRAK4, which were activated by TLR4, attenuated neointimal formation. Furthermore, the IRAK1/4 inhibitor suppressed the activation of the TLR4-mediated NF-κB pathway *in vivo* and *in vitro*.[16] However, the mechanism by which periodontal bacterial infection accelerates neointimal formation through TLR4 after arterial injury has not yet been elucidated. On the basis of these facts, the aim of this study is to investigate the involvement of TLR4 in periodontopathic bacteria-induced neointimal formation after arterial injury.

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## MATERIALS AND METHODS

### Animal protocol

Male C57BL/6 mice (wild-type; WT, 7 weeks, 20–25 g) were obtained from Japan Clea Co (Tokyo, Japan). Male TLR4 knockout (TLR4KO) mice in a C57BL/6 background were obtained from Oriental Yeast Co (Tokyo, Japan). The mouse strains were confirmed by genotyping, and age-matched mouse groups were used for experiments. The experimental procedures described here were approved by the Animal Welfare Committee and performed in accordance with the Animal Care Standards of Tokyo Medical and Dental University.

### Bacterial preparation

*P. gingivalis* strain A7A1-28 was grown on blood agar plates in an anaerobic chamber with 85% $N_2$, 5% $H_2$ and 10% $CO_2$. After incubation at 37 °C for 2–3 days, the bacteria were inoculated into a peptone yeast extract and incubated for a further week. The bacterial concentration was standardized to $10^8$ colony forming units (CFUs) $ml^{-1}$. The levels of anti-*P. gingivalis*-specific IgG in the plasma were determined by an enzyme-linked immunosorbent assay, as previously described.[17]

### Chamber model

Coil-shaped subcutaneous chambers were prepared from 0.5 mm stainless-steel wire and surgically implanted in the subcutaneous tissue of the back region of each mouse. During the period before inoculation, the outer incision healed completely, and the chambers became encapsulated by a thin-vascularized layer of fibrous connective tissue. Fourteen days after implantation, mice were inoculated with 0.1 ml of a suspension of *P. gingivalis* in phosphate-buffered saline. The non-infected group was only inoculated with phosphate-buffered saline. Mice were killed 14 days after arterial injury, and plasma was separated from blood obtained by retro-orbital bleeding.

### Wire injury model

In this study, we modified an arterial injury model.[18] Briefly, the femoral artery was looped and tied off with 6-0 silk sutures for temporary vascular control during the procedure. A transverse arteriotomy was made, and a flexible angioplasty guidewire (a curved 350-μm polished copper wire) was introduced and advanced 1 cm. Endothelial denudation of the artery was performed by withdrawal of the wire; three passes were made along the artery.[19,20] A sham operation (no wire injury) was also performed. Both WT and TLR4KO mice were divided into two groups: those injured by arterial surgery and inoculated with live *P. gingivalis* (0.1 ml of $10^8$ CFU $ml^{-1}$) and those injured by arterial surgery and inoculated with vehicle containing diluted medium (0.1 ml). Sham-operated vessels (no wire injury) with *P. gingivalis* infection in WT and TLR4KO mice were used as controls. Subcutaneous injections were performed once per week for 14 days. Fourteen days later, the mice underwent laparotomy and dissection.[19] Body weight was measured just before killing.[21] Figure 1 shows the time schedule of this study.

### Histological and morphometrical analyses

Histopathological analyses were performed as previously described.[18] The sections were stained with Elastica van Gieson (EvG). Complete transverse sections of arteries ~3 mm in length were obtained.[19] The persons who selected and measured the histological sections were blinded with respect to the intervention. The thickness of the intima, media and lumen within a cross-section of the artery in the slides stained with EvG was calculated using Image-Pro Express (Media Cybernetics, Silver Spring, MD, USA; $n=6$–10) software. The neointimal and medial areas of at least six sections per artery were measured. Some samples were excluded from statistical analysis when they included massive thrombus formation.

