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CUTTING EDGE

Cutting Edge: TLR2 Is Required for the Innate Response to *Porphyromonas gingivalis*: Activation Leads to Bacterial Persistence and TLR2 Deficiency Attenuates Induced Alveolar Bone Resorption[1]

Elia Burns, Gilad Bachrach,* Lior Shapira,† and Gabriel Nussbaum[2]**

***Periodontitis is a chronic inflammatory disease that leads to destruction of the attachment apparatus of the teeth. The presence of particular oral bacteria and the host inflammatory response contribute to disease progression.* Porphyromonas gingivalis *is a Gram-negative anaerobe considered to be a major periodontal pathogen. Isolated Ags from* P. gingivalis *activate innate immune cells through TLR2 or TLR4. We challenged TLR2- and TLR4-deficient mice with live* P. gingivalis *and studied the inflammatory response and bacterial survival. Wild-type and TLR4-deficient mice produced high levels of cytokines in response to* P. gingivalis *challenge, whereas cytokine levels were nearly absent or delayed in TLR2-deficient mice. Surprisingly,* P. gingivalis *was cleared far more rapidly in TLR2-deficient mice. In addition, TLR2-deficient mice resisted bone loss following oral infection with* P. gingivalis. *The Journal of Immunology,* 2006, 177: 8296–8300.**

Periodontal disease is a chronic inflammatory condition that leads to erosion of the attachment apparatus and supporting bone of the teeth. It is one of the most common chronic infectious diseases in humans (1) and is the most prevalent cause of tooth loss. Although the presence of oral bacteria is necessary for periodontitis to develop, tissue breakdown and alveolar bone resorption are considered to result from the barrage of inflammatory mediators secreted by the host (2). *Porphyromonas gingivalis* is a Gram-negative anaerobic bacterium implicated as a major periodontal pathogen (3). Surface components of *P. gingivalis*, such as LPS, lipoproteins, and fimbriae, interact with host expressed TLRs, key control elements in the innate immune response to a diverse array of microbe-associated molecules (4). TLRs are expressed on multiple cell types at the site of *P. gingivalis* encounter with the host, including epithelial cells, monocyte/macrophages, fibroblasts, and neutrophils (2). TLR activation leads to nuclear translocation of NF-κB and induction of inflammation-related genes (4). Elevated TLR expression in inflamed gingival tissue (5) suggests that exaggerated inflammation mediated by TLRs is a driving factor in periodontal disease. In fact, TLR4-deficient mice developed less alveolar bone loss than wild-type (WT)[3] mice following endodontal infection with a mixed culture of anaerobic bacteria (6).

Particular TLRs are required to respond to specific microbial structures, such as TLR4 for enterobacterial LPS and TLR2 for bacterial lipopeptides. Isolated components of *P. gingivalis* have been demonstrated to activate TLR2 and TLR4 on a variety of cell types. Native and recombinant surface fimbriae induce gingival epithelial cell IL-8 expression and monocyte chemokine and cytokine expression in a TLR2-dependent manner (7, 8). *P. gingivalis* LPS activates but also blocks TLR4 (9) and, unlike *Escherichia coli* LPS, studies show that *P. gingivalis* LPS also activates through TLR2 (10). Therefore, TLR2 and TLR4 are implicated in the response to *P. gingivalis*, although the contribution of each of these receptors during infection in vivo is uncertain. In addition, although TLR activation should benefit the host in the short term by the recruitment and activation of cells and factors that aid in pathogen clearance, some organisms exploit TLR signaling to evade the host response (reviewed in Ref. 11). Oral pathogens such as *P. gingivalis* have coevolved with the host and, therefore, may possess similar TLR-dependent evasion mechanisms that may not be apparent when studying the response to isolated components. In the present study we addressed these questions by studying the response of TLR-deficient mice to challenge with live *P. gingivalis*. Acute infection of s.c. chambers was used to characterize the inflammatory response and bacterial survival in the presence and absence of TLR2 or TLR4. Chronic oral exposure to *P. gingivalis* was used to determine the contribution of TLR signaling to the development of periodontal disease.

