Cyclooxygenase-2 inhibition increases lipopolysaccharide-induced atherosclerosis in mice

Jonathan M. Gitlin and Charles D. Loftin*

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 725 Rose Street, Room 414, Lexington, KY 40536-0082, USA

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**Aims** The risk of adverse cardiovascular events in humans is increased with chronic use of cyclooxygenase-2 (COX-2) inhibitors. However, the role of COX-2 in animal models of cardiovascular disease has been controversial. In humans and animal models, cardiovascular disease is increased by bacterial infection of the supporting tissue of the teeth, a condition known as periodontal disease. Periodontal disease may result in chronic exposure to pro-inflammatory mediators, such as bacterial lipopolysaccharide (LPS), thereby producing a systemic inflammatory response. The current study examined the role of COX-2 in atherosclerosis induced by LPS derived from the periodontal disease pathogen *Porphyromonas gingivalis*.

**Methods and results** *Porphyromonas gingivalis* LPS was administered by chronic infusion for 28 days and atherosclerosis development was examined in the aortic root of ApoE (apolipoprotein E)-deficient mice. The extent of atherosclerosis was compared between mice receiving control diet or diet containing the COX-2 inhibitor celecoxib. The role of COX-2 in *P. gingivalis* LPS-induced inflammatory cell activation was examined in peritoneal macrophages. *Porphyromonas gingivalis* LPS infusion significantly increased atherosclerosis development. In mice infused with *P. gingivalis* LPS, administration of the COX-2 inhibitor celecoxib further increased the extent of atherosclerotic lesion area. In peritoneal macrophages, *P. gingivalis* LPS increased the expression of COX-2 mRNA (messenger ribonucleic acid) and the production of prostaglandin (PG) E₂ (PGE₂), the latter of which was inhibited by celecoxib. *Porphyromonas gingivalis* LPS-induced expression of tumour necrosis factor alpha (TNFα) was enhanced by inactivation of COX-2 and was attenuated by treatment with PGE₂.

**Conclusion** The inhibition of COX-2-derived PGE₂ may enhance *P. gingivalis* LPS-induced atherosclerosis by increasing macrophage production of TNFα.

1. **Introduction**

   Periodontal disease is a chronic inflammatory condition that is associated with an increased incidence of atherosclerosis in humans.1,2 Periodontal disease results from chronic bacterial infection of the tissue surrounding and supporting the teeth. *Porphyromonas gingivalis* (*P. gingivalis*) is a bacterial pathogen often identified in adult periodontal disease and systemic exposure to pro-inflammatory factors from these bacteria, including lipopolysaccharide (LPS), may contribute to atherosclerosis development. LPS is an essential constituent of the cell wall of gram-negative bacteria and its recognition by the host’s innate immunity may account for a majority of the inflammatory response following bacterial infection.3,4 The detection of elevated serum levels of LPS or antibodies to *P. gingivalis* is associated with a greater risk of atherosclerosis development in humans.5 Therefore, although multiple factors contribute to atherosclerosis development, inflammation resulting from LPS exposure may exacerbate the disease.

   Animal models of atherosclerosis have shown that a chronic oral or systemic infection with *P. gingivalis* accelerates the disease in mice that are heterozygous- or homozygous-deficient in apolipoprotein E (ApoE).6,7 The mechanism for the enhanced atherosclerosis following *P. gingivalis* infection involves an increase in systemic pro-inflammatory factors, rather than alterations in plasma cholesterol levels. Furthermore, the atherosclerotic lesions in *P. gingivalis*-infected mice show evidence of enhanced vascular inflammation with increased macrophage infiltration.6,7 The release of LPS from the bacteria into the peripheral circulation may contribute to the increase in systemic markers of inflammation.6 In support of a systemic exposure mechanism, chronic administration of purified LPS has been shown to reproduce the proatherogenic and pro-inflammatory effects of chronic infection.8,9 Therefore, the increased...
atherosclerosis observed following oral infection with *P. gingivalis* may involve the release of LPS into the systemic circulation thereby enhancing the vascular inflammation contributing to the disease. 

LPS is an inducer of inflammation because of its potency for activating cells contributing to the inflammatory response. Macrophages are a primary cell type which respond to LPS detection by activation of intracellular pathways leading to production of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNFα). TNFα contributes to atherosclerosis by stimulating leucocyte adhesion and inflammatory cell recruitment. Therefore, macrophages are a significant source of pro-inflammatory mediators, such as TNFα, which may contribute to LPS-induced vascular inflammation during atherosclerosis development.

