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The interleukin-10 knockout mouse is highly susceptible to *Porphyromonas gingivalis*-induced alveolar bone loss

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Objective: Interleukin-10 is an anti-inflammatory cytokine that reduces periapical bone loss, but its role in periodontal bone loss is unclear. In the present study, we tested the hypothesis that endogenous interleukin-10 is a potent suppressor of *Porphyromonas gingivalis*-induced alveolar bone loss *in vivo*.

Methods: Interleukin-10 knockout (–/–) and wild-type mice were inoculated intraorally with *P. gingivalis*. Non-infected animals served as negative controls. Alveolar bone loss, gingival cytokine levels, and gingival gene expression were assessed using morphometric analysis, enzyme-linked immunosorbent assay (ELISA), and semiquantitative reverse transcription polymerase chain reaction (RT–PCR), respectively.

Results: P. gingivalis-infected interleukin-$10^{-/-}$ mice exhibited severe alveolar bone loss compared to non-infected interleukin-$10^{-/-}$ and wild-type mice by day 42. Surprisingly, bone resorptive cytokines interleukin-1α and tumor necrosis factor alpha (TNF-α) were not up-regulated in gingival tissues by *P. gingivalis*-infection. Although interleukin-1β was marginally increased, blockade of both interleukin-1 isoforms or interleukin-1 receptor type I with neutralizing antisera failed to reduce alveolar bone loss in interleukin-$10^{-/-}$ mice, indicating the operation of an interleukin-1-independent mechanism. No strong correlations between bone loss and other cytokines was observed, although interferon gamma (IFNγ), interleukin-6, interleukin-4, and prostaglandin E_2 were modestly up-regulated in infected interleukin-$10^{-/-}$ mice. *P. gingivalis* infection reduced the expression of cell markers in gingival tissue on days 7 and 14 in both interleukin-$10^{-/-}$ and wild-type animals, suggestive of bacteria-induced cytotoxicity or apoptosis. This was followed by up-regulated expression of receptor activator of nuclear factor kappa B ligand (RANKL) and CD40 ligand (CD40L on days 28 and 70 in infected interleukin-$10^{-/-}$ mice only.

Conclusion: The interleukin-$10^{-/-}$ mouse is highly susceptible to bone loss induced by the periodontal pathogen *P. gingivalis*, which is mediated via an interleukin-1-independent pathway.

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Periodontal disease is a chronic inflammatory condition caused by bacterial infection of the teeth and supporting tissues. This disease results in destruction of connective tissue and alveolar bone, and is a major cause of tooth loss. *Porphyromonas gingivalis* is a Gram-negative anaerobe that has been strongly implicated in the etiology of adult forms of periodontal disease, primarily in ecological studies in humans (1). Nevertheless, as *P. gingivalis* is frequently detected in periodontally healthy people, its colonization of the oral cavity is necessary, but not sufficient, to induce periodontal disease (2). This finding suggests that host factor(s) are also important in the induction and progression of periodontal diseases.

A number of pro-inflammatory cytokines, including interleukin-1, tumor necrosis factor (TNF), interleukin-6, interleukin-11, and interleukin-17 have been reported to stimulate bone resorption (3–8). Of these mediators, interleukin-1 has been implicated as having an important functional role in the bone and tissue destruction in periodontitis. Elevated levels of interleukin-1 were present in tissues from sites of active periodontal destruction (9). Neutralization of interleukin-1 *in vivo* with soluble interleukin-1 receptors but not soluble TNF receptors reduced ligature-induced periodontitis in non-human primates (10, 11). Treatment with interleukin-1 receptor antagonist reduced periapical bone loss (12).

Interleukin-1 production and activity may be modulated by a network of regulatory cytokines. The prototype Th1 cytokine interferon gamma (IFNγ) up-regulates inflammation and interleukin-1 expression (13–15), whereas Th2 cytokines are generally inhibitory. In previous studies from this laboratory, gene knockouts of interleukin-10 and to a lesser extent interleukin-6, but not interleukin-4, resulted in enhanced periapical bone destruction following infection of the dental pulp (16, 17). interleukin-10 is expressed in both healthy and inflamed human periodontal tissues, and is reported to be decreased in patients in which *Actinobacillus actinomycetemcomitans,* the agent associated with localized juvenile periodontitis, was detected (18). These findings suggest that interleukin-10 can be a protective cytokine in periodontal diseases, but this has not been established in an experimental model of periodontitis.

In the present study, we therefore tested the hypothesis that endogenous interleukin-10 is a key inhibitor of periodontal bone loss, using interleukin-10 knockout (–/–) mice and infection with the consensus periodontal pathogen *P. gingivalis*. The utility of the interleukin-$10^{-/-}$ mouse as a new and unique animal model of destructive periodontitis was also explored.

Material and methods

Animals

Interleukin-10 knockout (interleukin-$10^{-/-}$, background: C57BL/10) and wild-type C57BL/10 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were bred in the animal facility of the Forsyth Institute, and all animals were maintained under pathogen-free conditions. Animals were matched for age and gender in all studies.

