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Effects of Toll-like receptor 4 on *Porphyromonas gingivalis*-induced bone loss in mice

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Background and Objective: Toll-like receptor 4 (TLR-4)/myeloid differentiation protein-2 complex ligation by lipopolysaccharide induces production of pro-inflammatory cytokines and co-stimulatory molecules on antigen presenting cells. The aim of this study was to determine the role of the TLR-4 in bone loss-resistant C57BL mice and in bone loss-susceptible BALB/c mice after infection with *Porphyromonas gingivalis*.

Material and Methods: The BALB/c and C57BL/10 mice, either normal or TLR-4 deficient, were infected or sham-infected orally four times, at 4 day intervals, with 10^9 colony forming units of *P. gingivalis*. At 47 days, defleshed jaws were stained and photographed in a standardized position. We measured the surface area of the root trunk to assess the alveolar bone loss.

Results: Porphyromonas gingivalis-infected wild-type BALB/c mice lost 13.8% more bone than *P. gingivalis*-infected wild-type C57BL/10 mice. In contrast, *P. gingivalis*-infected TLR-4-deficient C57BL/10 mice lost 12.7% more bone than *P. gingivalis*-infected TLR-4-deficient BALB/c mice. *Porphyromonas gingivalis*-infected wild-type C57BL/6 and TLR-2 knockout C57BL/6 mice had similar bone levels to sham-infected control mice.

Conclusion: Toll-like receptor 4 is protective for C57BL/10 but detrimental to BALB/c mice, since its absence allowed C57BL/10 but not BALB/c mice to lose alveolar bone. Toll-like receptor 2 does not contribute to this protection in genetically similar C57BL/6 mice.

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Periodontitis is a group of infectious diseases, associated with various bacterial species, causing chronic inflammation of the soft tissue surrounding the teeth. Loss of connective tissue attachment, pocket formation and destruction of alveolar bone lead to tooth mobility and tooth loss (1). Diseased sites show elevated proportions of *Porphyromonas gingivalis*, *Tannerella forsythensis*, species of *Prevotella*, *Fusobacterium*, *Campylobacter* and *Treponema*, and other uncultivable species (2).

Risk for disease also depends on the host's response to the bacterial challenge (3). The host recognizes invading bacteria primarily via Toll-like receptors (TLRs), among other pattern recognition receptors (PRRs). Toll-like receptors are a conserved family of membrane proteins that recognize diverse microbial molecules produced by bacteria, viruses, fungi and protozoa (4). The Toll-like receptor 4 (TLR-4)/myeloid differentiation protein-2 complex, for example, recognizes lipopolysaccharide (LPS), lipoproteins and lipopeptides on gram-negative bacteria (5). *Porphyromonas gingivalis* LPS signals through TLR-2 and TLR-4 receptors to activate host cells (6). In *P. gingivalis*, variations in the lipid A structure of LPS and utilization of multiple TLRs may contribute to its potential to remain a persistent

colonizer of the oral cavity (7), as well as an inducer of an inflammatory response after crossing the epithelial barrier. Toll-like receptor 4 binding ultimately induces the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules, CD80 and CD86, on antigen presenting cells (APC). Thus, TLR binding initiates the innate phase of the immune response, with the production of inflammatory cytokines followed by activation of the humoral and cell-mediated adaptive immune responses (5).

In humans, the cytokine response to microbial challenges and susceptibility to periodontitis can be explained in part by the host's genetic make-up (8,9). As in humans, the genotype of inbred animals also contributes to the patterns of cytokine released after microbial challenge, and the susceptibility to microbial infections reflects such variability (10). For example, microbial challenges tend to elicit a T-helper 2 (Th2) response in BALB/c and a T-helper 1 (Th1) response in C57BL/6 mice (11). In a *P. gingivalis*-induced periodontitis model, BALB/c mice develop more alveolar bone loss than C57BL/6 mice (12). This dichotomy in the mouse model is important, since a gingival Th1 response in humans appears to protect against periodontitis while a Th2 response seems to be ineffective in controlling the microbial challenge and preventing disease progression (13). Since TLRs are among the first molecules to interact with the challenging microbe, we hypothesized that the TLR-4 pathway confers either disease susceptibility or resistance to infection by *P. gingivalis*.