The increased synthesis of prostaglandins (PG) is an important regulatory mechanism for limiting the potentially destructive effects of excessive TNFα production. PGs, particularly PGE2 (prostaglandin E2) attenuate TNFα gene expression and this anti-inflammatory effect of PGE2 occurs in macrophages without similar effects in endothelial or smooth muscle cells. The formation of PGE2 is dependent on activity of the PG G/H synthases, also known as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). The COX isoforms differ significantly in their expression characteristics with COX-1 being constitutively expressed in most tissues and COX-2 being induced by inflammatory stimuli. Following LPS stimulation, TNFα production is significantly increased by non-selective COX isoform inhibitors and by inhibitors selective for either COX-1 or COX-2. Therefore, either COX isoform may contribute to the suppression of TNFα expression following LPS stimulation of macrophages.

Although numerous studies in humans and animal models have shown the beneficial effects of COX-1 inhibition in reducing atherosclerosis development, the role of COX-2 in this disease remains controversial. Recently, questions have been raised about the safety of COX-2 inhibitor use in humans because of an increase in adverse cardiovascular events. Despite these findings of a potential antiatherogenic role of COX-2 in humans, there are a limited number of reports utilizing an animal model to show increased atherosclerosis following COX-2 inhibition. One report has shown that treatment of ApoE-deficient mice with the COX-2 inhibitor MF-tricyclic significantly increases early atherosclerosis development. Other reports utilizing hyperlipidaemic mouse models have shown either no effect or a reduction in atherosclerosis following COX-2 inhibition. Therefore, additional models where COX-2 inhibition increases atherosclerosis development are required to better characterize the adverse cardiovascular effects of COX-2 inhibitors. The findings in our current report show that COX-2 inhibition exacerbates atherosclerosis development following treatment of hyperlipidaemic mice with LPS derived from a periodontal disease pathogen.

2. Methods

2.1 Animals

Male ApoE-deficient mice (C57BL/6 background, Jackson Laboratory, Bar Harbor, ME, USA) were allowed to age up to 16 weeks before commencing the study. COX-2-deficient mice and their matching wild-type littermate controls, were a gift from R. Langenbach. Mice were housed under barrier conditions with food and water *ad libitum*. All animal studies were conducted under the approval of the University of Kentucky Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2 Lipopolysaccharide infusion

Mice were anesthetized using isoflurane, and *P. gingivalis* LPS (25 μg; InvivoGen, San Diego, CA, USA) was administered by Alzet osmotic mini-pumps (model 2004; Durect, Cupertino, CA, USA) implanted subcutaneously. There was no increase in mortality associated with the LPS administration. The mice were fed a standard mouse chow pelleted diet or the pelleted diet (Research Diets, Inc.) containing 1000 ppm celecoxib (LKT Laboratories Inc.) We have previously reported that 1000 ppm of celecoxib in the diet results in a plasma concentration of ~1.6 μg/mL, which approximates the reported therapeutic plasma concentration of celecoxib in humans. In the case of the mice treated with celecoxib, drug administration was started 7 days prior to pump implantation.

2.3 Tissue collection

At the study endpoint (28 days) mice were euthanized and perfused with PBS followed by formal-sucrose (4% paraformaldehyde/5% sucrose in PBS, pH 7.4). The hearts were removed and the organ immersed in formal-sucrose overnight before being stored at −80°C until sectioning.

2.4 Histological analysis of atherosclerosis, COX-2 and macrophage marker Mac-3

Atherosclerotic progression was assessed by quantitating lesions in the aortic root. Serial cryosections (10 μm) of the aortic root were prepared and every fourth section, for a total of four sections, were stained with oil red O (Sigma), counterstained with haematoxylin (Sigma), and digitally imaged to quantitate total lesion area. Immunohistochemical analysis of COX-2 (Cayman Chemical, Ann Arbor, MI, USA) and Mac-3 (BD Pharmingen, San Diego, CA, USA) was performed with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA).

