TLR2 Signaling and Th2 Responses Drive \textit{Tannerella forsythia}-Induced Periodontal Bone Loss

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TLR2 Signaling and Th2 Responses Drive *Tannerella forsythia*-Induced Periodontal Bone Loss

Srinivas R. Myneni,* Rajendra P. Settem,* Terry D. Connell,†‡ Achsah D. Keegan,§ Sarah L. Gaffen,* and Ashu Sharma*

Periodontal disease (PD) is a chronic inflammation of the tooth-supporting soft tissue and alveolar bone due to infection by a select group of Gram-negative microbes, which leads to tooth loss if untreated. Because mice deficient in CD4+ cells are resistant to infection-induced alveolar bone loss, Th cells have been implicated in bone-destuctive processes during PD. However, the extent to which different Th cell subtypes play roles in pathogenesis or host protection remains to be defined and is likely to vary depending on the dominant microorganism involved. By far, *Porphyromonas gingivalis* is the best-studied periodontal microbe in PD. Although the Gram-negative anaerobe *Tannerella forsythia* is also a vital contributor to periodontal bone loss, almost nothing is known about immune responses to this organism. Previous studies from our laboratory revealed that *T. forsythia* induces periodontal bone loss in mice and that this bone loss depends on the bacterially expressed BspA protein. In this study, we showed that *T. forsythia* activates murine APCs primarily through TLR2-dependent signaling via BspA. Furthermore, *T. forsythia* infection causes a pronounced Th2 bias, evidenced by T cell expression of IL-5, but not IFN-γ or IL-17, in draining lymph nodes. Consistently, deficiencies in TLR2 or STAT6 result in resistance to *T. forsythia*-induced alveolar bone loss. Thus, TLR2 signaling and Th2 cells play pathogenic roles in *T. forsythia*-induced alveolar bone destruction. The Journal of Immunology, 2011, 187: 501–509.

Periodontal disease (PD) is an inflammatory disease of the tooth-supporting tissue (periodontium) that frequently leads to tooth loss (1) and is the most common cause of inflammation-induced bone loss in humans. PD is caused by a select group of anaerobic Gram-negative bacteria that colonize subgingival spaces as biofilms. Periodontal bone destruction results mainly from the effects of the immune response to these biofilm bacteria (2, 3). The pathogenesis of PD is mediated by a polymicrobial consortium consisting of the Gram-negative pathogens *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, collectively known as the red complex (4–6). Although *T. forsythia* has been increasingly implicated in the development and severity of PD, it remains a highly understudied pathogen (7, 8). Its etiological role in PD has been recognized relatively recently, and the virulence mechanisms of *T. forsythia* are only just beginning to be defined (7). Unlike *P. gingivalis*, *T. forsythia* is a fastidious microbe with stringent growth requirements. We were the first, to our knowledge, to document the virulence potential of *T. forsythia* in a murine model of PD (9). In so doing, we found that the alveolar bone loss is dependent on the bacterially expressed virulence protein BspA (9). However, the immune response to *T. forsythia* remains almost entirely undefined.

Alveolar bone loss in response to oral infection by *P. gingivalis* is dependent on the host response. For example, SCID mice or mice specifically deficient in CD4+ T cells are resistant to alveolar bone loss due to *P. gingivalis* infection (10–12). Moreover, Th1 responses are associated with *P. gingivalis*-stimulated alveolar bone loss in mice (13). In humans, the role of T cells in PD pathobiology is complex, and data support a role for T cells in both protection and pathogenesis (6, 14–16). The existence of two distinct effector CD4 T cell subsets has been recognized since 1986 (17), with the description of Th1 (IFN-γ–producing) and Th2 (IL-4-, IL-5- and IL-13–producing) cells. The Th17 lineage of IL-17–producing T cells was recognized in 2005 (18). In *P. gingivalis* infection, both Th1 and Th2 cytokines are found in the periodontal lesion (6, 14–16). Prior to the discovery of Th17 cells, it was suggested that Th1 cells are characteristic of stable lesions, whereas Th2 cells are associated with disease (14). However, IL-17 was also documented in periodontitis patients with severe disease (19–22), and a significant number of CD4+ T cells isolated from gingival tissue of periodontitis patients express IL-17 (23). In *P. gingivalis* murine infections, IL-17 signaling is host protective by virtue of limiting infection via neutrophil mobilization (24).