Infection with *P. gingivalis*

P. gingivalis W83, a pathogenic strain isolated from a case of human periodontitis, was grown on tryptic soy broth agar plates with 5% sheep blood and subsequently in mycoplasma broth under anaerobic conditions (37°C, 80% N_2, 10% H_2, and 10% CO_2). The cells were harvested by centrifugation at 7000 *g* for 15 min and resuspended in prereduced anaerobically sterilized Ringer's solution (PRAS). The final concentration of the organism was determined spectrophotometrically, and adjusted to 10^{10} cells/ml in phosphate-buffered saline containing 2% methylcellulose. Periodontal infection with *P. gingivalis* was carried out following the regimen previously described (19–21). In brief, all animals received antibiotic treatment (Sulfatrim suspension, 20 ml/100 ml of drinking water) for 4 days to reduce the original oral flora, followed by 3 days of an antibiotic-free period, prior to infection with *P. gingivalis* W83. Each animal was infected with 10^9 *P. gingivalis* W83 delivered into the oral cavity and esophagus three times from day 0 at 2-day intervals. Non-infected animals (negative control) were given methylcellulose gavage without *P. gingivalis*.

Interleukin-1 and interleukin-1 receptor type I neutralization

Interleukin-1α, interleukin-1β, and interleukin-1 receptor type I (interleukin-1RI) were neutralized *in vivo* using antibody injections (17, 22). For interleukin-1 neutralization, animals were injected subcutaneously with a mixture of goat anti-mouse interleukin-1α and anti-interleukin-1β antibodies (R & D Systems, Minneapolis, MN, USA; 25 μg each antibody/mouse/time point in sterile phosphate-buffered saline) from day −1 relative to infection, until day 20 at 3-day intervals. This protocol has been shown to effectively block interleukin-1 activity *in vivo*, as determined by the absence of leukocyte priming and the development of disseminating dentoalveolar infections (22). For interleukin-1RI neutralization, a hamster monoclonal anti-mouse interleukin-1RI antibody (the generous gift of Immunex, Seattle, WA, USA; 100 μg/mouse/time point in sterile phosphate-buffered saline) was injected at 2-day intervals, following an initial loading dose (day −1, 0, 1) until day 20 (total of 12 doses). Isotype-matched normal immunoglobulin Gs (IgGs) served as negative controls. This antibody completely blocks interleukin-1α and interleukin-1β binding to interleukin-1RI at the indicated concentrations (H. Youssef and P. Stashenko, unpublished findings).

Sample preparation

Animals were killed by CO_2 inhalation at the indicated times. Mandibles and maxillae were removed and hemisected. One half mandible was used for protein extraction; the other half mandible and maxilla were used as a source of RNA. Gingival tissues were isolated under a surgical microscope, weighed, and kept

at −70°C for further analyses. After gingival tissue isolation, hemisected mandibles were defleshed in a dermestid beetle colony, bleached, and mounted on a microscope slide for bone loss measurements. For protein extraction, gingival tissue was ground using a sterile tissue homogenizer in 1 ml of lysis buffer as previously described (16, 23). The mixture was incubated at 4°C for 1 h, and the supernatant was collected after centrifugation and stored at −70°C until assay. Total gingival RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and genomic DNA contamination was eliminated by DNA-*free*™ (Ambion, Austin, TX, USA) following the manufacturer's protocols.

Bone loss measurements

Images of molar teeth and alveolar bone were captured using digital microscopy and saved as a TIFF file. The area of periodontal bone loss was determined using Adobe Photoshop™ (Adobe Systems, San Jose, CA, USA). The polygonal area enclosed by the cemento-enamel junction, the lateral margins of the exposed tooth root, and the alveolar ridge was measured using NIH Image (Wayne Rasband, NIH, Bethesda, MD, USA). An image of a precise ruler was captured at the same magnification and was used for the calibration. Results were expressed in mm^2.

Cytokine enzyme-linked immunosorbent assay (ELISA)

For interleukin-1α assay, a mouse sandwich ELISA system was used following the procedures described previously (23). ELISAs for other mediators in extracts employed commercially available kits as follows: interleukin-1β, interleukin-4, interleukin-6, interleukin-10, interleukin-12, IFNγ and TNF-α, all from BioSource International (Camarillo, CA, USA). The kit for prostaglandin E_2 (PGE_2) was purchased from R & D Systems. All assays using commercial kits were carried out according to the manufacturer's instructions. Results were expressed as pg cytokine/mg periapical tissue.

Gene expression analysis

Gene expression in gingival tissues was determined on days 0 (uninfected control), 7, 14, 28 and 70 after infection using reverse transcription–polymerase chain reaction (RT–PCR). Non-infected day-70 samples were also subjected to RT–PCR to assess age-related changes. cDNA was reverse transcribed using SuperScript™ II RT and oligo-$dT_{12–18}$ primer (both from Invitrogen). cDNA was amplified using the HotStarTaq System (Qiagen, Valencia, CA, USA). Sequences of specific primer sets were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5′-AACTTTGGCAT TGTGGAAGG-3′, antisense 5′-CACATTGGGGGTAGGAACAC-3′; receptor activator of nuclear factor kappa B ligand (RANKL): sense 5′-CGCTCTGTTCCTGTACTTTCG AGCG-3′, antisense 5′-TCGTGCTC CCTCCTTTCATCAGGTT-3′ (24); osteoprotegrin (OPG): sense 5′-GAAA GACCTGCAAATCGAGC-3′, antisense 5′-AAACAGCCCAGTGACCA TTC-3′; CD40 ligand (CD40L): sense 5′-CGGAATTCAGTCAGCATGAT AGAAAC-3′, antisense 5′-AAGTCG ACAGCGCACTGTTCAGAGT-3′ (25); CD23: sense 5′-CACTGGGAA ACGGAGAAG-3′, antisense 5′-CCTTAGATCCTCCTGGAGT-3′ (26). An optimized protocol of thermal cycling was used: 95°C for 15 min, followed by 27 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 10 min. The number of cycles was in the linear range of amplification for all PCR products.