2.5 Macrophage culture

For collection of peritoneal macrophages, male COX-2 (+/+) and COX-2 (−/−) mice were first anesthetized with a lethal dose of ketamine/xylazine and then euthanized by cervical dislocation. Peritoneal cavities were washed with 8 mL cold sterile saline and the collected macrophages were placed on ice. Individual samples were pooled from multiple mice, then centrifuged at 1500 rpm for 10 min to pellet the cells. Cells were resuspended in 1 mL RPMI (supplemented with 10% foetal bovine serum; Invitrogen, Carlsbad, CA, USA) and seeded at a density of 5 × 10⁵ cells per well. Macrophages were allowed to attach overnight, followed by addition of fresh media. Macrophage experiments utilized the following treatments: 10 ng/mL *P. gingivalis* LPS (InvivoGen), 1 μM celecoxib (LKT Laboratories), PGE2 (1 μM, Cayman Chemical), and/or antibodies generated against TLR2, TLR4, or control IgG (immunglobulin G) (eBioscience, Inc.) all at a concentration of 50 μg/mL. At the experimental endpoint, cell monolayers were lysed and total RNA was extracted using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA, USA).

2.6 Real-time polymerase chain reaction

Quantitative gene expression was performed by two-step RT–PCR (reverse transcription polymerase chain reaction) using Superscript II
2.7 Prostaglandin E₂ and TNFα enzyme-linked immunosorbent assay

For evaluating the effect of celecoxib on PGE₂ production, peritoneal macrophages were incubated with the COX-2 inhibitor (1 μM) in 10% serum media for 1 h prior to P. gingivalis LPS stimulation. PGE₂ levels were determined from cell extracts of peritoneal macrophages following 24 h of treatment (Prostaglandin E₂ Direct Biotrak Assay, Amersham, Piscataway, NJ, USA). TNFα levels in cell culture supernatants were determined after 24 h of treatment by ELISA (enzyme-linked immunosorbent assay) (R&D Systems, Minneapolis, MN, USA).

2.8 Statistical analyses

The mean and SEM were calculated for the total atherosclerotic lesion area with the sections from the aortic root of a single mouse considered as an n of 1. Significant differences among groups was determined using unpaired Student t-test, with differences being considered statistically different at P < 0.05. Each experiment was repeated a minimum of two times with similar results.

3. Results

3.1 Chronic infusion of P. gingivalis lipopolysaccharide increases atherosclerosis formation in apolipoprotein E-deficient mice

It has previously been reported that an oral infection with P. gingivalis increases early atherosclerosis development in ApoE-deficient mice. The current study utilized P. gingivalis LPS to determine whether this purified bacterial component also increases atherosclerosis development. To allow for chronic systemic exposure to P. gingivalis LPS, mice were administered LPS at a continual dose for 28 days using subcutaneous osmotic mini-pump infusion. As compared with saline-infused mice, P. gingivalis LPS infusion significantly increased the extent of atherosclerotic lesions in the aortic root (Figure 1). Therefore, similar to previous reports using live bacteria, LPS purified from P. gingivalis significantly increased atherosclerotic lesion development in ApoE-deficient mice.

3.2 Celecoxib administration enhances atherosclerosis formation following P. gingivalis lipopolysaccharide infusion

The role of COX-2 in contributing to atherosclerosis induced by P. gingivalis LPS was determined by examining the effect of COX-2-selective inhibition. The level of atherosclerosis was compared between mice receiving control diet without celecoxib (Figure 2A), and mice administered celecoxib in the diet at 1000 ppm (Figure 2B) beginning 1 week prior to initiation of the LPS infusion. The mice treated with celecoxib showed a significantly greater level of atherosclerosis development in the aortic root (Figure 2C). We determined the effectiveness of the celecoxib treatment for inhibiting PGE₂ production following LPS stimulation of whole blood. The level of PGE₂ was significantly lower in plasma from mice treated with celecoxib, as compared with plasma from mice on the control diet (Figure 2D). These findings suggest that PGs derived from the activity of COX-2 protect against P. gingivalis LPS-induced atherosclerosis development.

3.3 COX-2 expression in macrophage containing atherosclerotic lesions of P. gingivalis lipopolysaccharide-infused mice

To determine the localization of COX-2, we examined expression by immunohistochemistry in the aortic root of mice following P. gingivalis LPS infusion. A concentration of staining positive for COX-2 (Figure 3B) was detected in the region of the aortic root with lesion development as determined by oil red O staining (Figure 3A). The region staining positive for oil red O and COX-2 also showed evidence of macrophage infiltration, as determined by detection of the macrophage marker Mac-3 (Figure 3C).

3.4 P. gingivalis lipopolysaccharide induces COX-2 mRNA expression and prostaglandin E₂ formation in peritoneal macrophages

With macrophages being a primary cell type contributing to development of the atherosclerotic lesion, we examined the effect of P. gingivalis LPS on COX-2 mRNA expression in primary cultures of peritoneal macrophages. Macrophages were treated with a concentration of 10 ng/mL of P. gingivalis LPS to approximate the dose administered to the mice by subcutaneous osmotic mini-pump infusion. Six hours following treatment, cell lysates were collected for analysis of mRNA expression. Treatment of macrophages with the physiologically relevant concentration of 10 ng/mL P. gingivalis LPS significantly increased COX-2 mRNA expression (Figure 4A).