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[3] Abbreviations used in this paper: WT, wild type; μCT, micro-computed tomography.

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Materials and Methods

Bacterial growth

P. gingivalis (strain 381) was cultured for 48 h in Wilkins broth (Oxoid) under anaerobic conditions at 37°C. An OD value of 0.1 (650 nm) was determined to correlate to 10^{10} CFU per ml.

Mice

C57BL/6 mice were from Harlan Sprague Dawley. C57BL/10 and C57BL/10ScNJ-Tlr4$^{lps\text{-}del}$ (to be referred to as TLR4^{del} mice hereafter) were from The Jackson Laboratory. TLR2$^{-/-}$ mice backcrossed to the C57BL/6 background were a gift from Prof. S. Akira (Osaka University, Japan). We found no differences in the responses of C57BL/6 WT and C57BL/10 WT mice; therefore, both TLR-deficient strains were compared with C57BL/6 WT mice. All mice were housed at the specific pathogen-free unit of our university and all experiments were approved by the institutional animal care and use committee.

Subcutaneous chamber model

Two titanium coil chambers were inserted s.c. into anesthetized 6- to 8-wk-old female mice ($n \geq 6$) as previously described (12). After 10 days, live *P. gingivalis* (10^9 CFU in 100 μl of PBS) were injected into each chamber. Chamber exudates were collected at baseline (immediately before challenge) and at 2 and 24 h postinfection (each chamber was sampled only once). PBS was injected into chambers of control groups. Exudates were centrifuged and supernatants were collected for bacterial counts and cytokine analysis. Cell pellets were resuspended in 0.5% BSA/PBS for flow cytometry analysis.

Cytokine analysis

Levels of TNF-α, IL-10. and IFN-γ in chamber exudates were determined using mouse OptEIA sets (BD Biosciences) and IL-1β was determined using the mouse DuoSet (R&D Systems) according to the manufacturer's instructions.

Viable bacterial counts

Ten microliters of chamber exudates was serially diluted in triplicate in PBS and plated on tryptic soy agar containing sheep blood (Hylabs). Plates were grown under anaerobic conditions for 5–7 days at 37°C. *P. gingivalis* colonies were identified by their black pigment and by phase contrast microscopy.

Bacteremia

Anesthetized mice were bled from the retro-orbital venous plexus following intrachamber infection. Heparinized blood was immediately plated on tryptic soy agar containing sheep blood and grown for 5–7 days under anaerobic conditions. *P. gingivalis* colonies were identified as described above and selected colonies were confirmed by PCR using primers specific to *P. gingivalis* 16S RNA. Mice were considered bacteremic if more than three colonies of *P. gingivalis* were identified.

In vivo phagocytosis assay

P. gingivalis 381 was labeled with 0.1 mg/ml FITC (Sigma-Aldrich) in PBS for 20 min at room temperature. FITC-labeled *P. gingivalis* (10^9 CFU) bacteria were injected into s.c. chambers. Chamber exudates drawn 24 h postinfection were centrifuged and pellets were washed three times in PBS to remove nonadherent bacteria. Adherent bacteria were killed by incubating cells for 1 h in PBS containing 500 μg/ml metronidazole and 300 μg/ml gentamicin. Cells were then washed and resuspended in 0.5% BSA with PBS. The ability of antibiotic treatment to remove the fluorescence of extracellular bacteria was confirmed by confocal microscopy and FACS. A time period of 24 h was selected because of the dramatic difference in viable bacterial counts at this time point. Phagocytosis was measured using a FACScan flow cytometer equipped with CellQuest software (BD Biosciences).

