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Protective Role for TLR4 Signaling in Atherosclerosis Progression as Revealed by Infection with a Common Oral Pathogen

Chie Hayashi,*1 George Papadopoulos,*1 Cynthia V. Gudino,* Ellen O. Weinberg,* Kenneth R. Barth,* Andrés G. Madrigal,* Yang Chen,† Hua Ning,† Michael LaValley,‡ Frank C. Gibson, III,* James A. Hamilton,† and Caroline A. Genco*1,8

Clinical and epidemiological studies have implicated chronic infections in the development of atherosclerosis. It has been proposed that common mechanisms of signaling via TLRs link stimulation by multiple pathogens to atherosclerosis. However, how pathway-specific stimulation of TLR4 contributes to atherosclerosis progression remains poorly understood. In this study, atherosclerosis-prone apolipoprotein-E null (ApoE−/−) and TLR4-deficient (ApoE−/−TLR4−/−) mice were orally infected with the periodontal pathogen Porphyromonas gingivalis. ApoE−/−TLR4−/− mice were markedly more susceptible to atherosclerosis after oral infection with P. gingivalis. Using live animal imaging, we demonstrate that enhanced lesion progression occurs progressively and was increasingly evident with advancing age. Immunohistochemical analysis of lesions from ApoE−/−TLR4−/− mice revealed an increased inflammatory cell infiltrate composed primarily of macrophages and IL-17 effector T cells (Th17), a subset linked with chronic inflammation. Furthermore, enhanced atherosclerosis in TLR4-deficient mice was associated with impaired development of Th1 immunity and regulatory T cell infiltration. In vitro studies suggest that the mechanism of TLR4-mediated protective immunity may be orchestrated by dendritic cell IL-12 and IL-10, which are prototypic Th1 and regulatory T cell polarizing cytokines. We demonstrate an atheroprotective role for TLR4 in response to infection with the oral pathogen P. gingivalis. Our results point to a role for pathway-specific TLR signaling in chronic inflammation and atherosclerosis. The Journal of Immunology, 2012, 189: 000–000.

The identification of atherosclerosis as a chronic inflammatory disease has emphasized the fundamental role of the immune system in disease pathogenesis (1). Detection of endogenous and microbial ligands by immune competent cells occurs via germline encoded pattern recognition receptors including the innate immune TLRs (2). Engagement via TLRs initiates acute inflammatory responses that are critical in host defense (3). Resident cells in human atherosclerotic plaque express TLRs (4). Animal studies using hyperlipidemic mice have shown that TLR2, TLR4, and the downstream signaling molecule MyD88 play an important role in diet-induced atherosclerosis (5–9). Reduced atherosclerotic development has been observed in TLR4-deficient ApoE−/− mice on high-fat Western diet (5, 6, 8). We have previously shown that TLR2 also plays an important role in athero-

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Abbreviations used in this article: DC, dendritic cell; MRA, magnetic resonance angiography; Treg, regulatory T cell.
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ferential recognition by the TLR4 complex (29, 30). In the current study, we demonstrate the unique ability of *P. gingivalis* to evade TLR4 signaling while inducing TLR2-dependent proinflammatory responses reveals a protective role for TLR4 in chronic inflammatory atherosclerosis.

**Materials and Methods**

**Mice**

Male ApoE<sup>−/−</sup> and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR4<sup>−/−</sup> mice on C57BL/6 background were provided by S. Akira (Osaka University). ApoE<sup>−/−</sup>/TLR4<sup>−/−</sup> mice were generated in our laboratory. Mouse genotypes were confirmed by PCR, and experimental mice were age-matched. Mice were maintained under specific pathogen-free conditions and cared for in accordance with the Boston University Institutional Animal Care and Use Committee.

**Bacteria**

*P. gingivalis* strain 381 was grown anaerobically on blood agar plates (Becton Dickinson) and used to seed-inoculate brain heart infusion broth (Becton Dickinson), hemin (10 μg/mL; Sigma), and menadione (1 μg/mL). CFUs were standardized at an OD at 660 nm of 1 (equivalent to 1 × 10⁶ CFU/mL) by spectrometry (ThermoSpectronic Genesyss20). LPS from *P. gingivalis* 381 was isolated using a modified Tri-Reagent protocol (29).