We examined the effect of COX-2 inhibition on PGE₂ synthesis following stimulation with P. gingivalis LPS for 24 h. As compared with vehicle-treated controls, P. gingivalis LPS treatment significantly increased the level of PGE₂ detected in cell extracts (Figure 4B). The addition of the COX-2 inhibitor celecoxib (1 μM) in combination with P. gingivalis LPS significantly reduced the level of PGE₂ produced by the macrophages (Figure 4B). These findings

Figure 1 Chronic infusion of Porphyromonas gingivalis (P. gingivalis) lipopolysaccharide (LPS) increases atherosclerosis in ApoE (apolipoprotein E)-deficient mice. Quantification of aortic root lesion area stained positive for oil red O in control saline-infused mice (clear bar) and mice infused with P. gingivalis LPS. The mean ± SEM is shown for each group. n = 10–12 mice/group. ***P < 0.0001, unpaired Student t-test.
suggest that in peritoneal macrophages, \textit{P. gingivalis} LPS induces the expression of COX-2 resulting in the increased synthesis of PGE\(_2\).

### 3.5 COX-2 regulates \textit{P. gingivalis} lipopolysaccharide-induced TNF\(\alpha\) mRNA expression

TNF\(\alpha\) produced by macrophages contributes to the development of atherosclerosis, and PGE\(_2\) has been shown to regulate TNF\(\alpha\) production in different cell types. We examined the role of COX-2 and PGE\(_2\) on TNF\(\alpha\) mRNA expression in macrophages following stimulation by \textit{P. gingivalis} LPS. As compared with vehicle-treated controls, treatment with the physiologically relevant concentration of \textit{P. gingivalis} LPS of 10 ng/mL for 6 h significantly increased expression of TNF\(\alpha\) mRNA (Figure 5A). \textit{Porphyromonas gingivalis} LPS induction of TNF\(\alpha\) mRNA expression was significantly attenuated by the addition of PGE\(_2\) (1 \(\mu\)M) (Figure 5A).

The effect of endogenous PG synthesis on TNF\(\alpha\) mRNA expression was determined using macrophages from mice deficient in COX-2. As compared with wild-type macrophages, \textit{P. gingivalis} LPS induction of TNF\(\alpha\) mRNA was significantly greater in COX-2-deficient macrophages (Figure 5A). Similar to the COX-2 expressing wild-type macrophages, the addition of PGE\(_2\) to the COX-2-deficient macrophage cultures significantly attenuated \textit{P. gingivalis} LPS induction of TNF\(\alpha\) mRNA (Figure 5A). Therefore, COX-2-derived PGE\(_2\) may contribute to the attenuation of TNF\(\alpha\) mRNA expression following stimulation of macrophages by \textit{P. gingivalis} LPS.

### 3.6 COX-2 regulates \textit{P. gingivalis} lipopolysaccharide-induced TNF\(\alpha\) protein expression

With our findings of COX-2 and PGE\(_2\)-dependent regulation of TNF\(\alpha\) mRNA expression, we examined their role in regulating TNF\(\alpha\) protein production. Treatment with 10 ng/mL of \textit{P. gingivalis} LPS for 24 h significantly increased the level of TNF\(\alpha\) protein produced by COX-2 expressing macrophages (Figure 5B). The addition of PGE\(_2\) to the macrophage cultures eliminated the response to \textit{P. gingivalis} LPS (Figure 5B). In macrophages deficient in COX-2, the level of TNF\(\alpha\) protein induction in response to 10 ng/mL \textit{P. gingivalis} LPS was significantly greater than that observed in wild-type macrophages (Figure 5B). Similar to our findings with wild-type macrophages, PGE\(_2\) maintained its effectiveness in attenuating the induction of TNF\(\alpha\) protein expression in macrophages deficient in COX-2 (Figure 5B).
Therefore, COX-2-dependent production of PGE₂ may attenuate the increased TNF-α protein production that occurs following P. gingivalis LPS stimulation of macrophages.