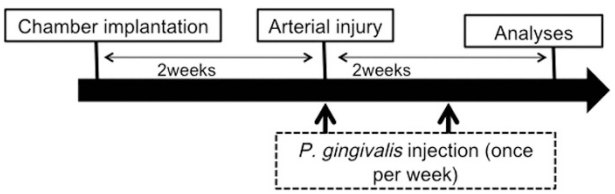


**Figure 1** Time schedule of this study. Live *P. gingivalis* was injected on days 0 and 7 after arterial injury. Samples were collected 14 days after arterial injury.

### Immunohistochemistry

For immunohistochemical staining, anti-MCP-1 antibody and anti-CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used ($n=5$, each). The number of immunohistochemically positive cells was counted per artery by a researcher who was blinded to the treatments of animals.

### Statistical analysis

All data are expressed as the mean ± s.e.m. An analysis of variance combined with the Kruskal–Wallis test was used to compare all groups. The differences between two selected groups were analyzed by Mann–Whitney's *U*-test. Statistical significance was accepted at $P<0.05$.

## RESULTS

### Quantification of antibacterial antibodies

The effects of the repeated injection of *P. gingivalis* or vehicle on plasma levels of anti-*P. gingivalis* IgG were determined using enzyme-linked immunosorbent assay 14 days after injury in WT and TLR4KO mice. In WT mice, the anti-*P. gingivalis* IgG level of the infected group ($n=8$) was significantly higher than that of the non-infected group ($n=8$). In TLR4KO mice, the anti-*P. gingivalis* IgG level of the infected group ($n=6$) was also significantly higher than that of the non-infected group ($n=6$; Figure 2).

### Quantitative analysis of intimal thickening after wire injury

Areas of the vascular wall were quantitatively analyzed in the non-infected mice and the *P. gingivalis*-infected mice 14 days after injury. In all of the vascular wall sections, internal and external elastic laminae were identifiable by EvG staining (Figure 3). In WT mice, the intima/media thickness ratio in the *P. gingivalis*-infected group ($n=10$) was significantly increased in comparison with the non-infected group ($n=10$). It is noteworthy that TLR4 deficiency

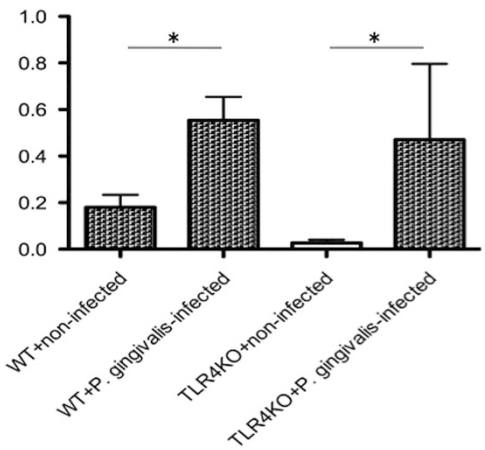


**Figure 2** The levels of anti-*P. gingivalis* IgG. In WT and TLR4KO mice, the effects of repeated injection of *P. gingivalis* or vehicle on the plasma levels of anti-*P. gingivalis* IgG were determined. Plasma samples were obtained from the non-infected group and the *P. gingivalis*-infected group 2 weeks after injury. The levels of anti-*P. gingivalis* IgG are expressed as the means ± s.e.m. $*P<0.05$, significant difference compared with the non-infected group.