**Oral infection**

Three independent experiments were performed with ApoE<sup>−/−</sup> (total n = 40) and ApoE<sup>−/−</sup>/TLR4<sup>−/−</sup> (total n = 30) mice, and data were pooled. Mice were fed a normal chow diet (Global 2018; Harlan Teklad, Madison, WI). Six-week-old male mice were given antibiotics (Sulfatrim; Hi-Tech Pharmacal ad libitum in the drinking water for 10 d, followed by a 2-d antibiotic-free period. One hundred microliters of *P. gingivalis* 381 (1 × 10⁶ CFU/mL) suspended in vehicle (2% bovine serum albumin in PBS) was topically applied to the buccal surface of the maxillary gingiva five times a week for 3 wk (34). Control mice received 100 μL of vehicle. Topical application of *P. gingivalis* to the buccal surface of the maxillary gingiva five times a week for 3 wk induces alveolar bone loss in ApoE<sup>−/−</sup> mice (28). Mice were euthanized 13 wk after the final oral challenge (24 wk of age). This time point is consistent with the time frame used in our prior studies (7, 28, 35).

**Magnetic resonance angiography**

Magnetic resonance angiography (MRA) of the infrarenal artery was performed with a vertical-bore Bruker 11.7 T Avance spectrometer (Bruker, Billerica, MA) as described (35). Mice were anesthetized with 0.5–2% isoflurane, placed into a 30-mm vertical probe (Micro 2.5) main-

**Flow cytometry**

Anti-mouse Abs included CD3 (no. 553062; BD Biosciences), CD4 (no. 553049; BD Biosciences), CD8 (no. 553034; BD Biosciences), Ly6G, CD45 (no. 550994; BD Biosciences) and isotype controls (BD Pharmingen) and IFN-γ (no. 48-7311; eBioscience), IL-17A (no. 51-7177; eBioscience), F4/80 (no. 12-4801; eBioscience), and TLR2 (no. 12-9022; eBioscience). Intracellular cytokine staining was performed using a mouse kit (no. 559311; BD Pharmingen). Samples were acquired on a BD LSR II flow cytometer (Becton Dickinson), and data were analyzed using FlowJo software (Tree Star).

**Cell culture**

For isolation of bone marrow-derived dendritic cells (DCs), bone marrow cells were cultured in RPMI 1640 containing 10% FBS, 1× nonessential amino acids (MP Biomedicals), 50 μM 2-mercaptoethanol (Life Technologies), 100 μg/ml streptomycin/100 IU penicillin (Cellgro), and 20 μg/ml recombinant mouse GM-CSF (Peprotech) for 11 d. DCs were greater than 95% positive for CD11c. Nonadherent DCs were collected and replated in 24-well dishes at 2 × 10⁵ cells/well in complete media without antibiotics before addition of *P. gingivalis* at multiplicity of infection of 25 and 50. After 24 h, culture supernatants were collected, and samples were clarified by centrifugation and stored at −80°C for ELISA.

**ELISA**

Concentrations of IL-6, IL-12p70, and IL-10 in cell culture supernatants were determined by ELISA (BD OptEIA). Plasma was collected from a subset of experimental mice 16 wks postinfection and assayed by ELISA for *P. gingivalis*-specific Ab isotypes IgG1, IgG2b, IgG2c, and IgG3 as described (36) as follows. Bacteria were washed three times in PBS and fixed overnight at 4°C in 4% paraformaldehyde. Fixed bacteria were washed five times in PBS and protein concentration estimated by bicine-

**Splenocyte restimulation assay**

Splenocytes (2 × 10⁶ per milliliter) were collected and stimulated with *P. gingivalis* soluble Ags (10 μg/mL) in the presence of 1 μg/mL anti-mouse CD28 (eBioscience) for 4 h and 10 μg/mL brefeldin A (eBioscience). Cells were harvested and stained with anti-mouse Abs (BD Pharmingen); CD3, CD4, and CD8. Intracellular cytokine staining for IL-17A (eBioscience) and IFN-γ (eBioscience) was performed using BD Cytofix/Cytoperm kit.

**Statistics**

Normality of data was determined by visually inspecting for bell-shaped probability density curves. A Mann–Whitney test was performed to compare two inde-

**Results**

TLR4 deficiency confers enhanced susceptibility to chronic and progressive atherosclerosis after infection with *P. gingivalis*

ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/TLR4<sup>−/−</sup> mice were orally infected with 10<sup>10</sup> CFU *P. gingivalis* strain 381. The predominant lipid A species...
expressed by \emph{P. gingivalis} 381 grown under standard laboratory conditions in the presence of excess heme was tetra-acylated nonphosphorylated (\textit{mlz} 1380), which is TLR4 inert and immunologically silent (data not shown) (32). The minor lipid A species produced by strain 381 was penta-acylated monophosphorylated (\textit{mlz} 1690) and has been demonstrated to act as both a weak TLR4 agonist and antagonist (29).