We also utilized neutralizing antibodies against TLR2 and TLR4 to determine their roles as receptors for P. gingivalis LPS. The addition of the TLR2 antibody eliminated P. gingivalis LPS induction of TNFα protein expression (Figure 5C). In contrast, there was no significant reduction of TNFα production following treatment with the neutralizing antibody against TLR4 or a control antibody against IgG (Figure 5D). Therefore, the induction of TNFα production by P. gingivalis LPS acts through a TLR2, rather than a TLR4-dependent pathway.

4. Discussion

Previous studies have used a variety of gene-targeted mouse models to examine the role of COX-2 in the development of atherosclerosis. In ApoE-deficient mice, the administration of the COX-2 inhibitor MF-tricyclic increases early atherosclerosis lesion area.17 Whereas, in female ApoE-deficient mice or male LDLR-deficient mice fed a Western diet, treatment with the COX-2 inhibitor rofecoxib reduces early atherosclerosis development.20,24 Additional reports utilizing other COX-2 inhibitors, including SC-236 or celecoxib, have shown no significant effect of these agents on atherosclerosis development at various stages of the disease.18,19 These previous reports showing either a decrease or no effect of COX-2 inhibition on atherosclerosis utilized mouse models where the primary induction of the disease resulted from hyperlipidaemia. In contrast, our current studies have utilized the pro-inflammatory mediator LPS for increasing atherosclerosis that results from hyperlipidaemia. Our findings show that in this model of the disease, COX-2 inhibition enhances early atherosclerosis development.

Periodontal disease is a common condition that has been associated with an increased risk of developing cardiovascular disease.1,2 Periodontal infection of gingival tissue with common pathogens such as P. gingivalis may lead to systemic exposure of LPS from the bacteria. In humans, detection of increased serum levels of LPS or antibodies specific for P. gingivalis, increases the risk of development of cardiovascular disease.5 Similarly, in mouse models of the disease, infection with P. gingivalis accelerates the level of atherosclerosis development.6,7 LPS is a major contributor to the inflammation that results from bacterial infection and the administration of purified LPS has been shown to produce an inflammatory response similar to infection with whole bacteria.25 Unlike LPS derived from other gram-negative bacteria such as Escherichia coli (E. coli), P. gingivalis LPS produces its inflammatory response in part through activation of TLR2. TLR2 has recently been shown to contribute to atherosclerosis development in hyperlipidaemic mice.5 In addition, deficiency of TLR2 only in bone marrow-derived cells significantly reduces atherosclerosis induced by a TLR2 agonist, suggesting the importance of TLR2 expression by macrophages.26 Our findings of the importance of COX-2 in attenuating atherosclerosis induced by P. gingivalis LPS, suggest a role for COX-2-derived PGs in attenuating TLR2-dependent atherosclerosis.
The infiltration of macrophages into the vessel wall is a key to the inflammatory response contributing to the initiation of atherosclerosis. During the development of atherosclerosis, macrophages are a significant source of COX-2-derived PGs. We examined the expression of COX-2 in atherosclerotic lesions of the aortic root following P. gingivalis LPS infusion. We observed a significant level of COX-2 expression by immunohistochemistry within the atherosclerotic lesions which co-localized with expression of the macrophage marker Mac-3 (Figure 3). Therefore, macrophage expression of COX-2 may function to limit vascular inflammation induced by P. gingivalis LPS.

Previous studies have shown that concentrations of LPS that are higher than physiologically relevant concentrations (>100 ng/mL) act through mechanisms independent of the LPS receptor. At supra-physiological concentrations (20 μg/mL), P. gingivalis LPS significantly increases COX-2 expression and resultant PGE2 production. Our current work examined the effect of a physiologically relevant concentration of P. gingivalis LPS of 10 ng/mL on the induction of COX-2 in macrophages. Significant induction of COX-2 mRNA expression was observed following 6 h of treatment with this relatively low P. gingivalis LPS concentration (Figure 4A). Therefore, at a concentration expected to produce LPS receptor-dependent activation, P. gingivalis LPS significantly increased macrophage expression of COX-2 mRNA.

PGE2 is a prominent PG produced by macrophages following E. coli LPS stimulation. PGE2 attenuates the pro-inflammatory effects of LPS stimulation in macrophages but does not produce this anti-inflammatory effect in LPS-stimulated endothelial or smooth muscle cells. The increased production of PGE2 by macrophages in response to E. coli LPS has been shown to result from increased activity of either COX-1 or COX-2. Our current studies show that P. gingivalis LPS significantly increased the level of PGE2 detected in cell extracts 24 h following treatment of the macrophages (Figure 4B). Furthermore, the induction of PGE2 production was eliminated by treatment with celecoxib. Therefore, the increased production of PGE2 in response to P. gingivalis LPS stimulation was dependent on the activity of COX-2.