Material and methods

Bacteria

Porphyromonas gingivalis strain ATCC 53977 (A7A1-28), obtained from the culture collection of Dr Pamela Baker at Bates College, ME, USA, was maintained frozen in skim milk at −80°C and grown anaerobically in 5% CO_2–10% H_2–85% N_2 in Todd Hewitt Base (THB) broth or on blood agar plates, both supplemented with 5.0 μg/mL hemin, 0.5 μg/mL menadione, at 37°C for 7 days. The number of colony forming units (c.f.u.) in a single *P. gingivalis* colony grown on supplemented blood agar was determined from a standard growth curve, estimated by change in optical density at 650 nm.

The control bacterium, *Lactobacillus murinus* (14), was transformed with plasmid pIL252 containing an erythromycin resistance gene and grown anaerobically in 5% CO_2,–10% H_2,–85% N_2 on deMan, Rogosa and Sharpe (MRS) broth or agar plates supplemented with 5 μg/mL erythromycin (ERM) and 100 μg/mL lincomycin (LIN). The number of colony forming units in a single *L. murinus* colony grown on ERM/LIN-supplemented MRS agar was determined from a standard growth curve, estimated by change in optical density at 560 nm.

Animals

The BALB/c background mouse strains (H-2^d haplotype) included wild-type BALB/cJ and TLR-4-deficient C.C3-Tlr4 < Lps-d > . The C57BL background mouse strains (H-2^b haplotype) included wild-type C57BL/6J, wild-type C57BL/10J, TLR-4-deficient C57BL/10ScNJ (Jackson Laboratory, Bar Harbor, ME, USA) and TLR-2 knockout C57BL/6 mice (15; Japan Science and Technology Corp., Osaka, Japan). Resistance to bone loss in C57BL/10J mice after *P. gingivalis* infection was confirmed when compared with the previously reported historical control C57BL/6J mice (12). When infected with bacteria, mice were maintained in biosafety level 2 containment in an Association for Assessment and Accreditation of Laboratory Animal Care-approved specific-pathogen-free facility. Animals were age- and sex-matched and were 8–12 weeks old at the start of experiments.

Oral infection

As described elsewhere (16), mice were given 20 μg/mL sulphamethoxazole-trimethoprim (Hi-Tech Pharmacal Co., Inc., Amityville, NY, USA) in deionized water *ad libitum* for 10 days, followed by 4 days without antibiotics. Each group of 15 mice was infected or sham-infected by oral gavage four times, at 4 day intervals, with 10^9 c.f.u. of live *P. gingivalis* or *L. murinus* in 100 μL of phosphate-buffered saline (PBS) with 2% carboxymethylcellulose. *Porphyromonas gingivalis* infection was tested by sampling the oral cavity of mice before the first gavage with *P. gingivalis* or *L. murinus* and 47 days later. Two sterile paper points was inserted along the buccal marginal gingiva of each mouse for 5 s and placed in 1 mL pre-reduced supplemented THB broth. After gentle vortexing, 100 μL of bacterial suspension was plated on pre-reduced supplemented THB blood agar. Plates were incubated anaerobically for 14 days. Among total colony forming units cultured in anaerobic conditions, small black-pigmented colonies were identified, subcloned and gram stained. *Porphyromonas gingivalis* were identified as gram-negative, 1–5 μm coccobacilli when harvested from subcultured colonies emanating the characteristic methyl mercaptan odour. Mice inoculated with *P. gingivalis* but not successfully infected on day 47 or control mice harbouring any bacteria growing as black pigmented colony forming units were excluded from the study. The efficiency of infection was consistently between 86 and 93% in wild-type or TLR-4-deficient BALB/c, C57BL/10 or C57BL/6 mice.