Oral sampling for *P. gingivalis*

To confirm *P. gingivalis* colonization, the oral cavity of each animal was sampled on day 14 after *P. gingivalis* infection with a sterile cotton swab immersed in PRAS. Swab samples were vortexed in 500 μl of PRAS. A 50-μl aliquot was plated onto tryptic soy broth agar plates in triplicate and incubated anaerobically for 1 week to determine the number of oral colony-forming units (CFU), and for 3–4 weeks to identify *P. gingivalis* by black pigmentation and Gram staining.

In selected experiments, genomic DNA of the normal oral flora was extracted from the remaining samples using DNAzol (Invitrogen), and subjected to rapid bacterial genotyping with *P. gingivalis*- and *A. actinomycetemcomitans*-specific primer sets prior to the infection; *P. gingivalis* rgpA gene: sense 5′-TCATATGAGCGGTC AGGCCGAGATTG-3′, antisense 5′-T CCTCGAGCTTGCCGTTGGCCTTGATCT-3′; *A. actinomycetemcomitans* OMP29 gene: sense 5′-TTC AGTTT GAACTCTGAT-3′, antisense 5′-GAA TGGGGTTTTCGCTAA-3′) were kindly provided by Drs Margaret Duncan, Tsute Chen (both in Department of Molecular Genetics, The Forsyth Institute) and Hitoshi Komatsuzawa (Department of Microbiology, Hiroshima University School of Dentistry, Japan). PCR was conducted as described above for 30 cycles.

Statistical analysis

Bone loss at 42 days was evaluated in a 2×2 factorial analysis of variance (ANOVA) of genotype by infection status in a model incorporating a genotype by infection status interaction term. In kinetic experiments, bone loss at day 70 was analyzed by 2×2 factorial analysis as described above. Bone loss at day 0 (uninfected) and days 7, 14 and 28 (infected) was evaluated in a two factor ANOVA (genotype and time) model. Cytokine levels at day 70 were also evaluated in a 2×2 factorial ANOVA for infected and uninfected mice by genotype. Cytokine kinetics from baseline (uninfected mice) over days 7, 14, 28 and 70 (infected mice only) were evaluated in a two factor (2×5) ANOVA of time, genotype, and time by genotype interaction. Post × baseline values for infected mice were compared to uninfected mice at baseline in a one-way ANOVA for each genotype, followed by Dunnet's test for comparison of multiple test groups against control with overall $\alpha = 0.05$. Student's *t*-test was applied to analysis of oral bacterial colonization and effect of blockade of interleukin-1 and interleukin-1RI.

Results

Effect of interleukin-10 deficiency on *P. gingivalis*-induced alveolar bone loss

In initial experiments, we tested the hypothesis that endogenous interleukin-10 is a potent suppressor of infection-stimulated periodontal bone loss. The oral cavities of interleukin-$10^{-/-}$ ($n = 12$: 6 males and 6 females) and wild-type mice ($n = 12$) were inoculated with live *P. gingivalis*, and alveolar bone loss was determined on day 42 after infection. As shown in Fig. 1, *P. gingivalis*-infected interleukin-$10^{-/-}$ mice exhibited severe alveolar bone loss ($p = 0.014$), compared to uninfected interleukin-$10^{-/-}$ controls ($n = 10$: 5 males and 5 females). In contrast, *P. gingivalis* infection did not stimulate significant bone loss in wild-type animals over the 6 week observation period. The severity of bone loss in interleukin-$10^{-/-}$ mice was illustrated by the observation that some interleukin-$10^{-/-}$ animals spontaneously lost the third molar (Fig. 2). These data indicate that bone loss in interleukin-$10^{-/-}$ mice is absolutely dependent on *P. gingivalis* infection and that endogenous interleukin-10 is a potent suppressor of *P. gingivalis*-induced alveolar bone loss *in vivo*.

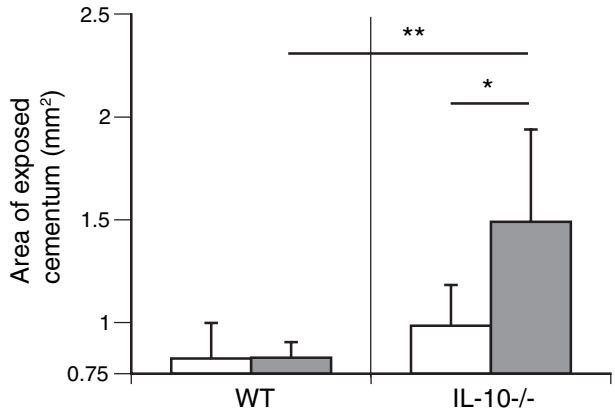

Fig. 1. Effect of *Porphyromonas gingivalis* infection on alveolar bone destruction. Alveolar bone loss was determined on day 42. Open column, non-infected ($n = 12$); closed column, infected ($n = 12$); vertical bars, standard deviation; $*p = 0.014$, infection effect; $**p < 0.0001$, genotype effect. The area of exposed cementum in non-infected animals represents the physiologic attachment site of gingival tissue. WT, wild-type mice; IL-$10^{-/-}$, interleukin-10 knockout mice.