The Th effector responses to *T. forsythia* are still poorly defined and may be different from responses to *P. gingivalis*. This could arise from differences in the nature of the pathogen-associated molecular patterns expressed on different periodontal pathogens, which ultimately shape the adaptive response. In support of this notion, *T. forsythia*, unlike *P. gingivalis*, is unable to block neutrophil recruitment during infection in a mouse model (25), and recent studies demonstrated that *T. forsythia*, but not *P. gingivalis*, preferentially activates TLR2. For instance, although *P. gingivalis*...
through its limbriae and LPS predominantly activate TLR2 (26), whole bacteria (27) and both minor and major fimbrial proteins activate TLR4/CD14/MD2 (28, 29). Thus, the role of CD4 effector responses in PD bone loss remains poorly defined (14, 30), particularly specific responses to T. forsythia.

TLRs can play protective or destructive roles, depending on the nature of the invading pathogen and its associated virulence determinants (31). T. forsythia, as well as its virulence factor BspA, induce proinflammatory cytokine and chemokine secretion through TLR2 (32–34). Signaling by TLR2 in APCs and expression of specific cytokines were suggested to favor Th2 responses (35–38). Moreover, the Th2-specific transcription factor STAT6 was linked to susceptibility to PD in mice (39). Accordingly, it was compelling to determine the role of TLR2 and STAT6-mediated responses in T. forsythia-induced bone destruction. We predicted that T. forsythia would favor a Th2 inflammatory response by activating TLR2. We further hypothesized that the Th2 response would exert a destructive role, based on our prior observation that T. forsythia causes alveolar bone loss in mice (9). As shown in the study, we found that TLR2−/− or STAT6−/− mice indeed showed markedly decreased susceptibility to T. forsythia-induced PD bone loss, associated with decreased Th2 responses. These results imply a critical involvement of a bone-destructive Th2 response following oral infection with T. forsythia, mediated via TLR2 signaling.

Materials and Methods

Mice

Specific-pathogen free BALB/c (wild-type [WT]) and STAT6−/− mice (BALB/c background) were from The Jackson Laboratory (Bar Harbor, ME). TLR2−/− mice on a BALB/c background used in this study represent progeny obtained after backcrossing the tl2 deletion (tl2−/−) originally on C57BL/6 to BALB/c background for six generations (T.D. Connell, unpublished observations). These mice were healthy and showed no signs of gross abnormalities. Mice were maintained in HEPA-filtered cages with autoclaved food, water, and bedding. All procedures were performed in accordance with protocols approved by the University at Buffalo Institutional Animal Care and Use Committee.

Bacterial strains and culture conditions

T. forsythia was cultured in T. forsythia broth or on T. forsythia-agar plates (1.5% agar in T. forsythia broth) under anaerobic conditions, as described previously (40).

Oral infection and alveolar bone loss assessment

Animals within groups were matched by age and sex (6–7 wk at the start of the experiment; n = 8–10 per group) and quarantined for 1 wk prior to the experiment. Mice were infected with T. forsythia, as previously described, with the following modifications (9): mice were treated with kanamycin (1 mg/ml) for 7 d ad libitum, followed by a 3-d antibiotic-free period. This was followed by infection with live bacteria (T. forsythia ATCC 43037) via oral gavage. Infection was given as 100-μl bacterial suspensions (100 CFU/ml) in 2% carboxymethyl cellulose three times at 48-h intervals for 2 wk. The control (sham-infected) mice received antibiotic pretreatment and 100 μl 2% carboxymethyl cellulose. Mice were sacrificed after 6 wk, and serum was collected by cardiac puncture. Jaws were autoclaved, defleshed, immersed overnight in 3% H2O2, and stained with 1% methylene blue. Horizontal bone loss was assessed morphometrically by measuring the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC). Measurements at 14 buccal sites per mouse (7 on the left and right maxillary molars) were made under a dissecting microscope (Brook-Anco, Rochester, NY) fitted with an Aquinto imaging measurement system (a4i America). Random, blinded bone measurements were taken by two independent evaluators. Data were analyzed on GraphPad Prism 5 software (GraphPad, San Diego, CA).