P. gingivalis *oral infection*

Alveolar bone loss was induced following *P. gingivalis* oral infection as previously described (13). Briefly, mice ($n \geq 8$) were treated with sulfamethoxazole (0.4% solution in drinking water) for 10 days followed by 3 days without antibiotics. WT and TLR2$^{-/-}$ mice were infected with live *P. gingivalis* in PBS (4×10^9 CFU) containing 2% carboxymethylcellulose ("vehicle") using a round-tipped feeding needle three times at 2-day intervals. Control groups were treated with vehicle alone. Six weeks after the first challenge mice were sacrificed and maxillae were scanned by micro-computed tomography (μCT; Scanco Medical). Three-dimensional alveolar bone loss was quantified as recently reported (14). Approximately 180 slices 12-μm wide were scanned for each sample, covering the entire buccopalatal aspect.

Statistical analysis

Data are reported as means ± SEM. Comparisons were made using the Student *t* test (SigmaPlot; Systat Software).

Results and Discussion

Impaired cytokine response to P. gingivalis *in TLR2*$^{-/-}$ *mice*

We analyzed the inflammatory exudate in s.c. chambers 2 and 24h following infection with *P. gingivalis* in WT, TLR2-deficient, and TLR4-deficient mice (Fig. 1). PBS injection into chambers of all strains did not elicit cytokine secretion in comparison to baseline levels (data not shown). We observed a rapid increase in TNF-α, IL-1β, IFN-γ, and IL-10 in both WT and TLR4-deficient mice 2 h following infection (Fig. 1). In fact, TNF-α levels were significantly higher in TLR4-deficient mice compared with those in WT mice ($p < 0.05$). In contrast, IL-1β and IFN-γ were absent in TLR2$^{-/-}$ mice at 2h (Fig. 1, *B* and *C*). TLR2$^{-/-}$ mice also produced significantly less TNF-α and IL-10 at 2 h (Fig. 1, *A* and *D*); however, there was a significant increase over baseline ($p < 0.05$). Twenty-four hours following infection TNF-α levels returned to baseline in

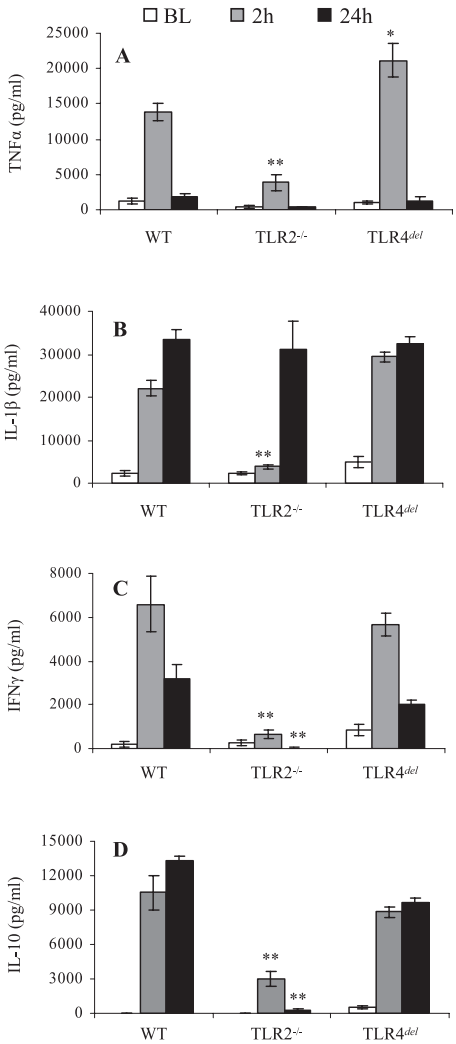

FIGURE 1. Cytokines following *P. gingivalis* challenge. TNF-α (*A*), IL-1β (*B*), IFN-γ (*C*), and IL-10 (*D*) were examined in chamber exudates at baseline (BL) and 2 and 24 h after *P. gingivalis* challenge. One representative experiment of three is shown. Significance shown represents comparisons to WT at the same time point. *, $p < 0.05$; **, $p < 0.01$.

all strains tested, and IFN-γ showed a significant decrease in WT and TLR4-deficient mice (Fig. 1, *A* and *C*). High levels of IL-1β and IL-10 were still observed at 24 h in the WT and TLR4 deficient mice. TLR2$^{-/-}$ mice achieved similar levels of IL-1β at 24 h postinfection despite its absence at 2 h; however, the small increase in IL-10 at 2 h returned to baseline by 24 h (Fig. 1, *B* and *D*). In summary, the cytokine response to live *P. gingivalis* in the presence of TLR2 signaling is faster (IL-1β), more varied (IFN-γ), and more robust (all cytokines tested) than in its absence.

