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J Immunol 2012; 189:3681-3688; Prepublished online 5 September 2012;
doi: 10.4049/jimmunol.1201541
http://www.jimmunol.org/content/189/7/3681

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Protective Role for TLR4 Signaling in Atherosclerosis Progression as Revealed by Infection with a Common Oral Pathogen

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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 pathogen-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 pathogen-specific TLR signaling in chronic inflammation and atherosclerosis. The Journal of Immunology, 2012, 189: 3681–3688.
ternal recognition by the TLR4 complex (29, 30). In the current study, we demonstrate the unique ability of \emph{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\textsuperscript{−/−} and C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR4\textsuperscript{−/−} mice on C57BL/6 background were provided by S. Akira (Osaka University). ApoE\textsuperscript{−/−}TLR4\textsuperscript{−/−} 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**

\emph{P. gingivalis} strain 381 was grown anaerobically on blood agar plates (Becton Dickinson) and used to seed-inoculate brain heart infusion broth (pH 7.4; Becton Dickinson) supplemented with yeast extract (Becton Dickinson), hemin (10 \mu g/mL; Sigma), and menadione (1 \mu g/mL). CFUs were standardized at an OD at 660 nm of 1 (equivalent to 1 × 10\textsuperscript{6} CFU/mL) by spectrometry (ThermoElectron Genways20). LPS from \emph{P. gingivalis} 381 was isolated using a modified Tri-Reagent protocol (29).

**Oral infection**

Three independent experiments were performed with ApoE\textsuperscript{−/−} (total \(n = 40\)) and ApoE\textsuperscript{−/−}TLR4\textsuperscript{−/−} (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 (Sulframit; Hi-Tech Pharmacal) ad libidum in the drinking water for 10 d, followed by a 2-d antibiotic-free period. One hundred microlits of \emph{P. gingivalis} 381 (1 × 10\textsuperscript{6} CFU) suspended in vehicle (2% carboxymethylcellulose 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 \mu L of vehicle. Topical application of \emph{P. gingivalis} to the buccal surface of the maxillary gingiva five times a week for 3 wk induces alveolar bone loss in ApoE\textsuperscript{−/−} 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 innominate 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) maintained at 23°C. Respiration was monitored using a monitoring and gating system (SA Instruments, Walthasha, WI). The unaged 3D gradient echo MRA was acquired with the following parameters: slab thickness = 1.5 cm; flip angle = 45°; repetition time = 20 ms; echo time = 2.2 ms; field of view = 1.5 × 1.5 × 1.5 cm; matrix = 128 × 128 × 128, in-plane; number of average = 4. Total scan time was ~25 min. Image reconstruction and analysis were performed using Paravision. The 3D reconstruction of the MRA images was achieved by maximum intensity projection. The cross sections were chosen at 0.3- to 0.5-mm distance below the subclavian bifurcation. Lumen area was manually defined and calculated with ImageJ (National Institutes of Health) by two independent observers. Measurement reproducibility had an interclass correlation coefficient of 0.92.

**Atherosclerotic plaque assessment**

Aortas were harvested and stained with Sudan IV as described (22). Digital images were acquired using a modified Tri-Reagent protocol (29).

**Immunohistochemistry**

Mice were euthanized (\(n = 4\) / group), perfused with 4% paraformaldehyde, and aortic arch with heart tissue was embedded in OCT freezing compound. Five-micrometer serial cryosections were collected every 50 \mu m in the innominate artery and aortic sinus. In the innominate artery, cryosections were obtained from the region corresponding to the greatest plaque size as revealed by MRA, ~0.3 mm below the bifurcation of the innominate and subclavian arteries as described (35). Immunohistochemistry was performed using rat anti-mouse F4/80 (no. MCA497R; Serotec, Oxford, U.K.), rat anti-mouse CD4 (BD Biosciences no. 550278 and Caltag Lab-
expressed by \textit{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 of ApoE \textsuperscript{-/-} mice at 16 wk \((p < 0.001)\), infected ApoE \textsuperscript{-/-} mice exhibited a progressive decline in luminal area compared with infected ApoE \textsuperscript{-/-}/TLR4 \textsuperscript{-/-} mice (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 \textit{P. gingivalis}. Innominate arteries were imaged by MRA at baseline (week 0) and at 12 and 16 wk after first oral infection. (A) The temporal change in luminal area (mm\textsuperscript{2}) was calculated for individual mice (\(n = 5\)/group). Inset, Representative MRA image indicating the innominate artery (yellow box), where measurements were taken. Uninfected ApoE \textsuperscript{-/-} (blue); \textit{P. gingivalis}-infected ApoE \textsuperscript{-/-} (red); uninfected ApoE \textsuperscript{-/-}/TLR4 \textsuperscript{-/-} (green); \textit{P. gingivalis}-infected ApoE \textsuperscript{-/-}/TLR4 \textsuperscript{-/-} (purple). (B) Representative hematoxylin staining from each group in innominate artery with F4/80 staining (macrophages stain brown). Scale bar, 20 \(\mu\)m. (C) Visualization of intima, media, and adventitia of representative images. Areas indicated in (B) (blue box). Scale bar, 5 \(\mu\)m. (D) Plaque area within the innominate artery measured from histological images using IPLab software (Becton Dickinson) (\(n = 5\)/group). Black bar, Uninfected; gray bar, \textit{P. gingivalis} infected. **\(p < 0.01\), ***\(p < 0.001\).
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 aortic lesions from P. gingivalis-infected mice