ELISA

T. forsythia-specific ELISAs were performed as described (9). Briefly, 96-well Immuno-Maxisorp plates (Nalgene Nunc International, Rochester, NY) were coated with formalin-fixed T. forsythia (105 cells/well). Sera were added in 2-fold serial dilutions, and T. forsythia-specific IgG was detected using HRP-conjugated goat anti-mouse IgG (Bethyl Laboratories, Montgomery, TX). Specific serum IgG isotype Ab was detected by addition of biotinylated isotype-specific secondary Ab (rat anti-mouse IgG1 or IgG2a; Southern Biotechnology, Birmingham, AL) followed by streptavidin-conjugated HRP (Southern Biotechnology). ELISA wells were color developed with TMB Microwell enzyme substrate (Kirkgaards and Perry, Gaithersburg, MD). After stopping the enzyme reaction by adding 0.1 N H2SO4, plates were read at 495 nm. The titer was defined as the log2 of the highest dilution with a signal that was 0.1 OD units above background.

Isolation of dendritic cells and macrophages and stimulation

Bone marrow-derived dendritic cells (BMDCs) were generated as described (41). Briefly, femurs and tibias were flushed with PBS. Erythrocytes were lysed using a-M_FORCE buffer (R&D Systems, Minneapolis, MN), and cells were suspended in RPMI 1640 supplemented with 10% FBS, 1% antibiotic mixture (penicillin (50 U/ml), streptomycin (50 μg/ml), l-glutamine (2 mM), 2-ME (50 μM), sodium pyruvate (1 mM), sodium bicarbonate (1.5 mg/ml), and HEPES (25 mM)). BMDCs were cultured in 24-well plates at 105 cells/ml/well. GM-CSF (R&D Systems) was added at 20 ng/ml. Media + GM-CSF were replaced on days 2, 4, and 6. Cells were restimulated on day 7. This protocol yielded BMDCs with dendritic morphology and >85% purity by CD11c staining. Peritoneal macrophages were prepared as described (42).

Cytokine ELISA

Cytokines were measured in triplicate with ELISA kits from eBiosciences. Detection limits were 15 pg/ml for IFN-γ, 4 pg/ml for IL-10, 10 pg/ml for IL-12 p40, and 8 pg/ml for IL-17.

Intracellular staining and FACS analysis

The following Abs and isotype controls were purchased from eBioscences: PE-conjugated anti–IFN-γ and rat IgG2a; FITC-conjugated anti–IL-4; PE-Cy5-labeled anti–CD4; anti-CD80 (16-10A1) and anti-CD83 (Michel-17); rat IgG1, IgG2a, and IgG2b; PE-conjugated anti–CD11c (N418); hamster IgG; PE-conjugated anti–IL-17A (eBio17B7); and rat IgG2a. For intracellular staining, cell suspensions from cervical lymph nodes (cLNs) were stimulated with anti-CD3 and anti-CD28 Abs (eBioscience) for 48 h, followed by FMA (50 ng/ml), ionomycin (1 μg/ml), and brefeldin A (10 μg/ml) for 6 h. Cells were washed, incubated with FcR block (1 μg/ml), and stained for CD4. Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin (Sigma), and stained for IL-4, IL-17, or IFN-γ. Cells were analyzed on a FACSCalibur (BD Biosciences) with FCS Express software (DeNovo Software).

Histological staining

Murine maxillary and mandibular bones (n = 4) were fixed in 10% phosphate-buffered formalin and decalcified in 10% EDTA. Samples were embedded in paraffin, and sections at 4 μm were prepared and stained for tartrate-resistant acid phosphatase (TRAP; Sigma). Slides were digitally scanned with a ScanScope CS system (Aperio) to minimize color fading and viewed with ImageScope viewing software (Aperio). The right maxillary and mandibular interdental areas (average of 10 higher-power fields/slide) of the crestal alveolar bone from the first molar to third molar were used to quantify osteoclasts.

CD45 staining

The right and left halves of the maxillary and mandibular bones were removed, fixed, and embedded in paraffin, and 4-μm sections were cut and mounted. They were deparaffinized in xylene and hydrated in graded ethanol. After Ag retrieval by incubating at 90°C for 10 min with BD Retrievagen A (BD PharMingen), specimens were sequentially incubated in blocking solution containing 0.1% Triton-X-100 in 0.1 M PBS for 1 h at room temperature and then monoclonal rat anti-mouse CD45 (BD PharMingen) (1:30 dilution in PBS containing 0.1% Triton-X-100) for 1 h at room temperature. The slides were incubated with a biotinylated goat anti-rat secondary Ab, followed by an avidin–biotin complex developed with 3,3-diaminobenzidine (Vector Labs, Burlingame, CA); the counterstain was hematoxylin. After each step, slides were rinsed in PBST (three times for 10 min). Slides were analyzed with a ScanScope CS system and viewed with ImageScope viewing software (both from Aperio). The interdental areas from the first to third molar were used to quantify inflammatory cells.