TLR2$^{-/-}$ mice rapidly clear P. gingivalis

To test the impact of TLR-mediated inflammation on bacterial survival, we determined viable *P. gingivalis* CFU from the chambers at 2 and 24 h postinfection. There was no significant decrease in bacterial viability compared with the inoculum at either time point in WT and TLR4 deficient mice (Fig. 2*A*). Surprisingly, 2 h postinfection significantly less *P. gingivalis* was recovered from TLR2$^{-/-}$ mice and by 24 h no viable *P. gingivalis* could be cultured (Fig. 2*A*). To determine the extent of intrachamber bacterial survival in the presence of intact TLR2 signaling, *P. gingivalis* was cultured from chambers of WT mice up to 8 days postinfection (Fig. 2*B*). Bacterial survival gradually decreased beginning 48 h postinfection; however, even at 8 days the number of viable *P. gingivalis* in WT chambers did not fall below the number recovered from TLR2$^{-/-}$ mice at 2 h. The presence of TLR2 signaling therefore significantly prolongs bacterial persistence in the host. The rapid disappearance of bacteria from the chambers of TLR2$^{-/-}$ mice and the lack of a significant early cytokine response suggested that *P. gingivalis* may escape the chamber and spread systemically. To rule out this possibility we tested for bacteremia at various time points following challenge. Surprisingly, WT and TLR4-deficient mice were bacteremic for several days after infection, whereas no bacteremia was detected in TLR2$^{-/-}$ mice at any time point (Table I). No traces of *P. gingivalis* were found in the spleens of any mouse strain by PCR (data not shown).

Phagocytosis of P. gingivalis i*s more efficient in the absence of TLR2 activation*

We established a flow cytometry method to track intrachamber interactions of host cells with *P. gingivalis* following challenge. Using labeled Abs we found similar ratios of neutrophils and monocytes in the chambers of WT and TLR-deficient mice at baseline. No significant differences in the cell population recruited to the chamber following infection were observed in WT and TLR-deficient animals (data not shown). Next, we challenged mice with FITC-labeled *P. gingivalis* to follow phagocytosis in vivo. Cells were drained from the chamber, surface-attached *P. gingivalis* was removed, and phagocytosis was measured by FACS. Three levels of fluorescence were observed among cells from chambers of WT mice (defined as M1, M2, and M3), signifying variable uptake of *P. gingivalis* (Fig. 3*A*). In contrast, cells from chambers of TLR2$^{-/-}$ mice behaved in a homogeneous manner and fluoresced at the highest of the three levels (Fig. 3*B*). Phagocytosis in TLR4-deficient mice was similar to that found in WT mice (data not shown). A quantitative measure of the percentage of cells at each of the fluorescence levels is shown in Fig. 3*C*. Significantly more TLR2$^{-/-}$ cells ($p < 0.05$) are found at the highest fluorescence level (M3), whereas significantly more WT cells ($p < 0.01$) are found at the