Assessment of alveolar bone loss

At day 47, mice were killed by CO_2 inhalation. Mandibles were dissected, boiled in distilled water for 10 min, defleshed, placed in 1 N NaOH for 3 h to remove the remaining keratinized gingiva, and stained with 1% methylene blue for 1 min. Left and right jaws were mounted, lingual side up, on a pliable mold (Plumber's Putty, W. H. Harvey Co., Omaha, NE, USA) and photographed at ×40 magnification under a stereomicroscope (Nikon SMZ800) equipped with a digital camera (DC290, Kodak). In the main study, to minimize image distortion related to sample–objective angle, the lingual view of the jaws was standard-

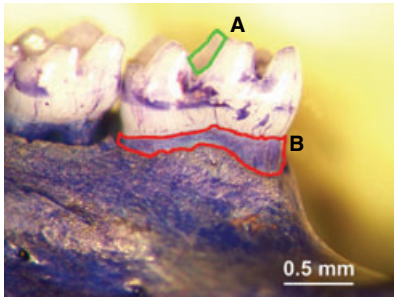

Fig. 1. Standardized photograph of lingual surface of mandibular first molar. The coronal outline (perimeter A) defines the standardizing 10 000 pixel areas, and the root trunk outline (perimeter B) is an indirect measure of crestal alveolar bone level.

ized so that the projected area between the distal edge of the buccal middle cusp and the distal edge of the lingual middle cusp was 10 000 ± 500 pixels (Fig. 1, perimeter A). Alveolar bone loss was measured indirectly as the number of pixels in the surface area of the visible root trunk (Fig. 1, perimeter B). This area was measured with the magnetic lasso tool of Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA) by tracing the cementum–enamel junction (CEJ), the distal edge of the visible root, the marginal bony crest and the mesial edge of the visible root. Tooth size was compared among groups by measuring the distance between the mesial and distal CEJ of first molars.

Statistical analysis; validation of alveolar bone loss assessment

We collected eight measurements per mouse: two jaws per mouse; two photos per jaw; and two measurements per photo. The average of the eight measurements was used as the dependent variable in an analysis of variance (ANOVA). Mouse strain, TLR-4 deficiency and infection with *P. gingivalis*, as well as their interactions, were independent variables.

To test whether standardizing jaw position reduces the measurement variability, a pilot study and the main *P. gingivalis* study measurements were compared. The pilot study used six wild-type BALB/cJ and six wild-type C57BL/6J mice, none of which was infected. Four sources of variation in bone level measurements were considered: (1) variation in area of root trunk among mice within a group [σ^2_{mouse}]; (2) variation between jaws of the same mouse [σ^2_{jaw}]; (3) inconsistent positioning of the jaws, altering the photographic image [σ^2_{photo}]; or (4) inconsistent measurements from a given photograph [σ^2_{rep}]. The contributions of each variance component were analysed using a Bayesian mixed linear model and comparing the variance components from the pilot and main studies.

Results

Components of variation of crestal alveolar bone loss measures

The proportion of total variance contributed by the four sources of variation, i.e. σ^2_{mouse}, σ^2_{jaw}, σ^2_{photo} and σ^2_{rep}, is shown in Table 1. In the pilot study, variations between photos attributed to the inconsistent positioning of the jaws (σ^2_{photo}) were the largest source of variation, at 45.3%. Owing to the standardization of the jaw position in the main study, σ^2_{photo} was significantly ($p > 0.99$) smaller, accounting for only 19.5% of the variation (Table 1). Mouse-to-mouse variation (σ^2_{group}) was the largest source of variation in the *P. gingivalis* main study, since this factor included infected and sham-infected mice.

Porphyromonas gingivalis infection induces bone loss in wild-type BALB/cJ but not in C57BL/10J or C57BL/6 mice

At 47 days after initial inoculation, gram-negative, 1–5 μm coccobacilli growing on hemin menadione THB blood agar were determined to be *P. gingivalis*. Bacterial colonies and cells with these characteristics were absent in all mice prior to oral inoculation with *P. gingivalis* and in sham-infected control mice at the end of the experiment.

Wild-type BALB/cJ mice infected with *P. gingivalis* showed 17.1% more bone loss compared with sham-infected control animals ($p = 0.0001$). Infection of wild-type C57BL/10J or C57BL/6 mice with *P. gingivalis* caused only 3.3 or 4% more bone loss, respectively, than in sham-infected animals ($p = 0.44$ and $p = 0.18$, respectively, Figs 2A and 4). *Porphyromonas gingivalis* induced 13.8% more bone loss in BALB/cJ than in C57BL/10J mice after correcting for tooth size in the two strains ($p = 0.03$; Fig. 3, open bars). Infection with the commensal microorganism, *L. murinus*, did not induce alveolar bone loss in either wild-type BALB/cJ ($p > 0.05$) or C57BL10J mice ($p > 0.05$).