Data analysis

Data were analyzed on GraphPad Prism software (GraphPad). Comparisons between groups were made using a Student t test (between two groups) or
ANOVA (multiple group comparisons), as appropriate. Statistical significance was defined as \( p < 0.05 \).

**Results**

*T. forsythia*-induced cytokine expression in APCs is dependent on TLR2

*T. forsythia* and its major virulence protein BspA induce cytokine expression in epithelial cells through activation of TLR2 (33, 34). To determine the contribution of TLR2 in APCs in the recognition of and response to *T. forsythia*, BMDCs and macrophages were challenged with *T. forsythia*, and cytokines were assayed by ELISA. IL-6, a bone-resorptive cytokine, contributes to alveolar bone loss in the context of *P. gingivalis* infection (11), and IL-10–deficient mice are increasingly susceptible to *P. gingivalis*-induced bone loss (43). IL12p70, IL-12p40, and IL-23p19 were assayed as T effector-polarizing cytokines. BMDCs and peritoneal macrophages were purified by negative selection and confirmed to be 90–95% pure by staining for CD11c, MHC class II, and CD86 (BMDCs) or CD11b (macrophages) by flow cytometry (data not shown). Recombinant *Escherichia coli* LPS (eLPS) was used as a TLR4 agonist. *T. forsythia* whole cells induced significantly greater amounts of IL-6 and IL-10 in WT (BALB/c) BMDCs and macrophages compared with TLR2\(^{-/-}\) cells (Fig. 1A). The reduced secretion of IL-6 and IL-12p40 in TLR2\(^{-/-}\) BMDCs was quite intriguing, considering that eLPS is a TLR4 agonist. Future investigations ongoing in our group will address this issue in detail. As expected, BspA and Pam\(_{3}CSK_{4}\) induced low levels of cytokine secretion in TLR2\(^{-/-}\) cells, whereas eLPS induced potent production of these cytokines (Fig. 1A). Interestingly, only BMDCs secreted IL-12p40 and IL-12p70 in response to *T. forsythia* challenge, which was largely independent of TLR2 (Fig. 1B). These results suggested that *T. forsythia* induces differential responses in APCs with respect to the Th1 cytokine IL-12. This also suggested that other surface receptors or intracellular pattern-recognition receptors (e.g., TLR9 or nucleotide-binding oligomerization domain-like receptors) might be involved after the bacteria are taken up by dendritic cells (DCs). In contrast, the Th17-related cytokine IL-23p19 was not detected in supernatants from WT or TLR2\(^{-/-}\) DCs or macrophages stimulated with *T. forsythia* (Fig. 1B).

**TLR2 deficiency attenuates *T. forsythia*-induced alveolar bone loss**

We previously reported that *T. forsythia* induces alveolar bone loss in BALB/c mice, which is dependent on BspA (9). As a starting point in this study, we confirmed that both purified BspA or intact bacteria activate BMDCs and macrophages through TLR2 (Fig. 1).