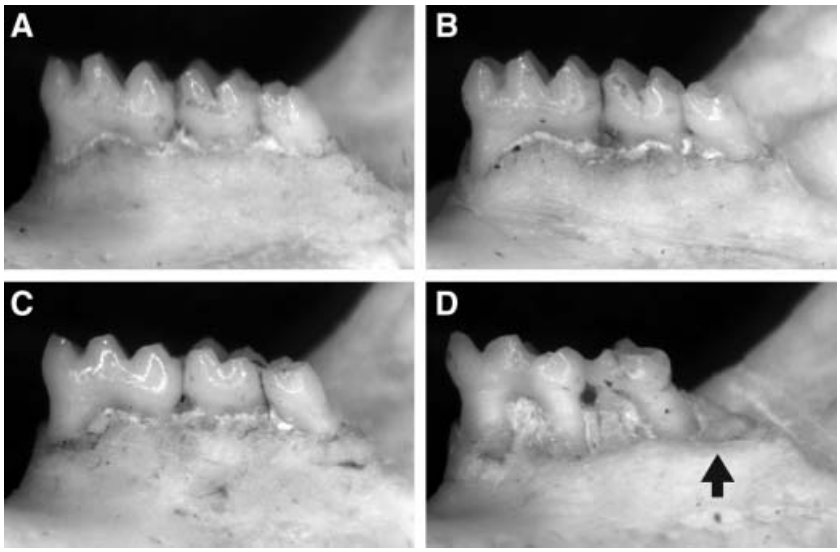

Fig. 2. Representative mandibles from *Porphyromonas gingivalis*-infected and non-infected mice. Images of mandibular molars and alveolar bone were captured on day 42. A, non-infected wild-type mouse; B, infected wild-type; C, non-infected interleukin-10 knockout (IL-$10^{-/-}$); D, infected IL-$10^{-/-}$. Arrow, spontaneous loss of third molar in infected IL-$10^{-/-}$.

Kinetics of infection-stimulated alveolar bone loss

The kinetics of *P. gingivalis*-induced alveolar bone loss was determined. Using an identical infection regimen as employed in the preceding experiment, alveolar bone loss was assessed on days 7, 14, 28, and 70 ($n = 6$/group: 3 males and 3 females). Non-infected groups analyzed on days 0 and 70 were established as negative controls. As shown in Fig. 3, *P. gingivalis*-infected interleukin-$10^{-/-}$ mice exhibited significant bone loss only on day 70 (+27%, $p = 0.0006$), whereas wild-type mice exhibited only slightly increased bone loss (+15%) within the same observation period, which was not statistically significant. Of note, non-infected day-70 interleukin-$10^{-/-}$ mice did not experience alveolar bone loss, indicating that these animals do not harbor oral microorganisms capable of inducing bone resorption, nor do they undergo age-related bone loss within this time frame.

Oral flora in interleukin-$10^{-/-}$ and wild-type animals

Persistent oral colonization by *P. gingivalis* was confirmed in infected animals ($n = 4$: males). As shown in Table 1, Gram-negative black-pigmented bacteria were recovered from most infected animals on day 14, whereas no pigmented colonies were isolated from non-infected animals. We also determined that interleukin-$10^{-/-}$ and wild-type mice did not harbor *P. gingivalis*, or another periodontal pathogen, *A. actinomycetemcomitans*, in their oral cavities prior to deliberate infection. Genomic DNA was extracted from

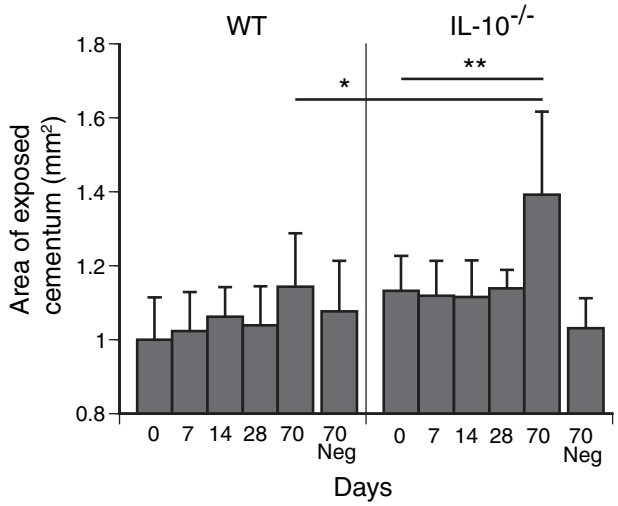

Fig. 3. Kinetics of *Porphyromonas gingivalis*-induced alveolar bone destruction. Alveolar bone destruction was determined on days 0 (non-infected), 7, 14, 28, and 70 ($n = 6$ in each group). The non-infected day 70 group (70 Neg) served to assess any age-related effects. Vertical bar, standard deviation; $*p = 0.012$; $**p = 0.0006$. WT, wild-type mice; IL-$10^{-/-}$, interleukin-10 knockout mice.