TNFα is thought to contribute to the inflammatory response that occurs during atherosclerosis development in humans and mouse models of the disease. The increased synthesis of PGs is an important mechanism for limiting TNFα production. Although different PGs have been shown to attenuate TNFα production in response to a variety of stimuliants, the inhibitory effect of PGE2 is well characterized. Our results show that addition of PGE2 significantly attenuated the TNFα mRNA induction that occurred following 6 h of stimulation by P. gingivalis LPS (Figure 5A). Treatment with PGE2 also eliminated P. gingivalis LPS induction of TNFα protein following 24 h of stimulation (Figure 5B). Therefore, PGE2 may limit the pro-inflammatory effects that result from TNFα following P. gingivalis LPS-dependent macrophage activation.

Although the inhibitory effect of exogenous PGE2 on LPS-induced TNFα expression is well described in peritoneal macrophages, there have been conflicting reports describing the effect of PGs produced from endogenous sources. Our current study utilized macrophages from mice deficient in COX-2 for determining the effect of PGs produced endogenously by COX-2 on P. gingivalis LPS-induced TNFα production. Following stimulation by P. gingivalis LPS, TNFα mRNA and protein levels were significantly greater in COX-2-deficient macrophages, as compared with COX-2 expressing macrophages from wild-type mice (Figures 5A and B). Therefore, in peritoneal macrophages, COX-2 is responsible for the production of PG(s) which function to attenuate P. gingivalis LPS-induced TNFα expression.

Our findings of increased TNFα production in macrophages deficient in COX-2 suggest the importance of COX-2 for the synthesis of PG(s) which attenuate TNFα production. However, it has previously been reported that the genetic inactivation of COX-2 down-regulates PGE2 receptor expression, suggesting that the increased TNFα production that we observed could potentially result from reduced PGE2 receptor expression rather than the elimination of COX-2-derived PG(s). Therefore, to confirm that the expression of PGE2 receptors was maintained following COX-2 deficiency,
we examined the effect of exogenous addition of PGE2 on *P. gingivalis* LPS-induced TNFα expression in COX-2-deficient macrophages. The addition of exogenous PGE2 attenuated the increased levels of TNFα mRNA and protein that we observed in the COX-2-deficient macrophages (Figures 5A and B). Thus, the effectiveness of PGE2 for down-regulation of TNFα expression is maintained in macrophages deficient in COX-2.

Exposure of murine macrophages to *P. gingivalis* LPS produces a response distinct from that elicited by LPS derived from *E. coli*.

Differences in the signalling mechanisms between *E. coli* LPS and *P. gingivalis* LPS have been reported to result from the utilization of different TLRs. Although *E. coli* LPS activation of a variety of cell types requires TLR4-dependent signalling, *P. gingivalis* LPS activation has been shown to primarily utilize TLR2. However, LPS preparations containing varying lipid A constituents from *P. gingivalis* have been shown to activate human and mouse TLR2, as well as TLR4. Therefore, we examined the TLR binding characteristics of the preparation of *P. gingivalis* LPS used in the current studies. Our findings show that disruption of TLR2 signalling by addition of a neutralizing antibody effectively eliminated the stimulation of TNFα production by *P. gingivalis* LPS (Figure 5C). In contrast, there was no significant effect of a neutralizing antibody against TLR4 on *P. gingivalis* LPS induction of TNFα (Figure 5D). These findings indicate that the *P. gingivalis* LPS stimulation of macrophage TNFα production that we observed utilized a TLR2 pathway, rather than a TLR4 pathway.

In addition to contributing to the inflammation associated with atherosclerosis, increased TNFα production is also important for stimulating defence mechanisms which limit the severity of an infection. The antibacterial function of TNFα could potentially reduce *P. gingivalis* infections by limiting growth of the bacteria. However, our current model using *P. gingivalis* LPS infusion for increasing atherosclerosis would not be influenced by potential antibacterial effects of TNFα.

In summary, the current report is the first to show that chronic infusion of *P. gingivalis* LPS increases the extent of atherosclerosis in mice, and in this model, COX-2 inhibition increases severity of the disease. Furthermore, in macrophages, COX-2-derived PG(s) function to attenuate the induction of TNFα production following TLR2-dependent activation by *P. gingivalis* LPS. These findings suggest that COX-2 inhibition may exacerbate the increased risk of atherosclerosis development that is associated with periodontal disease.

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