To determine the contribution of TLR2-mediated responses to alveolar bone loss, we evaluated the susceptibility of TLR2\(^{-/-}\) mice to *T. forsythia*. In this model, mice are pretreated with antibiotics to reduce normal flora and then subjected to infection with *T. forsythia* by oral gavage (9). Analysis of 16S rDNA by PCR indicated that all WT and TLR2\(^{-/-}\) (\( n = 6 \)) mice infected with *T. forsythia* were positive for a 620-bp *T. forsythia*–specific 16s rDNA, as well as 1.4-kb universal eubacteria-specific 16s rDNA PCR products, verifying the presence of *T. forsythia* and common resident bacteria in the oral flora (Supplemental Fig. 1). As expected, sham-infected mice (WT and TLR2\(^{-/-}\)) were positive for the 1.4-kb universal 16s product but not the 620-bp product. Currently, reliable methods to quantify periodontal bacteria from the mouse oral cavity are unavailable (44), and estimating bacteria by oral swabbing is not thought to reflect the...
bacterial load. Periodontal bacteria, and particularly *T. forsythia*, invade buccal and gingival epithelial cells and form biofilms (45–47), thereby avoiding detection. As another confirmation of infection, *T. forsythia*-specific serum IgG titers in *T. forsythia*-infected mice were increased over sham-infected mice. The net Ab response, defined as the titer of sham-infected mice subtracted from *T. forsythia*-infected mice, increased in both WT and *TLR2*−/− mice (Fig. 2A). Although there was a low titer, even in sham-infected mice, these Abs presumably represent cross-reactive nonspecific Abs against normal resident bacteria. TLR2-deficient mice elicited lower levels of *T. forsythia*-specific Abs compared with WT mice following infection (Fig. 2A). Thus, both WT and *TLR2*−/− mice were productively infected with *T. forsythia*. Conceivably, TLR2 deficiency directly impairs Ab production against *T. forsythia* by affecting cellular pathways associated with Ab maturation. Alternatively, this occurs indirectly by reducing *T. forsythia* proliferation and survival, resulting in suboptimal humoral responses. In support of the latter, it was suggested that *P. gingivalis* survival is impaired in TLR2−/− mice (48). The lack of suitable methods to quantify bacteria precluded us from determining whether TLR2 deficiency indeed causes lower *T. forsythia* loads.

After 6 wk, alveolar bone loss was measured as the distance between the CEJ and ABC at 14 buccal sites per mouse (horizontal bone loss, see Fig. 2B). As expected, significant alveolar bone loss was observed in *T. forsythia*-infected WT mice compared with controls (Fig. 2B, 2C). Strikingly, TLR2−/− mice infected with *T. forsythia* showed bone loss at fewer sites compared with sham-infected *TLR2*−/− mice. Although the baseline ABC–CEJ distances showed a trend for higher CEJ–ABC distances in TLR2−/− mice compared with WT mice, this did not reach statistical significance. Nevertheless, the average net alveolar bone loss induced by *T. forsythia* (measured as total ABC–CEJ for sham-treated mice subtracted from *T. forsythia*-infected mice) in TLR2−/− mice was significantly lower than was the average net bone loss observed for WT mice (Fig. 2C). These results implied that TLR2 signaling stimulates bone loss following *T. forsythia* infection, which is ameliorated in TLR2−/− mice.

**Stat6 deletion attenuates *T. forsythia*-induced alveolar bone loss**

TLR2 was suggested to stimulate primarily Th2 responses (35–38), although there are exceptions to this finding. To test the hypothesis that Th2 responses are responsible for inflammatory alveolar bone destruction, mice deficient in STAT6 were assessed. Notably, *stat6* gene expression has been linked to alveolar bone loss susceptibility in response to *P. gingivalis* infection, although its role in *T. forsythia* infection has never been evaluated (39). One week after the final infection, all mice tested positive for the 620-bp *T. forsythia* rDNA band by PCR (Supplemental Fig. 2). Similarly, the presence of increased *T. forsythia*-specific IgG titers in sera of animals confirmed that all animals were productively infected.

**FIGURE 2.** TLR2 promotes *T. forsythia*-induced alveolar bone loss. *A*, Oral infection with *T. forsythia* elicits serum IgG response in WT and *TLR2*−/− mice. After 6 wk of infection (sham or *T. forsythia*), sera from WT and *TLR2*−/− mice were analyzed for *T. forsythia*-specific IgG by ELISA. Net Ab response in each group (WT or *TLR2*−/−) was determined by subtracting the Ab titers of sham-infected mice from that of *T. forsythia*-infected mice. Data represent means and standard deviations for each group (n = 8–10). *p* < 0.05, unpaired *t* test. *B* and *C*, TLR2−/− mice exhibit reduced net alveolar bone loss in response to *T. forsythia* infection. WT and *TLR2*−/− mice (n = 8–10) were infected with *T. forsythia* or were sham infected. Alveolar bone destruction was assessed after 6 wk by measuring the distance from the ABC to the CEJ at 14 maxillary buccal sites per mouse (R1–R7, right jaw; L1–L7, left jaw). *B*, Representative maxillary phenotypes of male BALB/c and *TLR2*−/− mice. Maxillary jaws were stained with methylene blue, and images were acquired with a Nikon SMZ 1000 microscope (original magnification ×3). *C*, Average alveolar bone loss at 14 buccal sites for BALB/c and *TLR2*−/− mice. Net bone loss shows ABC–CEJ distance of *T. forsythia*-infected sites minus the mean ABC–CEJ distance of sham-treated sites. Data were analyzed by unpaired *t* test; SDs are shown. *p* < 0.05, **p < 0.01, ***p < 0.001, *T. forsythia* infection versus sham infection.
(Fig. 3A). Moreover, T. forsythia-specific Ab titers in STAT6−/− mice were lower than those in WT mice, indicating that Stat6 deficiency reduces the humoral response to T. forsythia (Fig. 3A). Following infection, average net bone loss in STAT6−/− mice was significantly lower than in WT mice (Fig. 3B, 3C), indicating that STAT6 is essential for alveolar bone destruction caused by T. forsythia. Because STAT6 is important in Th2 differentiation, these data implied a role for Th2 cells in driving alveolar bone loss associated with T. forsythia.