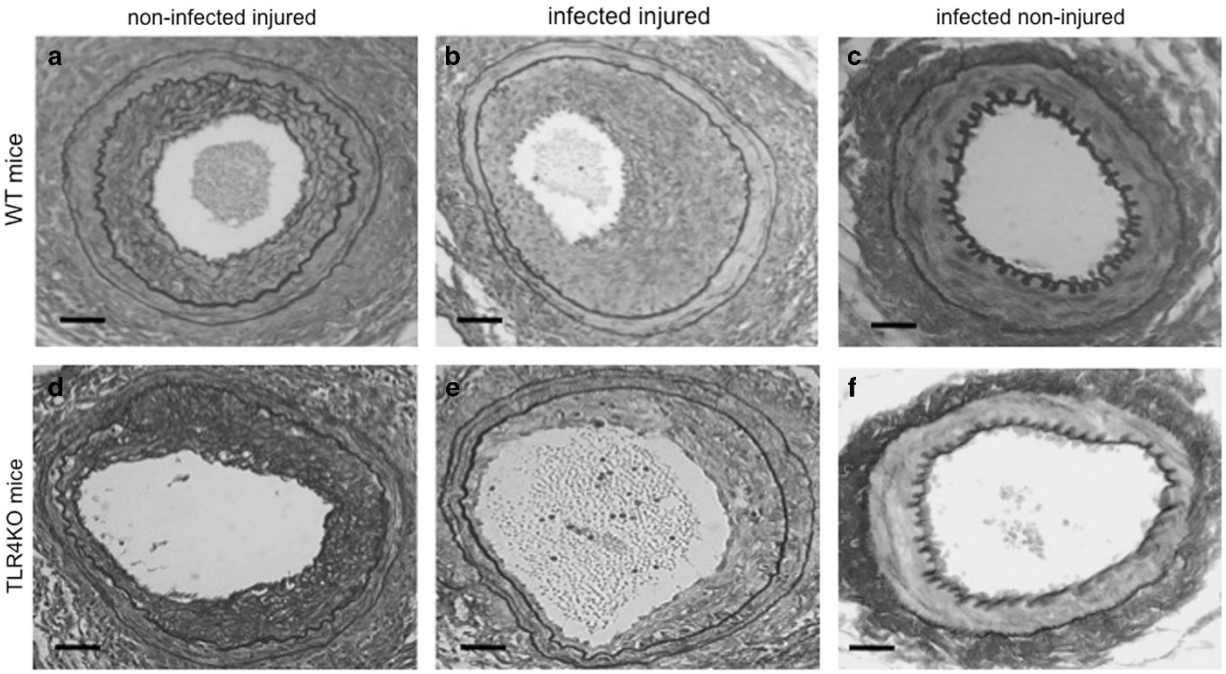


**Figure 3** Pathological findings of injured arteries. Representative EvG-stained arterial sections in WT mice (upper panels) and TLR4KO mice (lower panels) are shown. The left panels show arteries from non-infected, injured mice, middle panels are arteries from *P. gingivalis*-infected and injured mice and the right panels are *P. gingivalis*-infected and non-injured mice. Injured arteries in WT mice infected with *P. gingivalis* (**b**) showed significantly thickened intima, while the degree of thickening was less severe in the injured arteries of TLR4KO mice infected with *P. gingivalis* (**e**). Sham-operated vessels of WT (**c**) and TLR4KO (**f**) mice infected with *P. gingivalis* showed no intimal thickening. Scale bars, 50 μm. A full color version of this figure is available at the *Hypertension Research* journal online.

negated *P. gingivalis*-induced neointimal formation ($n=10$) compared with the WT mice. In TLR4KO mice, there was no significant difference in the intima/media thickness ratio between non-infected mice ($n=6$) and *P. gingivalis*-infected mice ($n=6$). Sham-operated vessels in *P. gingivalis*-infected WT and TLR4KO mice showed no intimal thickening (Figures 3 and 4). The body weight showed no significant difference between the groups (Table 1).

### Immunohistochemistry

In WT mice, the *P. gingivalis*-infected group ($n=5$) showed more MCP-1 positive cells in the neointimal area compared with the non-infected group ($n=5$, $P<0.05$) 14 days after injury. Among the *P. gingivalis*-infected groups, TLR4KO mice significantly suppressed the number of MCP-1 positive cells ($n=5$, $P<0.05$) compared with the *P. gingivalis*-infected WT mice. In TLR4KO mice, the numbers of MCP-1 positive cells were comparable between the non-infected and *P. gingivalis*-infected groups (Figures 5 and 6). To identify reendothelialization after arterial injury, we performed CD31 immunohistochemistry. We showed that all analyzed injured arteries had completely reendothelialized by day 14; there was no significant difference in CD31 staining among the groups (Figure 7).

## DISCUSSION

Periodontitis has been reported to be a significant independent risk factor for peripheral vascular disease.[22] Recently, we reported that *P. gingivalis* infection accelerated neointimal formation after arterial injury.[13] It has also been reported that *Chlamydia pneumonia* infection induced neointimal formation after arterial injury.[23] Inflammation after infection is now considered a factor that worsens cardiovascular disease.