Toll-like receptor 4 protects C57BL/10J but not BALB/cJ mice from alveolar bone loss after *P. gingivalis* infection

The BALB/c [TLR-4 ^{deficient}] infected mice showed similar bone loss to sham-infected control animals ($p = 0.38$;

Table 1. Components of variation in the pilot study procedure and the main study standardized procedure

Components of variation	Proportion of variance (%)		Estimate of SD (units of 1000 pixels)		Ratio of SD main to SD pilot	Probability pilot SD > main SD
	Pilot	Main	Pilot	Main		
Treatment/strains (σ^2_{mouse})	15	49	3.5	6.9	1.97	0.11
Jaw (σ^2_{jaw})	26	27	5.1	5.1	0.99	0.51
Photo (σ^2_{photo})	45	19	6.8	4.3	0.63	> 0.99
Repeated measurements (σ^2_{rep})	14	5	3.8	2.2	0.57	> 0.99

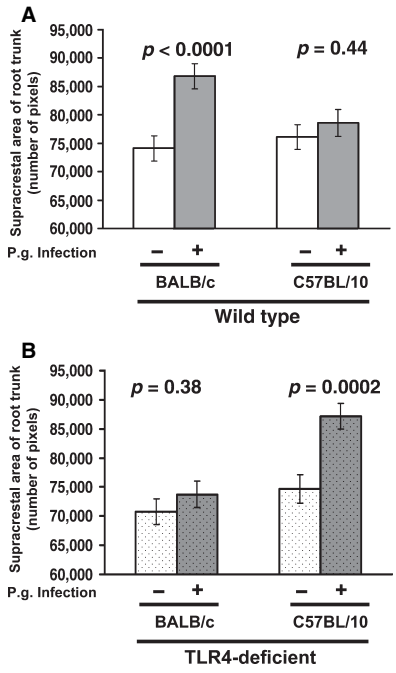

Fig. 2. Mean ± SD for root trunk surface area detected in BALB/cJ and C57BL/10J mice (A) and in TLR-4-deficient strains BALB/c or C57BL (B) at 47 days after infection or sham-infection with *P. gingivalis* (P.g.). Values of *p* were generated by *post hoc* tests in the context of ANOVA.

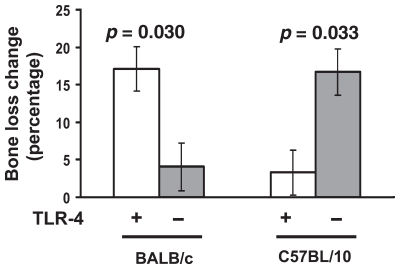

Fig. 3. Alveolar bone level changes observed in the wild-type BALB/cJ and C57BL10J strains (open bars) and in the TLR-4-deficient strains (grey bars) infected with *P. gingivalis*. Values of *p* are from *post hoc* tests in the context of ANOVA. Test for three-way interaction identifies significant opposite effects of TLR-4 in the two strains (ANOVA, $p = 0.0026$).

Fig. 2B). However, C57BL/10 [TLR-4^{deficient}] mice infected with *P. gingivalis* showed 16.7% more alveolar bone loss than sham-infected control animals ($p = 0.0002$; Fig. 2B). Alveolar bone loss in *P. gingivalis*-infected wild-type BALB/cJ mice was significantly greater than in BALB/c [TLR-4^{deficient}] mice ($p = 0.03$; Fig. 3). Alternatively, in *P. gingivalis*-infected wild-type C57BL/10J the alveolar bone loss was significantly lower than in C57BL/10 [TLR-4^{deficient}] mice ($p = 0.033$) (Fig. 3). In addition, C57BL/10 [TLR-4^{deficient}] mice lost 12.7% more bone than BALB/c [TLR-4^{deficient}] mice after correcting for tooth size ($p = 0.035$). The effect of the TLR-4 deficiency was significantly different between strains and in opposite directions ($p = 0.0026$ for three-way interaction in ANOVA).