To assess whether Th1- or Th2-biased immune responses were elicited in response to T. forsythia, we evaluated levels of IgG1 and IgG2a isotypes in serum. In mice, IgG2a and IgG1 Ig isotypes are considered to represent Th1 and Th2 phenotypes of immune responses, respectively (17). WT mice produced high titers of both T. forsythia-specific IgG1 and IgG2a isotypes (Fig. 4). In contrast, as expected, STAT6−/− mice with a Th1 bias to T. forsythia infection induced significantly higher titers of IgG2a compared with IgG1 (Fig. 4). TLR2−/− mice produced low levels of each isotype, which were not significantly different (Fig. 4). One might expect to observe increased T. forsythia-specific IgG1 levels in WT mice given the apparent Th2 bias during infection; however, in light of the fact that IL-12 is also secreted following T. forsythia challenge in BMDCs (Fig. 1), it is not surprising that the IgG2a response may develop with time. Thus, the isotypes elicited against T. forsythia seemed somewhat linked to Th responses, and the results further supported data in the field that IgG1 and IgG2a subtypes do not functionally play any role in alveolar bone loss.

**Th2 cell polarization in bone loss-susceptible mice**

To gain insight into the nature of Th cell responses associated with PD loss, we analyzed in vivo-primed Th cells from draining lymph nodes of PD-susceptible (WT) and -resistant (TLR2−/− and STAT6−/−) strains. Mice were infected three times at 48-h intervals with T. forsythia; 72 h after the last infection, cells from cLNs were stimulated with anti-CD3 and -CD28 Abs to induce cytokine production. Cells were treated with PMA-ionomycin, stained for cell surface CD4, and stained intracellularly for IL-5, IFN-γ, and IL-17 to detect Th2, Th1, and Th17 cells, respectively. In WT (susceptible) mice, the number of Th2 cells increased, whereas the number of Th1 and Th17 cells decreased following infection compared with controls (Fig. 5). Conversely, the frequency of Th2 cells was not altered in TLR2−/− or STAT6−/− mice (Fig. 5). Following infection, the number of Th1 cells decreased significantly in TLR2−/− mice but increased in STAT6−/− mice; conversely, Th17 cells remained unchanged in STAT6−/− mice but were reduced significantly in TLR2−/− mice. Because T. forsythia-induced net bone loss is reduced in TLR2−/− and STAT6−/− mice, these results implied a destructive capacity for Th2 responses in T. forsythia-induced PD. In contrast, Th1 and Th17 responses might play protective roles, which would be consistent with previous observations in the context of P. gingivalis-induced bone loss and systemic responses (24) (see also Discussion).