Table 1. Detection of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* before and after oral infection.

			Percentage of animals with etiologic agents	
Infection	Detection method	Mouse strain	*P. gingivalis*	*A. actinomycetemcomitans*
Before	PCR	Wild-type (C57BL/10J)	0	0
		IL-$10^{-/-}$	0	0
After (day 14)	Pigmentation and Gram staining	Wild-type	75	Not done
		IL-$10^{-/-}$	100	Not done

IL-$10^{-/-}$, interleukin-10 knockout mice; PCR, polymerase chain reaction.

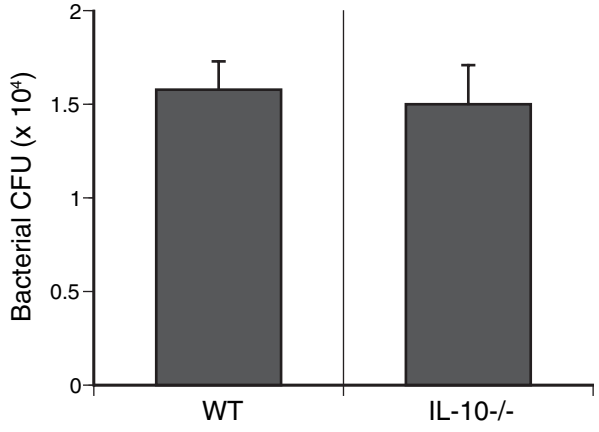

Fig. 4. Oral bacterial colonization in wild-type (WT) and interleukin-10 knockout (interleukin-$10^{-/-}$) mice. The oral cavities were sampled prior to antibiotic treatment ($n = 4$), and colony forming units (CFU) were determined after 7 days anaerobic culture. Vertical bar, standard deviation. No significant difference was observed.

cultured oral samples, and *P. gingivalis*-specific and *A. actinomycetemcomitans*-specific PCRs were carried out. Neither *P. gingivalis* nor *A. actinomycetemcomitans* were detected in the normal oral flora prior to *P. gingivalis* infection.

The number of bacterial CFUs present in the oral cavities of uninfected wild-type and interleukin-$10^{-/-}$ mice were examined in the same experiment, to determine if interleukin-$10^{-/-}$ mice have increased microbial colonization compared to wild-type animals, possibly influencing their increased susceptibility to alveolar bone loss. Oral swab samples were collected from the lingual surfaces of all molars and were grown on blood agar plates under anaerobic conditions. As shown in Fig. 4, interleukin-$10^{-/-}$ mice and wild-type mice had similar numbers of total CFU recoverable from their oral cavities. Taken together, these data demonstrate that interleukin-$10^{-/-}$ and wild-type mice have similar levels of background oral flora, lack periodontal pathogens, and can be persistently colonized with *P. gingivalis* under the infection regimen utilized.

Expression of bone resorptive cytokines in gingival tissues

Several cytokines that are induced by infection have been implicated in alveolar bone destruction, most notably interleukin-1 and TNF-α (27). We therefore assessed the levels of bone resorptive mediators in extracts of gingival tissues from interleukin-$10^{-/-}$ and wild-type mice before and after infection with *P. gingivalis*, over a 70-day experimental period. As shown in Fig. 5, uninfected interleukin-$10^{-/-}$ mice expressed approximately 50% higher amounts of interleukin-1α than wild-type mice in gingival tissues on day 0 and 25% higher levels on day 70. However, interleukin-1α levels were not further increased by *P. gingivalis* infection in either strain. Uninfected interleukin-$10^{-/-}$ mice exhibited lower levels of interleukin-1β compared to wild-type mice on day 0 ($p = 0.001$), but this difference disappeared following

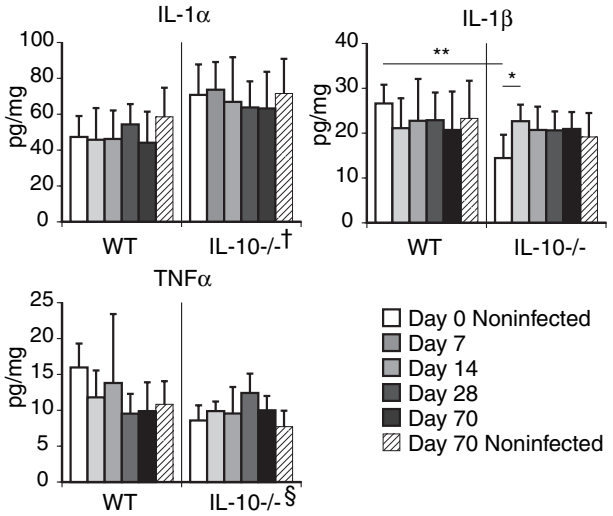

Fig. 5. Kinetics of expression of bone resorptive cytokines in gingiva. Gingival levels of interleukin (IL)-1α, IL-1β, and tumor-necrosis factor alpha (TNF-α) were determined at the indicated times by enzyme-linked immunosorbent assay (ELISA). Non-infected day 70 group (70 Neg) served to assess any age-related effects. Vertical bars, standard deviation; *significant difference by Dunnet's test ($\alpha = 0.05$); **$p = 0.001$, genotype effect at day 0; †$p < 0.0001$, genotype effect by two factor ANOVA. §$p = 0.006$, genotype effect by two factor ANOVA. WT, wild-type mice; IL-$10^{-/-}$, IL-10 knockout mice.