Progression of atherosclerosis in the innominate artery of individual mice was examined in vivo by MRA. The innominate artery exhibits a high degree of lesion progression and expresses features of human disease including vessel narrowing, perivascular inflammation, and plaque disruption (35). The luminal area of the innominate artery of \emph{P. gingivalis}-infected ApoE\textsuperscript{−/−} and ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice decreased between baseline and 12 wk compared with uninfected ApoE\textsuperscript{−/−} and ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice (Fig. 1A) illustrating vessel narrowing and disease progression in infected mice. In \emph{P. gingivalis}-infected ApoE\textsuperscript{−/−} mice, the luminal area remained unchanged between 12 and 16 wk. However, \emph{P. gingivalis}-infected ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice exhibited a progressive decline in luminal area at 0, 12, and 16 wk after the first oral infection, indicative of progressive atherosclerosis. The luminal area was significantly smaller in ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice compared with ApoE\textsuperscript{−/−} mice at 16 wk (\textit{p} = 0.03).

Assessed by microscopy in postmortem sections, the innominate artery of uninfected ApoE\textsuperscript{−/−} and ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice appeared as tightly packed layers of smooth muscle cells with uniform distribution about the circumference of the artery with no apparent lipids and inflammatory cells (Fig. 1B: ApoE\textsuperscript{−/−}, upper left; ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−}, upper right). Brown staining indicates the presence of macrophages. Plaque formation in the innominate artery of \emph{P. gingivalis}-infected ApoE\textsuperscript{−/−} mice was modest and superficial, appearing as fatty streaks at the intimal surface (Fig. 1B, lower left). In \emph{P. gingivalis}-infected ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice, we observed a significant increase in arterial plaque, which accumulated within subendothelial layers and coincided with the infiltration of inflammatory cells, including macrophages (Fig. 1B, lower right). In contrast to fatty streaks in infected ApoE\textsuperscript{−/−} mice, plaques in infected ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice protruded into the arterial lumen. Higher resolution reveals the structure of the intima, media, and adventitia in the innominate arteries of each group (Fig. 1C). Corresponding sections from MRA analyses revealed an increase in plaque area within the innominate artery of infected ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice compared with infected ApoE\textsuperscript{−/−} mice (Fig. 1D, bar graph). No significant differences in plaque area between uninfected ApoE\textsuperscript{−/−} and ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice were observed.

In the absence of infection, no differences in en face total aortic lesion area, assessed by lipid staining, were observed between uninfected ApoE\textsuperscript{−/−} and ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice (Fig. 2). Consistent with our previous studies (22), infected ApoE\textsuperscript{−/−} mice developed significantly more plaque than uninfected ApoE\textsuperscript{−/−} controls. Aortas from infected ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} mice also demonstrated significantly more plaque than their uninfected, genotype-matched ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} controls; however, plaque area was significantly

\textbf{FIGURE 1.} TLR4 deficiency confers enhanced susceptibility to atherosclerosis in the innominate artery after infection with \emph{P. gingivalis}. Innominate arteries were imaged by MRA at baseline (week 0) and at 12 and 16 wk after first oral infection. (\textbf{A}) The temporal change in luminal area (\textit{mm}²) was calculated for individual mice (\textit{n} = 5/group). Inset, Representative MRA image indicating the innominate artery (yellow box), where measurements were taken. Uninfected ApoE\textsuperscript{−/−} (blue); \emph{P. gingivalis}-infected ApoE\textsuperscript{−/−} (red); uninfected ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} (green); \emph{P. gingivalis}-infected ApoE\textsuperscript{−/−}:TLR4\textsuperscript{−/−} (purple). (\textbf{B}) Representative hematoxylin staining from each group in innominate artery with F4/80 staining (macrophages stain brown). Scale bar, 20 \textmu m. (\textbf{C}) Visualization of intima, media, and adventitia of representative images. Areas indicated in (\textbf{B}) (blue box). Scale bar, 5 \textmu m. (\textbf{D}) Plaque area within the innominate artery measured from histological images using IPLab software (Becton Dickinson) (\textit{n} = 5/group). Black bar, Uninfected; gray bar, \emph{P. gingivalis} infected. **\textit{p} < 0.01, ***\textit{p} < 0.001.
greater in infected ApoE−/−TLR4−/− mice compared with infected ApoE−/− mice. Whereas the increase in lesion area in infected ApoE−/− mice largely localized to the atherosclerosis-prone regions in the aortic arch, lesions in infected ApoE−/−TLR4−/− mice occurred in the proximal as well as the distal aorta.