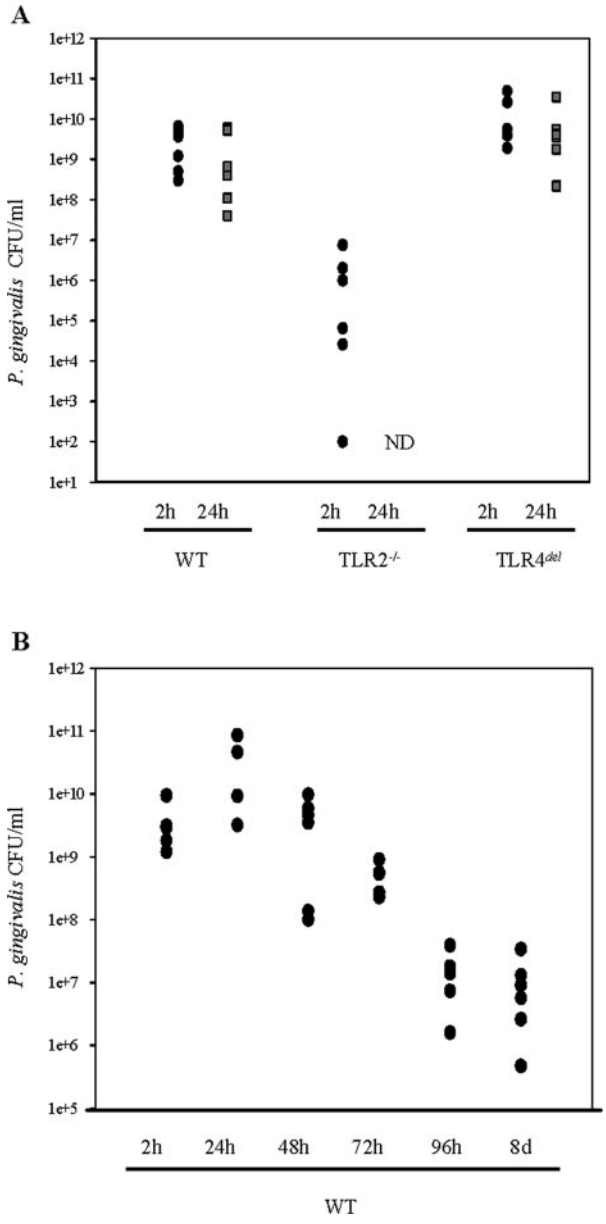

FIGURE 2. *P. gingivalis* survival in chamber exudates. *A*, Viable CFU from chamber exudates of WT, TLR2$^{-/-}$, and TLR4^{del} mice were determined 2 h (●) and 24 h (▨) after intrachamber challenge with *P. gingivalis.* Each symbol represents an individual mouse ($n = 6$ per strain). One representative experiment of three is shown. ND, None detected. *B*, Three groups of six WT mice were used to follow intrachamber *P. gingivalis* survival postinfection. Each circle represents one mouse.

lowest florescence (M1). Because the fluorescence level reflects the number of labeled *P. gingivalis* bacteria phagocytosed per cell, efficient bacterial clearance (in TLR2$^{-/-}$ mice; Fig. 2*A*) depends, at least in part, on the consistent phagocytosis of multiple organisms per cell. The intracellular fate of *P. gingivalis* in WT and TLR-deficient cells following phagocytosis and the effect on neutrophil apoptosis are currently under study. Delayed neutrophil apoptosis has been suggested to play a role in certain bacterial infections (15), and LPS from *P. gingivalis* has been shown to prevent apoptosis of HL60-derived neutrophils in vitro (16).

Because TLR2 is not a phagocytic receptor, differences in *P. gingivalis* phagocytosis between WT and TLR2$^{-/-}$ mice likely

Table I. *Percentage of bacteremia following* P. gingivalis *challenge in WT and TLR-deficient mice*

	WT (%)	TLR2$^{-/-}$ (%)	TLR4^{del} (%)
24 h	80	0	66
96 h	40	0	40
8 days	0	0	0

reflect differences in a factor secreted either by the phagocytes themselves or by other cells present in the chamber. Cytokines such as IL-10 have been shown to reduce neutrophil-dependent bacterial killing, suggesting that the net effect of the cytokine milieu might benefit *P. gingivalis* survival (17). Alternatively, TLR2-dependent activation of β_1 integrins on the surface of monocytes and neutrophils by *P. gingivalis* (18, 19) may enhance bacterial survival, because integrin-mediated uptake enables *P. gingivalis* to persist intracellularly (20).Thus, *P. gingivalis* may evade immune clearance through several TLR2-mediated mechanisms.