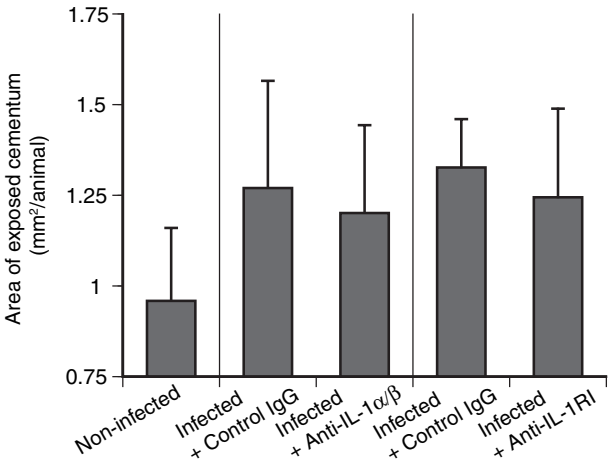

Fig. 6. Effect of blockade of interleukin (IL)-1 or IL-1 receptor type I (IL-1RI) on *Porphyromonas gingivalis*-induced alveolar bone loss in IL-10 knockout (IL-$10^{-/-}$) mice. IL-1α and IL-1β ($n = 5$), or IL-1RI ($n = 10$) was neutralized by antibody injections in infected IL-$10^{-/-}$ mice. Non-infected animals ($n = 5$) served as negative controls, and mice given control immunoglobulin G (IgG) ($n = 5$ for IL-1α/β; $n = 10$ for IL-1RI) served as positive controls. Alveolar bone destruction was determined on a per animal basis on day 42. Vertical bar, standard deviation. No significant differences were observed.

infection. On the other hand, TNF-α was higher in wild-type mice at baseline, but was not changed by *P. gingivalis* infection. interleukin-$10^{-/-}$ mice had lower levels of TNF-α ($p = 0.006$), which were not significantly altered by *P. gingivalis* infection.

Effect of interleukin-1 blockade on periodontal bone loss

Although these data indicated that *P. gingivalis*-induced bone loss in interleukin-$10^{-/-}$ mice does not correlate with the expression of these bone resorptive cytokines, it remained possible that the higher constitutive expression of interleukin-1α and modest up-regulation of interleukin-1β in interleukin-$10^{-/-}$ mice was a necessary but not sufficient condition for the observed extensive alveolar bone loss in this model. We therefore determined whether *P. gingivalis*-induced alveolar bone loss in interleukin-$10^{-/-}$ mice was interleukin-1 dependent, using well-established *in vivo* antibody neutralization protocols (17, 22). Endogenous interleukin-1 was neutralized with a mixture of polyclonal goat anti-mouse interleukin-1α and anti-interleukin-1β antibodies, whereas the interleukin-1RI was blocked with an antireceptor monoclonal antibody. Antibody injections were given from days 1–21 relative to *P. gingivalis* infection, and alveolar bone loss was determined on day 42. As shown in Fig. 6, *P. gingivalis*-induced bone loss was unaffected by the neutralization of interleukin-1α and interleukin-1β, or by interleukin-1RI blockade, compared to unreactive control IgG treated groups. These data strongly suggest that *P. gingivalis*-induced alveolar bone loss in interleukin-$10^{-/-}$ mice is induced by an interleukin-1 independent mechanism(s).

Expression of regulatory mediators

The expression of regulatory cytokines and mediators was also examined (Fig. 7). The prototype Th1 cytokine IFNγ was higher in uninfected wild-type animals compared to uninfected interleukin-$10^{-/-}$ mice ($p = 0.004$), but was significantly elevated in interleukin-$10^{-/-}$ mice on day 70 ($p < 0.037$) when bone loss was maximal. The Th2 mediators interleukin-6 and interleukin-4 (not shown) were both higher in wild-type mice prior to infection. interleukin-4 was modestly up-regulated in interleukin-$10^{-/-}$ mice after infection, whereas interleukin-4 and interleukin-6 were down-regulated in wild-type animals ($p < 0.05$ in interleukin-6). PGE_2, which is an inflammatory mediator induced by interleukin-6 as well as by interleukin-1 and TNF-α, was also up-regulated in interleukin-$10^{-/-}$ mice ($p = 0.029$). Regulatory

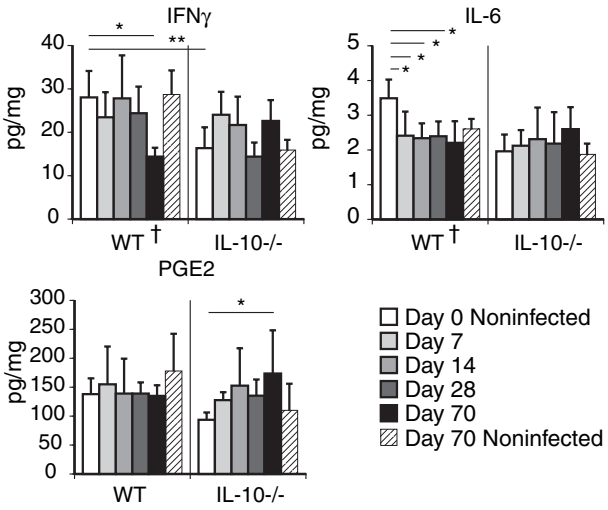

Fig. 7. Kinetics of expression of regulatory cytokines in gingiva. Gingival levels of regulatory cytokines were determined at the indicated times by enzyme-linked immunosorbent assay (ELISA). Non-infected 70 group (70 Neg) served to assess any age-related effects. Vertical bars, standard deviation; *significant difference by Dunnet's test ($\alpha = 0.05$); **$p < 0.005$; †$p = 0.003$, genotype effect by two factor ANOVA. WT, wild-type mice; IL-$10^{-/-}$, interleukin-10 knockout mice; IFNγ, interferon gamma; IL-6, interleukin-6; PGE2, prostaglandin E_2.