TLR4 deficiency is associated with increased macrophage infiltration and expression of TLR2 in atherosclerotic lesions after infection with P. gingivalis.

The increased atherosclerotic plaque observed in infected ApoE−/−TLR4−/− mice was accompanied by a significantly increased accumulation of macrophages within the aortic sinus, whereas macrophage accumulation was not significantly increased in infected ApoE−/− mice (Fig. 3A, 3C, left). In agreement with previous findings (22), P. gingivalis infection resulted in increased expression of TLR2 within the aortic sinus of infected ApoE−/− mice, as well as in ApoE−/−TLR4−/− mice, in areas where macrophages were found (Fig. 3B, 3C, right). TLR2 expression was also significantly higher in infected ApoE−/−TLR4−/− mice compared with ApoE−/− mice (Fig. 3C, right).

Greater plaque area and infiltration of macrophages into plaque in ApoE−/−TLR4−/− mice cannot be attributed to differences in plasma cholesterol or triglycerides, as these were similar among all groups (cholesterol, mean ± SE: uninfected ApoE−/−, 476 ± 22; infected ApoE−/−, 449 ± 24; uninfected ApoE−/−TLR4−/−, 500 ± 36; infected ApoE−/−TLR4−/−, 512 ± 24 mg/dl; triglycerides, mean ± SE: uninfected ApoE−/−, 237 ± 21; infected ApoE−/−, 245 ± 18; uninfected ApoE−/−TLR4−/−, 225 ± 24; infected ApoE−/−TLR4−/−, 206 ± 22 mg/dl).

TLR4 deficiency promotes Th1/Th17 regulatory T cell imbalance in atherosclerotic lesions after infection with P. gingivalis.

In infected ApoE−/− mice, we observed no increase in CD8+ T cells, CD4+ T cells, or IL-17+ cells in the innominate artery compared with uninfected ApoE−/− mice (Fig. 4A, 4C). Accumulation of CD4+ and CD8+ cells within the innominate artery of infected ApoE−/−TLR4−/− mice was dramatically increased compared with infected ApoE−/− mice. The abundance of T cells was accompanied by increased numbers of IL-17-expressing cells and markedly diminished numbers of Foxp3+ expressing regulatory T cells (Tregs) (Fig. 4B, 4C). The marked increase in CD4+, CD8+, and IL-17+ cells and the diminution of Foxp3+ Tregs in infected mice in the absence of TLR4 expression (ApoE−/−TLR4−/− mice) reveal that in the presence of TLR4 expression,
TLR4 may be protective after *P. gingivalis* infection, which serves to prevent the infiltration of IL-17+ T cells and enhance the numbers of Foxp3+ Tregs in the inflammatory lesion.

IgG humoral immunity and Th1 responses are altered in the absence of TLR4

Infection with *P. gingivalis* induced a robust IgG1 response in both ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>-TLR4<sup>−/−</sup> mice, indicating preservation of IgG1-mediated humoral immunity in the absence of TLR4 (Fig. 5A). However, *P. gingivalis*-infected ApoE<sup>−/−</sup>-TLR4<sup>−/−</sup> mice produced significantly reduced IgG2b (Fig. 5B) and IgG3 (Fig. 5D) responses compared with ApoE<sup>−/−</sup> mice—IgG subclasses that are associated with Th1 responses (37). IgG2c levels were increased to a similar level in infected ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>-TLR4<sup>−/−</sup> mice (Fig. 5C).

We restimulated splenocytes from experimental mice with *P. gingivalis* soluble Ags and identified responsive cells that express the effector cytokines IFN-γ and IL-17. T cells from uninfected mice did not exhibit cytokine expression in response to *P. gingivalis* Ags. We observed a high percentage of IFN-γ-expressing CD4<sup>+</sup> (Fig. 6A, 6C) and CD8<sup>+</sup> (Fig. 6B, 6C) T cells from *P. gingivalis*-infected ApoE<sup>−/−</sup> mice. In contrast, the majority of responsive CD4<sup>+</sup> (Fig. 6A, 6D) and CD8<sup>+</sup> T cells (Fig. 6B, 6D) from infected ApoE<sup>−/−</sup>-TLR4<sup>−/−</sup> mice expressed IL-17. A small subset of CD8<sup>+</sup> (14%) T cells from infected ApoE<sup>−/−</sup>-TLR4<sup>−/−</sup> mice also expressed IFN-γ. Although the number of reactive T cells indicates that these responses may not be Ag specific, they were specific to *P. gingivalis* infection, as T cells from uninfected mice failed to respond to stimulation with Ags.
These results suggest that in the absence of TLR4, *P. gingivalis* infection results in impaired Th1 immunity and IL-17 skewing.