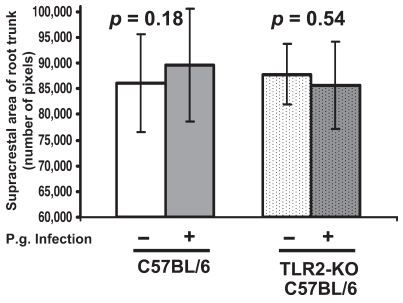

Fig. 4. Mean ± SD root trunk surface area detected in C57BL/6J and TLR-2 knockout (TLR2-KO) C57BL/6J mice 47 days after infection or sham-infection with *P. gingivalis* (P.g.). Values of *p* were generated by *post hoc* tests in the context of ANOVA.

Toll-like receptor 2 does not contribute or protect from bone loss in C57BL/6J

As expected, wild-type C57BL/6J mice showed resistance to bone loss because *P. gingivalis*-infected and sham-infected mice had similar bone levels 47 days after the initial *P. gingivalis* inoculum ($p = 0.18$). Likewise, *P. gingivalis*-infected and sham-infected TLR-2 knockout C57BL/6J mice showed similar bone levels at day 47 ($p = 0.54$; Fig. 4). The bone levels in wild-type and TLR-2 knockout C57BL/6J mice, either infected or sham-infected, where essentially similar ($p = 0.51$ for three-way interaction in ANOVA).

Discussion

We developed a reliable technique that measures bone loss around murine teeth and compared bone loss in two different strains of mice, BALB/cJ and C57BL/10J, after infection either with a putative periodontal pathogen, *P. gingivalis*, or with a murine commensal microorganism, *L. murinus*. As previously reported (12), we confirmed that BALB/cJ mice are susceptible to bone loss after infection with *P. gingivalis*. In contrast, when C57BL/10J mice are infected with *P. gingivalis*, bone loss does not differ from the sham-infected control animals. Ultimately, *P. gingivalis*-infected BALB/cJ mice show 13.8% more bone loss than *P. gingivalis*-infected C57BL/10J mice, which is again consistent with previous work in the C57BL/6J strain (12). Susceptibility to bone loss in the two strains is hypothesized to be mediated by the phenotype of T-helper cells, since ablation of $CD4^+$ T cells abrogates susceptibility to bone loss in BALB/c mice (17).

Naive T cells differentiate into two distinct phenotypes, Th1 and Th2, which have distinct cytokine profiles (18). The C57BL strain generates a dominant cell-mediated response to infections with interleukin (IL)-2, interferon-γ and tumour necrosis factor-α production (Th1), whereas the BALB/c strain develops a dominant antibody response mediated by IL-4, IL-10 and IL-13 (Th2; 11,19). Our results are consistent with data in C57BL mice showing that B cells activated in the presence of Th1 cytokines (interferon-γ) inhibit osteoclastogenesis by acting directly on osteoclasts or indirectly through B cells (20).

Our investigation also tested the role of TLR-4 in the pathogenesis of alveolar bone loss. We demonstrated that C57BL mice lacking TLR-4 became susceptible to bone loss, just like wild-type BALB/c mice, indicating that TLR-4 signalling is protective in wild-type C57BL mice. In contrast, BALB/c mice lacking TLR-4 lose less bone than wild-type BALB/c mice, indicating that TLR-4 signalling is detrimental to the BALB/c strain, allowing progression of bone loss after *P. gingivalis* infection. In essence, our findings suggest, surprisingly, that TLR-4 signalling has significantly opposite effects in BALB/c and C57BL/10 mice.