**Susceptibility to alveolar bone loss correlates with osteoclastic activity and lymphocytic infiltration**

To evaluate inflammatory and osteoclastic activity, maxillae bones were stained for CD45+ cells and TRAP (a marker of osteoclasts). CD45 staining was used as a marker for inflammatory cells in the gingival tissues (49, 50), whereas TRAP staining was used as a marker for osteoclastic activity and a measure of bone resorption in the jaw bones due to infection (51, 52). The results indicated scattered CD45+ inflammatory cells in sham-injected WT mice.
Not surprisingly, *T. forsythia* infection was associated with an increased infiltration of CD45+ cells in gingival tissue of WT mice. However, there was no significant increase in CD45+ infiltration in TLR2<sup>−/−</sup> and STAT6<sup>−/−</sup> mice following infection with *T. forsythia*. Standard deviations are shown. Statistically significant differences in *T. forsythia*-infected samples compared with sham-infected samples of the same strain were determined by the unpaired *t* test. ***p < 0.001.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** *T. forsythia* infection induces mixed Th1 and Th2-regulated Abs in WT mice and selectively induces Th1-regulated Abs in STAT6<sup>−/−</sup> mice. Sera from mice described were analyzed for *T. forsythia*-specific IgG subclass (IgG1, IgG2a) Ab levels in WT, TLR2<sup>−/−</sup>, and STAT6<sup>−/−</sup> mice by ELISA following infection with *T. forsythia*. Standard deviations are shown. Statistically significant differences in *T. forsythia*-infected samples compared with sham-infected samples of the same strain were determined by the unpaired *t* test. ***p < 0.001.

**Discussion**

Periodontitis, characterized by alveolar bone destruction of the maxillary jaw, is by far the most common form of bacterially induced inflammation that affects bone. This study shows for the first time, to our knowledge, that *T. forsythia*, a relatively understudied periodontal pathogen, mediates the alveolar bone loss process via the TLR2/Th2 pathway. This is the first detailed analysis of immune responses to *T. forsythia*.

Although initiated by bacteria, the bone pathology in PD is mediated almost entirely by the host response. No single bacterial species has been implicated as the primary pathogen in PD, and all available evidence is consistent with a polymicrobial disease etiology. A bacterial consortium known as the red complex, consisting of *P. gingivalis*, *T. denticola*, and *T. forsythia*, is strongly implicated in the onset and the severity of PD (4). Although there is a plethora of studies on the nature of *P. gingivalis*-associated host response and its role in disease pathology, similar information...
infection versus sham infection.

matory cells were quantified as number of CD45+ following infection of WT, TLR2−/−, and STAT6−/− mice (original magnification ×400). B. Inflammatory cells were quantified as number of CD45+ cells/mm². *p < 0.05, **p < 0.001, T. forsythia infection versus sham infection.

FIGURE 6. Alveolar bone loss correlates with lymphocytic infiltration. A, CD45 staining for inflammatory infiltrate cells in gingival tissue 3 wk following infection of WT, TLR2−/−, and STAT6−/− mice (original magnification ×400). B, Inflammatory cells were quantified as number of CD45+ cells/mm². *p < 0.05, **p < 0.001, T. forsythia infection versus sham infection.

regarding T. denticola or T. forsythia is evidently lacking (5). Previous studies have largely been restricted to P. gingivalis as a model periodontal pathogen. Although informative, such studies are limited to the nuances of this organism.

Although initiated by bacteria, bone loss is mediated by the immune response. A destructive role for T and B cells in PD bone loss is well documented (10–12). In contrast, impaired neutrophil and macrophage recruitment due to deficiencies in P-selectin and ICAM-1 result in increased susceptibility to P. gingivalis-induced alveolar bone loss (53). Thus, although lymphocytes can drive bone loss, a robust innate immune response controls infection and, hence, limits overall bone destruction. Despite the potential bone-destructive capacity of IL-17 and Th17 cells seen in the context of rheumatoid arthritis (54), a deficiency in IL-17 signaling in PD results in increased susceptibility to P. gingivalis-induced alveolar bone loss. It was also demonstrated that TLR2-mediated inflammatory responses are critical to P. gingivalis-induced alveolar bone loss through promoting pathogen survival and burden in the host (48). Taken together, these studies demonstrated that both innate and adaptive arms of immunity play critical roles in PD. However, the contribution of individual T cell responses (i.e., Th1, Th2, or Th17) needs to be explored further in PD (6, 55).