**Th1 and Treg polarizing cytokine production after *P. gingivalis* infection is impaired in DCs from TLR4-deficient mice**

Activation of TLRs on DCs triggers the release of cytokines that play decisive roles in modulating T helper subset differentiation from naive CD4+ cells (38). To investigate the role of *P. gingivalis*-induced TLR4 activation in DC production of T cell polarizing cytokines, DCs from wild-type and TLR4-/- mice were infected with *P. gingivalis*, and expression of T cell polarizing cytokines was examined. *P. gingivalis* induced the production of IL-12 (Fig. 7A), IL-10 (Fig. 7B), and IL-6 (Fig. 7C) in DCs from ApoE-/- mice. Production of these cytokines was markedly reduced in DCs from ApoE-/-/TLR4-/- mice. These results suggest that TLR4 is necessary for production of these cytokines after *P. gingivalis* infection. The abrogated DC production of T cell polarizing cytokines in the absence of TLR4 may be responsible for impaired development of Th1/Treg effector immunity as well as the enhanced IL-17 expression in T cell populations within plaques of ApoE-/-/TLR4-/- mice.

**Discussion**

Common chronic infections may contribute to up to 40% of newly developed atherosclerotic cases (39). A role for *P. gingivalis*-mediated periodontal disease as a risk factor for atherosclerotic cardiovascular disease is well documented (10, 11, 14–18). The observation that innate immune signaling triggered by *P. gingivalis* is dysregulated within atherosclerotic lesions has sparked interest in the association between oral infection and induction of innate immune cascades in atherosclerosis progression (40). Most experimental studies have focused on the proatherogenic consequence of TLR signaling in mouse models of atherogenesis; many involving the influence of high-fat diet (5, 6, 8, 12). In contrast to studies reporting diminished high-fat diet-induced atherosclerosis in TLR4-deficient mice, we report the unexpected finding that TLR4-deficient mice are markedly more susceptible to atherosclerosis after infection with *P. gingivalis*. Live animal imaging demonstrated that enhanced disease severity occurred progressively, long after cessation of the infectious stimulus and at two anatomically relevant sites, in large (aortic sinus) and medium (innominate artery) size vessels. Enhanced atherosclerosis progression in ApoE-/-/TLR4-/- mice compared with ApoE-/- mice is unlikely to be due to differences in plasma cholesterol or triglycerides, which were similar among all groups. Minimal atherosclerotic lesion area in the innominate artery was observed in uninfected ApoE-/- mice, and this is likely due to the fact that animals were fed a normal chow diet. In our recent study in which atherosclerosis progression was examined using MRA in the innominate artery of uninfected and *P. gingivalis*-infected ApoE-/- mice, animals were fed a high-fat diet for the duration of the study (35). High-fat diet enhances atherosclerosis progression in ApoE-/- mice. In the absence of high-fat diet and infection, plaque accumulation within the aorta and innominate artery progresses more slowly and is minimal at the time point examined in the current study. Effective control of immune-mediated pathol-
In P. gingivalis-infected ApoE−/− mice coincided with an increase in Tregs within the innominate artery. In contrast, the exacerbated inflammatory pathology in P. gingivalis-infected ApoE−/−TLR4−/− mice was associated with increased lesion macrophage numbers and T cell infiltration and enhanced expression of IL-17. Tregs play a critical role in maintaining immunological tolerance and controlling the extent of immune-mediated pathology, especially in cases of chronic infection (41, 42). Our studies indicate that in the absence of TLR4, mice fail to mediate pathology, especially in cases of chronic infection (41, 12). A separate report demonstrated a protective role for TLR4 deficiency in diet-induced atherogenesis (8). It is important to note that these results could not be recapitulated under germ-free conditions (50), indicating a potential interaction between hyperlipidemia and indigenous microbes. On the basis of these observations, it was proposed that common mechanisms of signaling via TLR2, TLR4, and MyD88 link stimulation by multiple pathogens and endogenous ligands to atherosclerosis, and that therapeutic TLR4 antagonism could prove beneficial in the treatment of chronic atherosclerosis (8, 51, 52). Our results clearly point to a critical role for specific TLR signaling, in particular, TLR4, in chronic inflammation and atherosclerosis induced by P. gingivalis. Our results raise caution for the safety and efficacy of TLR4 antagonists for the treatment of atherosclerosis, especially in patients with comorbid conditions including periodontal disease and other infectious diseases.

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**Disclosures**

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