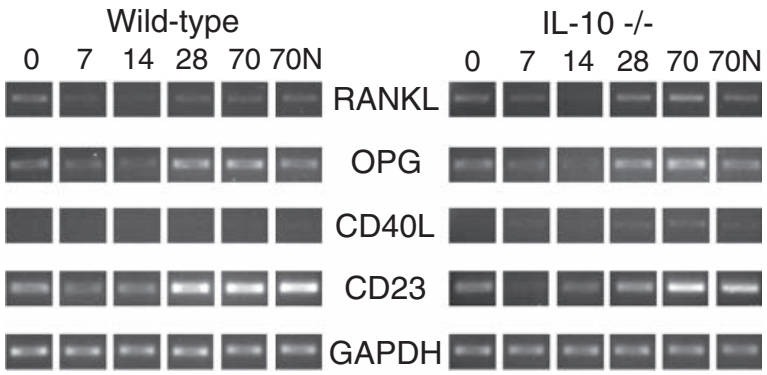

Fig. 8. Kinetics of expression of cell markers in gingiva. Total RNA was extracted from gingivae at the indicated times and was analyzed by semiquantitative reverse transcription polymerase chain reaction (RT–PCR). The non-infected day 70 group (70N) served to assess any age-related effects. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. WT, wild-type mice; IL-$10^{-/-}$, interleukin-10 knockout mice; RANKL, receptor activator of nuclear factor kappa B ligand; OPG, osteoprotegrin; CD40L, CD40 ligand.

cytokines therefore had an expression pattern that was distinct from the pattern of resorptive cytokines, with elevations of both Th1 and Th2 mediators seen in interleukin-$10^{-/-}$ vs. wild-type mice on day 70. However, no changes in cytokine expression correlated closely with the observed dramatic increases in bone loss.

Expression of cell activation markers

The gene expression profiles of several cell activation markers were compared in wild-type and interleukin-$10^{-/-}$ mice by RT–PCR. As shown in Fig. 8, gingival RANKL was modestly up-regulated on days 28 and 70 in interleukin-$10^{-/-}$ vs. wild-type mice, which correlated with the observed alveolar bone loss. OPG, the decoy receptor for RANKL, was modestly up-regulated in both interleukin-$10^{-/-}$ and wild-type mice. CD40L expression, a marker of T cell activation, was increased only in interleukin-$10^{-/-}$ on days 28 and 70. The IgE receptor CD23 is expressed by activated B cells and macrophages. It was more highly expressed by wild-type animals at all time points compared to interleukin-$10^{-/-}$ mice. Of interest, RANKL, OPG, and CD23 all had transiently reduced expression in gingival tissues at 7 and 14 days after *P. gingivalis* infection, suggesting possible inhibition of cell activation, inhibition of local production of chemoattractant factors, or induction of apoptosis by this organism.

Discussion

P. gingivalis has been strongly implicated as an etiological agent of adult periodontitis in humans (1). In this study, we tested the hypothesis that interleukin-10 is a potent endogenous suppressor of *P. gingivalis*-induced periodontal bone loss *in vivo*. We demonstrate that *P. gingivalis* infection induced significant periodontal bone loss in interleukin-$10^{-/-}$ mice, whereas bone loss did not occur in uninfected interleukin-$10^{-/-}$ or in comparably infected wild-type animals. Of interest, bone loss in interleukin-$10^{-/-}$ did not appear to be mediated by interleukin-1, as determined by ligand and receptor blocking studies. These data support the hypothesis that the interleukin-$10^{-/-}$ mouse is highly susceptible to periodontal bone loss induced by an implanted consensus human periodontal pathogen, and are consistent with our previous demonstration that interleukin-$10^{-/-}$ mice exhibit severe infection-stimulated periapical bone destruction (16).

The interleukin-$10^{-/-}$ mouse provides several major advantages over previously described mouse models (20, 21, 28). These include rapid disease development (over 6 weeks), the severity of disease as exemplified by spontaneous loss of the third molar in some animals, the existence of a non-pathogenic indigenous oral flora, and the requirement for an exogenous etiological agent such as *P. gingivalis* for disease induction. With respect to the latter point, we have also found that implantation of *A. actinomycetemcomitans*, the putative etiological agent of localized juvenile periodontitis, also induces bone loss in interleukin-$10^{-/-}$ mice (T. Kawai and H. Sasaki,

unpublished). Although increased alveolar bone loss was recently reported in interleukin-10$^{-/-}$ mice, this was observed in the absence of deliberate oral infection, but did not become significant until approximately 30 weeks of age, long after the time frame in these studies (6–16 weeks), and appeared to be a manifestation of a generalized osteoporosis (29). The advantages of this model indicate that the interleukin-10$^{-/-}$ mouse will be useful for understanding pathways of infection-stimulated bone destruction in a model of enhanced inflammation, for assessing putative virulence genes in pathogenic bacteria, and for testing preventive and therapeutic measures including antimicrobials and vaccines.