The mechanisms by which T. forsythia induces inflammation and alveolar bone loss are poorly understood. We previously demonstrated that T. forsythia expresses a TLR2-activating molecule, BspA (34), which is required for inducing alveolar bone loss in mice (9). Because both BspA and intact T. forsythia signal through TLR2 (33, 34), the focus of this study was to evaluate the role of TLR2-mediated responses in alveolar bone loss. Moreover, because TLR2-mediated responses were shown to favor Th2 development (31, 35, 36, 38), we predicted that Th2 responses would be elicited and dictate the alveolar bone loss associated with T. forsythia infection. Indeed, our results showed that TLR2 plays a significant role in stimulating DCs and macrophages to elicit cytokine responses via BspA (Fig. 1). DCs or macrophages stimulated with bacteria or BspA did not express IL-23, suggesting that Th17 cells are not induced in response to T. forsythia. Interestingly, DCs, but not macrophages, produced IL-12 (Th1 cytokine) in response to bacteria or BspA. It is possible that engagement of other pathways, such as through C-type lectin receptors, intracellular nucleotide-binding oligomerization domain-like receptors, or TLR9, might be operative in DCs. Indeed, we showed that T. forsythia DNA is a strong inducer of TLR9 (56). Consistently, Mycobacterium tuberculosis induces strong IL-12 expression in DCs through the TLR9 pathway but weak IL-12 and strong IL-10 expression in macrophages through engagement of TLR2 (57). TLR2 activation inhibits IL-12 by dampening the Th1 IFN-γ amplification loop in DCs and promotes induction of Th2 and Th17 responses (38). Furthermore, IL-10, produced primarily via TLR2, inhibits IP-10 and IL-12p35; therefore, it is considered a Th1-suppressing cytokine. Although IL-10 is primarily an inhibitor of Th1, recent studies indicated that it also suppresses Th2 and Th17 cell responses (58). The failure of T. forsythia to induce IL-23 in DCs or macrophages (Fig. 1B) indicated that Th17 responses are likely not induced in this setting, which was confirmed by our findings in T. forsythia-infected mice (Fig. 5). Also consistent with our hypothesis, the net bone loss caused by T. forsythia in TLR2−/− mice was attenuated. This finding indicated that TLR2 activation plays a destructive role. This finding is similar to what has been observed for P. gingivalis infections, where TLR2−/− mice are resistant to P. gingivalis-induced alveolar bone loss (48). It is likely that T. forsythia, which depends on the availability of host factors, such as peptides, heme, and sialic acid for growth, exploits TLR2-mediated inflammation for its growth and survival.

FIGURE 7. Alveolar bone loss correlates with osteoclastic activity. A. Representative histological sections showing TRAP+ cells (arrows) from T. forsythia- and sham-infected WT, TLR2−/−, and STAT6−/− mice (original magnification ×400). B. Average number of TRAP+ cells in 10 high-power magnification fields/slide (n = 4 mice/group). *p < 0.05, **p < 0.001, T. forsythia infection versus sham infection.
To explore the possibility of polarization of Th2 responses downstream of TLR2, STAT6−/− mice were assessed. In STAT6−/− mice T. forsythia-induced alveolar bone loss was significantly reduced (Fig. 3). Consistent with our findings, the stat6 gene was shown to be associated with increased susceptibility in P. gingivalis-induced PD (39). We observed increased osteoclastic activity following infection only in alveolar bone loss-susceptible WT mice. Consistently, WT mice presented increased inflammatory cells in the connective tissue surrounding alveolar bone following infection, which correlated with orthoclastic activity. These results strongly suggested that the TLR2–Th2 inflammatory axis plays a significant role in T. forsythia-induced alveolar bone loss.

Our observations suggested that STAT6 does not play a bone-protective role during T. forsythia-induced alveolar bone loss. However, STAT6 is considered important for dampening the activity of osteoclasts via IL-4 and IL-13 in vitro (59, 60), so other mechanisms are likely to be involved whereby Th2 mediates bone loss. In that respect, transgenic expression of IL-4 in mice was shown to suppress osteoclast activity, leading to general osteoporosis (61). Thus, it is possible that Th2 cytokines suppress local osteoclast activity in the jaw. Another possibility is that this occurs via increased levels of RANKL expression, driving osteoclast differentiation. However, RANKL expression is thought to be limited mainly to Th1 (62) and Th17 (63) cells, although this may vary depending on the infection stimulus, and it was not assessed in our study. Alternatively, it is tempting to speculate that Th2 responses induce RANKL-mediated osteoclastogenesis through effects on B cells, which also express RANKL, and can drive osteoclastogenesis in inflamed periodontium (64, 65).

In summary, we presented evidence for the roles of TLR2 signaling and Th2 differentiation in mediating T. forsythia-induced alveolar bone destruction. We showed that T. forsythia-induced TLR2 activation resulted in alveolar bone destruction. Furthermore, Th2 development downstream of TLR2 activation was associated with alveolar bone destruction caused by T. forsythia.

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