TLR2$^{-/-}$ mice resist alveolar bone loss following repeated oral challenge with P. gingivalis

P. gingivalis is an oral pathogen and a major etiological factor in periodontal disease. To examine the contribution of TLR2 to

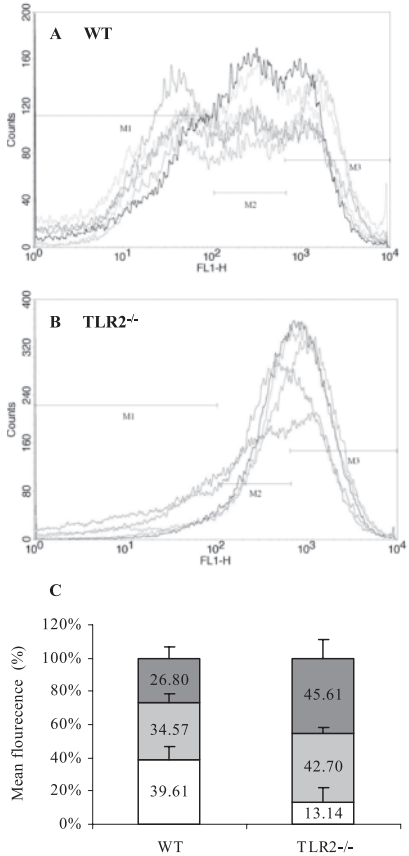

FIGURE 3. In vivo phagocytosis. Following challenge with FITC-labeled bacteria, cells from chambers of WT (*A*) and TLR2$^{-/-}$ (*B*) mice were analyzed for FL-1 fluorescence. Negative fluorescence was set by analysis of cells from chambers challenged with nonlabeled bacteria (not shown). Each line represents cells drawn from one mouse. M1, M2, and M3 were determined according to the three cell populations found in WT mice. The percentage of cells at each level of fluorescence is shown in *C*.

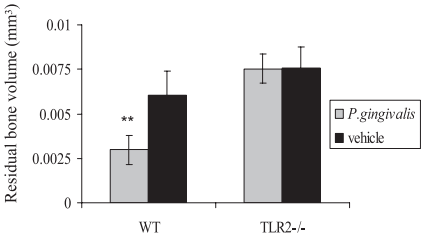

FIGURE 4. Alveolar bone loss in WT and TLR2$^{-/-}$ mice. Six weeks following oral challenge with *P. gingivalis*, alveolar bone loss was determined by μCT. For each strain, *P. gingivalis* infected mice (▨) were compared with vehicle-treated mice (■). **, $p = 0.0022$.

periodontitis, we measured the degree of alveolar bone loss in the mandible following repeated oral infection with *P. gingivalis*. Bone loss was measured by μCT to allow quantification of the volume of absorbed bone in three dimensions (14). Oral infection with *P. gingivalis* induced significant bone loss in WT compared with control mice treated with vehicle alone. In contrast, no differences were found between infected and vehicle-treated TLR2$^{-/-}$ mice (Fig. 4). Thus, in the oral cavity, TLR2 is required for *P. gingivalis* to cause disease. It remains to be determined if differences in host response factors or bacterial survival, such as those observed in the s.c. chambers of WT and TLR2$^{-/-}$ mice, underlie the resistance to bone resorption.

Gram-negative bacteria are generally recognized by the innate immune system through TLR4 (21). Although the discrepancy regarding *P. gingivalis* LPS recognition through TLR2 vs TLR4 continues (reviewed in Ref. 10), our results demonstrate that TLR2 governs the in vivo response to live *P. gingivalis*. We therefore show in this study an unusual recognition of a Gram-negative bacterium by TLR2. TLR-dependent host recognition of pathogens usually enhances their clearance. Thus, TLR2$^{-/-}$ mice are far more susceptible to infections with bacteria that activate host immunity through TLR2 (22). We find the exact opposite with *P. gingivalis* — bacterial survival and the ability to cause disease are enhanced in the presence of robust TLR-dependent host recognition. This phenomenon may explain the persistence of other pathogens implicated in chronic infectious/inflammatory lesions.

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Disclosures

The authors have no financial conflict of interest.

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