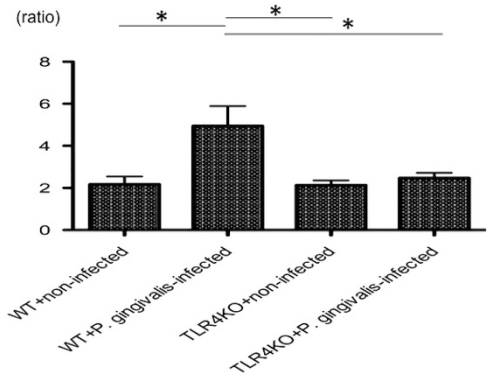


**Figure 4** Quantitative results of neointimal formation. The graph indicates the quantitative intima/media thickness. The amount of thickened intima was comparable between the non-infected WT and non-infected TLR4KO mice. However, the amount of thickened intima in the injured artery from WT mice infected with *P. gingivalis* was significantly higher than that from *P. gingivalis*-infected TLR4KO mice. Results are expressed as the means ± s.e.m. $^*P<0.05$.

This study showed that repeated injection of *P. gingivalis* upregulated the anti-*P. gingivalis* IgG titer in not only WT mice but also in TLR4KO mice, which concurs with the findings of a previous report.[24] This means that *P. gingivalis* was recognized even in TLR4KO mice. Although *P. gingivalis* infection induced neointimal hyperplasia after arterial injury in WT mice, *P. gingivalis*-induced neointimal hyperplasia was suppressed in TLR4KO mice. Pi *et al.* revealed that increased TLR4 and proinflammatory cytokines were observed in wire injury-induced carotid neointima. The TLR4 deficiency protected the

**Table 1 Body weight and vessel numbers**

| | *WT non-infected injured* | *WT infected injured* | *TLR4KO non-infected injured* | *TLR4KO infected injured* |
|---|---|---|---|---|
| Body weight (g) | 26.6±0.1 | 26.3±0.3 | 26.5±0.7 | 25.3±0.8 |
| Vessel numbers | 10 | 10 | 7 | 11 |