It has previously been reported that several wild-type mouse strains, including AKR/J, DBA/2J, BALB/cByJ, and BALB/cJ, are susceptible to *P. gingivalis*-induced bone loss (20). However, bone loss in wild-type strains occurs quite slowly and its extent is modest. More dramatic bone loss has been reported in certain genetically engineered mutant mice, particularly those with cell adhesion molecule deficiencies, including animals lacking both P and E-selectin inter cellular adhesion molecule-1 (ICAM-1) (21, 28, 30). P/E-selectin mice have a 10-fold elevation of bacterial colonization of their oral cavities (28), suggesting that the deficit in leukocyte migration may permit *P. gingivalis* to persistently colonize host tissues. Of interest, no similar elevation in oral microbial colonization was seen in interleukin-10$^{-/-}$ mice, indicating that pathologic changes are likely related to increased inflammation rather than increased bacterial load (Fig. 4).

Interleukin-1α is a prominent cytokine in infection-stimulated bone resorption (31), but was not up-regulated in gingival tissues by *P. gingivalis* infection in interleukin-10$^{-/-}$ mice. This result is in contrast to our previous findings that periapical tissue levels of interleukin-1α were increased by infection up to eightfold in interleukin-10$^{-/-}$ mice (16). Moreover, the 50% higher tissue levels of interleukin-1α in non-infected interleukin-10$^{-/-}$ mice on days 0 and 70 did not appear to mediate significant bone loss in the absence of *P. gingivalis* infection. Although interleukin-1β was slightly up-regulated by *P. gingivalis* infection, the neutralization of both interleukin-1α and interleukin-1β, or the blockade of interleukin-1RI, also failed to suppress *P. gingivalis*-induced bone loss. These findings indicate that interleukin-1 is neither necessary nor sufficient for bone loss in interleukin-10$^{-/-}$, and suggest that some other pathway is operative in stimulating bone loss. Along these lines, it has been reported that periodontal fibroblasts can stimulate bone resorption *in vitro* by an interleukin-1-independent mechanism (32).

A survey of other cytokines herein showed that *P. gingivalis* infection modestly increased the levels of interleukin-6, IFNγ, and PGE_2 in *P. gingivalis* infected interleukin-10$^{-/-}$ mice on day 70, although the increased levels were generally similar to those expressed in wild-type mice that showed no increased bone loss. interleukin-6 has been linked to increased resorption *in vivo* (19), although we find that its action is predominantly anti-inflammatory in a periapical bone loss model (17). There is some evidence that IFNγ produced by CD4(+) T cells contributes to infection-stimulated bone destruction in both a murine model of periodontal disease and in human patients (19, 33). The macrophage-specific STAT3 knockout mouse, which lacks interleukin-10 signaling in macrophages, exhibits a polarized Th1 response and chronic enterocolitis similar to the standard interleukin-10$^{-/-}$ mouse (34, 35). This phenotype can be eliminated by deletion of interleukin-12 (36). Thus, interleukin-12 and/or IFNγ could have a role in the induction of inflammatory disease in the interleukin-10$^{-/-}$ mouse. Arguing against this possibility is the finding in the periapical model that the deletion of IFNγ or interleukin-12 did not significantly reduce periapical bone destruction (23). At this time therefore, no strong correlation can be made between any resorptive or regulatory cytokine with the dramatic loss we have observed. Further studies are needed to determine if other resorption-stimulating factors such as interleukin-11 or interleukin-17 may be involved, as well as to establish the precise roles of members of the cytokine network in the periodontal pathology in this model.

Analyses of cell associated molecules showed that CD40L, a marker of activated T cells, and RANKL, the key stimulator of osteoclast development and activation, were both up-regulated on days 28 and 70 in interleukin-10$^{-/-}$ vs. wild-type animals, whereas the B cell marker CD23 was not. RANKL produced by activated CD4(+) T cells may have pathogenic consequences, and has been found to increase joint destruction in immune complex arthritis (37, 38), as well as in alveolar bone destruction *in vivo* (39). *P. gingivalis* infection also induced a transient reduction in the expression of RANKL, OPG, CD40L, and CD23 in gingival tissues from both interleukin-10$^{-/-}$ and wild-type animals in the first few weeks after infection. This could represent a generalized inhibition of cell activity or possibly induction of apoptosis by *P. gingivalis*, either of which could further contribute to pathogenicity. *P. gingivalis* has been shown to induce apoptosis in host cells both *in vitro* (40–46) and more importantly *in vivo* (47, 48). Although there is one report that *P. gingivalis* lipopolysaccharide is pro-apoptotic (45), most studies implicate the R and K gingipains in apoptosis induction, possibly the result of detachment of adherence-dependent connective tissue cells from the extracellular matrix (40, 42, 43, 49–51). It remains to be demonstrated whether cysteine proteinase-dependent mechanisms also cause apoptosis in nonadherent cells (e.g. T and B cells) *in vivo*, thus accounting for the observed decreases in inflammatory cell markers.

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