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 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−/− and ApoE−/−TLR4−/− mice, indicating preservation of IgG1-mediated humoral immunity in the absence of TLR4 (Fig. 5A). However, *P. gingivalis*-infected ApoE−/−TLR4−/− mice produced significantly reduced IgG2b (Fig. 5B) and IgG3 (Fig. 5D) responses compared with ApoE−/− mice—IgG subclasses that are associated with Th1 responses (37). IgG2c levels were increased to a similar level in infected ApoE−/− and ApoE−/−TLR4−/− 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+ (Fig. 6A, 6C) and CD8+ (Fig. 6B, 6C) T cells from *P. gingivalis*-infected ApoE−/− mice. In contrast, the majority of responsive CD4+ (Fig. 6A, 6D) and CD8+ T cells (Fig. 6B, 6D) from infected ApoE−/−TLR4−/− mice expressed IL-17. A small subset of CD8+ (14%) T cells from infected ApoE−/−TLR4−/− 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−/− mice, we report the unexpected finding that TLR4−/− 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 TLR4-deficient 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-
ogy in \textit{P. gingivalis}-infected ApoE\(^{-/-}\) mice coincided with an increase in Tregs within the innominate artery. In contrast, the exacerbated inflammatory pathology in \textit{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 develop protective Th1 immunity and are unable to regulate adaptive immune responses mediated by Th17 cells after \textit{P. gingivalis} infection. We propose that this results in a breakdown of immunological tolerance, owing to impaired Treg function, leading to unrestricted activation of pathogenic T cells that mediate arterial inflammation. The unique TLR4 evasive properties of \textit{P. gingivalis} lipid A position this organism to disrupt effector T cell mechanisms at the level of DC activation, the interface of innate and adaptive immunity.

TLR2 expression was increased markedly in aortic lesions by \textit{P. gingivalis} infection in ApoE\(^{-/-}\) mice and further increased in ApoE\(^{-/-}\)-TLR4\(^{-/-}\) mice. It is plausible that enhanced TLR2 expression in ApoE\(^{-/-}\)-TLR4\(^{-/-}\) mice may have contributed to increased vascular inflammation and atherosclerosis in ApoE\(^{-/-}\)-TLR4\(^{-/-}\) mice. Thus, the increase in atherosclerosis in ApoE\(^{-/-}\)-TLR4\(^{-/-}\) mice may be a result of not only TLR4 deficiency but also high TLR2 expression. This increased TLR2 expression in activated macrophages and the endothelium may reflect the development and maintenance of a hyperinflammatory state in the absence of TLR4 expression. This observation was an unexpected finding of this study, as was the finding that plaque development was enhanced in the absence of TLR4.

Our results also showed that after in vitro restimulation with Ag, T cells from \textit{P. gingivalis}-infected ApoE\(^{-/-}\)-TLR4\(^{-/-}\) mice predominantly produced IL-17, whereas IFN-\(\gamma\) was the predominant cytokine produced by T cells from infected ApoE\(^{-/-}\) mice. We also demonstrate that TLR4 deficiency was associated with markedly inhibited production of the Th1-polarizing cytokine 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 develop protective Th1 immunity and are unable to regulate adaptive immune responses mediated by Th17 cells after \textit{P. gingivalis} infection. We propose that this results in a breakdown of immunological tolerance, owing to impaired Treg function, leading to unrestricted activation of pathogenic T cells that mediate arterial inflammation. The unique TLR4 evasive properties of \textit{P. gingivalis} lipid A position this organism to disrupt effector T cell mechanisms at the level of DC activation, the interface of innate and adaptive immunity.

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**Disclosures**

The authors have no financial conflicts of interest.

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