The propensity of normal C57BL mice to develop a Th1 response (11) is important in the inhibition of osteoclastogenesis, since interferon-γ accelerates degradation of the adaptor molecule, tumour necrosis factor receptor associated factor 6 (TRAF6), in osteoclast precursors (21). The adaptor molecule TRAF6 is a central player of a cascade initiated by receptor-activator of nuclear factor-κB (RANK) ligation. The RANK receptor ligation drives differentiation of osteoclast progenitors (reviewed in reference 22). Therefore, reducing transduction efficiency of the RANK pathway by interferon-γ via accelerated TRAF6 degradation decreases overall osteoclastic activity, with ultimate inhibition of bone destruction. In our model, the propensity of C57BL mice to release interferon-γ after microbial challenge (11) supports its protective role and ultimately the resistance to bone loss. This speculation is plausible, since TLR-4 signalling in antigen presenting cells drives naive T cells to a Th1 phenotype (22,23). Most probably, in our model, the lipid A moieties of *P. gingivalis* LPS do signal through both TLR-2 and TLR-4 receptors (6). The TLR-2 and TLR-4 bind a shared intracellular adaptor molecule named myeloid differentiation primary-response protein 88 (MyD88). Toll-like receptor 4 can also transduce LPS binding through a MyD88-independent pathway, which ultimately induces interferon-β expression (24). Like interferon-γ, interferon-β is also a negative regulator of osteoclast differentiation (25). Therefore, from the current literature we can infer that in the C57BL mouse that is resistant to bone loss and prone to interferon-γ production (11), the absence of TLR-4 signalling presumably decreases the production of not only interferon-γ (22,23) but also interferon-β (24). It is mechanistically plausible that in C57BL mice lacking TLR-4, low levels of these two negative regulators of osteoclastogenesis tilt the balance towards osteoclast differentiation. Moreover, in C57BL mice, the protective effect of TLR-4 as a transducer of LPS binding is strengthened by the finding that the gram-positive *L. murinus* had no effects on alveolar bone loss of either C57BL or BALB/c wild-type mice.

In BALB/c mice, however, the bone-destructive role of TLR-4 is consistent with the preferential Th2 response initiated by TLR-4 ligation, and with the delayed and reduced expression of Th1-related message (10). In BALB/c mice lacking MyD88, the TLR-4 ligation is transduced independently of MyD88 by the Toll/interleukin-1 receptor-domain containing adaptor inducing interferon/Trif-related adapter molecule pathway. This alternative pathway promotes a Th2 rather than Th1 response. In BALB/c mice, this finding is important because it explains why, without TLR-4, the MyD88-independent Th2 response is abrogated (26). It is therefore plausible to correlate the destructive role of TLR-4 in our BALB/c mice to induction of an ineffective Th2 response preferentially mediated by the MyD88-independent pathway.

Lipid A moieties from *P. gingivalis* LPS signal TLR-4 as well as TLR-2 (6). Toll-like receptor 2 plays a major role in the expression of IL-6 signal in peritoneal macrophages (27) or inducing chemokine CXCL8 production in human gingival epithelial cells after *P. gingivalis* infection (28). Despite these significant effects, in our model of oral infection with *P. gingivalis*, TLR-2 does not appear to contribute to the resistance against alveolar bone loss, because TLR-2 absence in disease-resistant C57BL/6 mice did not have a substantial effect on alveolar bone levels after *P. gingivalis* infection. We concluded, therefore, that at the mucosa of the oral cavity, TLR-4 and not TLR-2 is important in protecting C57BL mice from alveolar bone loss.

The role of Th1 and Th2 in periodontal disease is not completely understood. It appears that Th1 and Th2 cytokines and their mRNA are present simultaneously in periodontitis lesions (29–31). Nonetheless, deep periodontal pockets have walls infiltrated primarily with a Th2-induced cellularity with a high proportion of B cells and plasma cells (32–34). Not surprisingly, high levels of IL-4 and IL-6 protein are detected in such periodontitis lesions (35,36). In contrast, healthy/gingivitis sites show primarily a T cell infiltrate, very low numbers of tartrate-resistant acid phosphatase $(TRAP)^+$ $CD163^+$ macrophages (37) and a prevalence of Th1 proteins and mRNAs (38,39). This pattern of cytokine expression and macrophage distribution/activation in gingivitis sites supports the hypothesis that a Th1 response prevents bone loss by inhibiting osteoclastogenesis. In periodontitis lesions, given the infrequent bursts of disease activity and the limited data on the cytokine pattern before an episode of attachment loss (34), it is reasonable to expect a preponderance of Th1 cytokines coexisting with Th2 cytokines. A plausible hypotheis is that failure of the interferon-γ-mediated protective response allows maturation of preosteoclasts. Low interferon-γ may allow a Th2 response that promotes the generation of osteoclasts and B cell differentiation into plasma cells (37), ultimately unable to control the pocket biofilm.

To conclude, we demonstrated that that TLR-4 protects Th1-prone C57BL mice from alveolar bone loss induced by *P. gingivalis*. In C57BL mice, this protective role seems confined to TLR-4 and not attributable to TLR-2. In contrast, in Th2-prone BALB/c mice, TLR-4 does contribute to the susceptibility to alveolar bone loss induced by *P. gingivalis*.

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