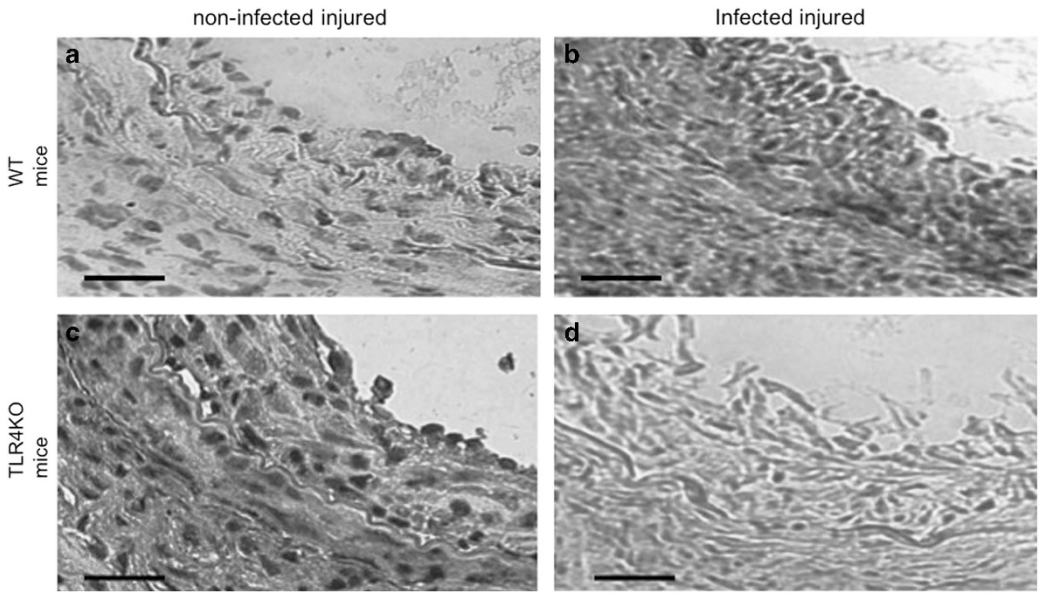


**Figure 5** Immunohistochemical analysis. Representative images of immunohistochemical detection of MCP-1 are shown. (**a**) An artery from a non-infected WT mouse and (**b**) an artery from a *P. gingivalis*-infected WT mouse. (**c**) An artery from a non-infected TLR4KO mouse and (**d**) an artery from a *P. gingivalis*-infected TLR4KO mouse. Scale bars, 10 μm. A full color version of this figure is available at the *Hypertension Research* journal online.

injured carotid artery from neointimal formation via suppression of reactive oxygen species.[25] Zhang *et al.* also showed that increased TLR4 and proinflammatory cytokines were observed in wire injury-induced carotid neointima. They revealed that PPAR-gamma inhibited vascular smooth muscle cell proliferation and migration by suppressing TLR4-mediated inflammation and attenuated intimal hyperplasia after carotid injury.[26] There are many virulence factors of *P. gingivalis*, such as LPS, fimbriae, gingipains, proteases and hemagglutinin.[27–29] Previous studies indicated that *P. gingivalis* LPS triggered inflammatory pathways through the production of chemokines via the TLR4 pathway in human aortic endothelial cells[30] and human gingival fibroblasts.[31]

MCP-1 is a principal factor in initiation and progression of neointimal formation.[32] MCP-1 is well known to have a critical role in the pathogenesis of coronary restenosis after percutaneous coronary intervention (PCI). Oshima *et al.* demonstrated that MCP-1 production at stented coronary arterial sites is associated with an increased risk of restenosis after clinical stent implantation. Therefore, we examined serum MCP-1 levels after arterial injury.[33] MCP-1 is also known to be a critical factor for vascular smooth muscle cell proliferation.[34] *P. gingivalis* LPS induces MCP-1 gene expression in human gingival fibroblasts.[35] MCP-1 production is activated by TLR ligands, such as LPS and bacterial lipoprotein.[36] We have shown that *P. gingivalis* infection increased the number of MCP-1 positive cells in the neointimal area in WT mice.[13] In this study, however, the *P. gingivalis*-infected TLR4KO mice significantly suppressed the number of MCP-1 positive cells in the neointimal area compared with the *P. gingivalis*-infected WT mice. TLR4 ligand stimulation induced MCP-1

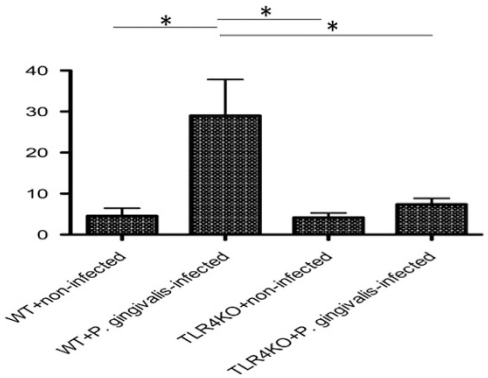


**Figure 6** Quantitative results of MCP-1 positive cell numbers. The bar graph demonstrates the number of MCP-1 positive cells in the neointimal area. Results are expressed as the means±s.e.m. $*P<0.05$.

production in murine macrophage-like cells.[37] These findings suggest that *P. gingivalis* infection induced MCP-1 expression via TLR4 stimulation and promoted neointimal hyperplasia after arterial injury.

Studies have demonstrated that MCP-1 has an important role in the mobilization or recruitment of endothelial progenitor cells, which may have beneficial effects on endothelial repair after injury. Fujiyama *et al.* showed that MCP-1-activated bone marrow-derived CD34-/CD14+ monocyte lineage cells (BM-MLCs) adhered onto injured endothelium, differentiated into endothelial cell-like cells and

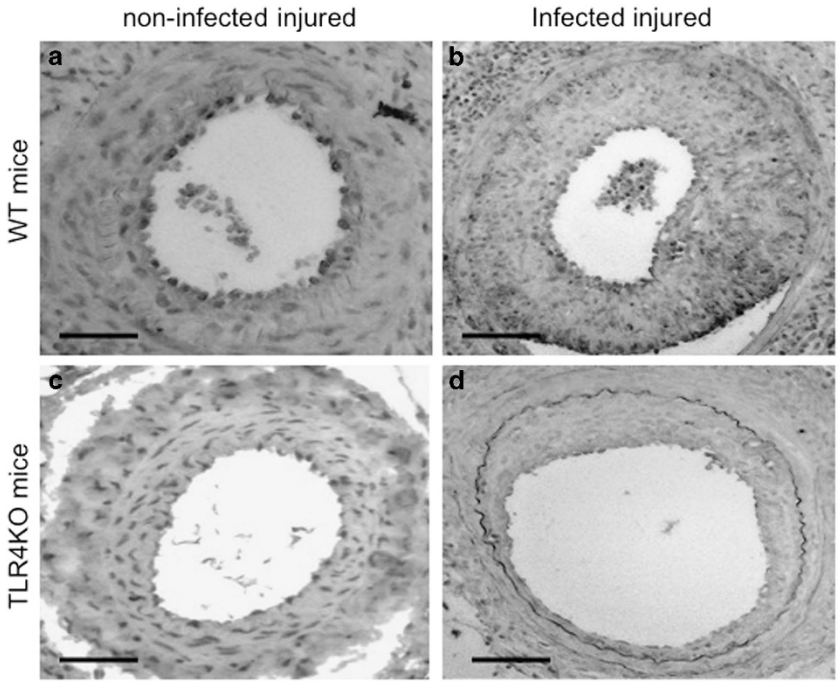


**Figure 7** Representative images of immunohistochemical detection of CD31 are shown. (**a**) An injured artery from a non-infected WT mouse and (**b**) an injured artery from a *P. gingivalis*-infected WT mouse. (**c**) An injured artery from a non-infected TLR4KO mouse and (**d**) an injured artery from a *P. gingivalis*-infected TLR4KO mouse. Scale bars, 10 μm. A full color version of this figure is available at the *Hypertension Research* journal online.

inhibited neointimal hyperplasia. BM-MLCs can function as endothelial cell progenitors, which have the ability to adhere to injured endothelium in an MCP-1-dependent manner, leading to reendothelialization associated with inhibition of intimal hyperplasia.[38]

Lucas *et al.* revealed the effect of chemokine blockade on plaque growth induced by *P. gingivalis* infection after aortic balloon injury in mice. They used a virus-derived anti-inflammatory protein, M-T7, which binds a broad spectrum of C, CC and CXC chemokines. Although *P. gingivalis* infection significantly increased monocyte invasion and arterial plaque growth after balloon injury, M-T7 treatment significantly blocked the pathological changes by modifying expression of TLR4. The results suggest a central role for chemokine-mediated inflammation after arterial injury in *P. gingivalis*-infected mice.[39] Because sham-operated vessels with *P. gingivalis*-infected WT and TLR4KO mice showed no intimal thickening in our study, *P. gingivalis* itself has no effect on positive arterial remodeling.

We previously reported that a study of TLR2KO mice showed similar results to this TLR4KO study.[14] Although we cannot compare these results directly, the reduced rate of intimal thickening seems to be comparable. Because signal cascades from these TLRs and from MCP-1 via NF-κB are related, similarities may exist between studies of different TLRKO mice; however, these may not be the only signaling cascades that impact intimal thickening.

Because reendothelialization after arterial injury is an important pathological phenomenon, we performed CD31 immunohistochemistry in this study.[40] We showed that there was no difference in CD31 staining among the groups on day 14. Thus, examination of vessels at earlier time points may clarify the role of reendothelialization in arterial remodeling in *P. gingivalis*-infected mice with a TLR4 deficiency.

In summary, *P. gingivalis* induced neointimal hyperplasia after arterial injury in WT mice; however, TLR4KO mice suppressed neointimal hyperplasia induced by *P. gingivalis* after arterial injury. This suggests that *P. gingivalis*-induced neointimal hyperplasia is mediated by TLR4 signaling.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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