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Accelerated alveolar bone loss in mice lacking interleukin-10: late onset

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Objective and background: Interleukin-10 (IL-10) is an anti-inflammatory cytokine regulating immune responses. We have previously reported that IL-10(–/–) mice experience accelerated alveolar bone loss. The purpose of the present study was to examine the timing of the manifestation of accelerated alveolar bone loss in IL-10(–/–) mice.

Materials and methods: Twenty-four IL-10(–/–) and 21 IL-10(+/+) age-matched male 129/SvEv mice were used. Sacrifice times occurred at 1, 3 and 9.5 months of age. Alveolar bone loss was determined morphometrically on defleshed jaws. Enzyme-linked immunosorbent assay (ELISA) was used for determination of serum concentration of type I collagen C-telopeptide, a systemic marker of bone resorption.

Results: Alveolar bone loss for the entire IL-10(–/–) group was significantly different than for the IL-10(+/+) group ($p = 0.025$). There was no significant difference in alveolar bone loss between IL-10(–/–) and IL-10(+/+) mice at 1 and 3 months of age. At 9.5 months of age, IL-10(–/–) mice exhibited 39% greater alveolar bone loss than IL-10(+/+) mice ($p = 0.018$). For IL-10(–/–) mice, alveolar bone loss significantly increased with age. Serum C-telopeptide levels significantly decreased with age in both groups. IL-10(–/–) mice had consistently higher C-telopeptide levels than IL-10(+/+) mice and the difference between the two groups reached statistical significance ($p = 0.011$) for the 9.5-month-old mice.

Conclusions: These results suggest that the accelerated alveolar bone loss observed in IL-10(–/–) mice is a late-onset condition and that lack of IL-10 may have an effect on bone homeostasis.

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The study of the function of interleukin (IL)-10, a potent anti-inflammatory cytokine regulating immune responses (1–4), has been significantly advanced by the development of IL-10(–/–) mice, i.e. mice lacking the IL-10 gene (5). Animal studies implicate IL-10 in the development and progression of arthritis (6–9) and chronic colitis (10, 11). IL-10 has a critical role in the *in vivo* regulation of pro-inflammatory cytokine levels; e.g. interleukin-1 (IL-1) and tumor necrosis factor production in response to diverse inflammatory stimuli is increased in the absence of IL-10 and decreased by IL-10 administration (3, 8, 9).

Periodontitis, a chronic inflammatory condition of infectious nature, is characterized by loss of connective tissue attachment and alveolar bone destruction (12). Host factors, in general, and pro-inflammatory cytokines, such as IL-1 and tumor necrosis factor, in particular, contribute to periodontal alveolar bone loss (13, 14). This fact, in conjunction with the significant effect of IL-10 on the *in vivo* levels of such pro-inflammatory cytokines (3, 8, 9), led us to hypothesize that lack of IL-10 would lead to increased alveolar bone resorption. We recently reported that IL-10(–/–) mice exhibit accelerated alveolar bone loss when compared to age-, sex-, and strain-matched control mice, in the absence of any manipulation of the

normal oral flora of the animals (15). However, the time of onset of this condition has not been established, since only 7-month-old animals were originally examined (15). The purpose of the present study was to examine the natural history of alveolar bone loss in IL-10(–/–) mice compared to age-, sex-, and strain-matched IL-10(+/+) control animals and to determine the systemic levels of type I collagen C-telopeptide, a bone resorption marker.

Materials and methods

Experimental animals

Twenty-four IL-10(–/–) and 21 IL-10(+/+) male mice of the 129/SvEv strain were used. All animals were raised and housed in group cages under identical, conventional, specific mouse pathogen-free conditions at the breeding colony of DNAX Research Institute. The genotype of the mice was confirmed by polymerase chain reaction using DNA extracted from tail tip digests both prior to the commencement of the experiments and after killing. All live animal work occurred at DNAX Research Institute, and the IACUC of DNAX approved the study protocol.

At the age of 1 month, 3 months, and 9.5 months 16 [eight IL-10(–/–); eight IL-10(+/+)], 14 [seven IL-10(–/–); seven IL-10(+/+)], and 15 [nine IL-10(–/–); six IL-10(+/+)] animals were killed, respectively. Animals were killed by CO_2 inhalation and decapitated. Animal heads were kept frozen (−70°C), until further processing. Whole blood was obtained by cardiac puncture at death. Blood was allowed to clot at 4°C overnight, serum was collected after centrifugation, aliquoted, and stored at −70°C until testing.

Alveolar bone loss measurements

Animal heads were defleshed mechanically, treated with sodium hypochlorite to remove all organic material and bleached by hydrogen peroxide treatment. Skulls were stained by methylene blue, to help identify the cemento-enamel junction. Jaw images were digitally captured after positioning on a dissecting microscope stage (16). All measurements were performed using a computer-assisted image analysis system (16), and the sole operator performing the measurements was blinded regarding type and age of animal.

Alveolar bone loss was measured as exposed molar root surface area (mm^2) on the lingual aspect of the right and left mandible. In a modification of the previously reported method (16), only the exposed root surface area corresponding to the first and second molar was measured, because in a significant number of 1-month-old and 9.5-month-old animals the third molars were lost during processing. This was attributed to the developmental stage of the third molar (1-month-old animals) or the extensive alveolar bone loss around the teeth (9.5-month-old animals). The coefficient of variation for replicate measurements using this method has been determined with both mouse (15) and rat jaws (16) and has been found to be less than 5%. The two mandibular measurements were averaged to calculate mean animal alveolar bone loss. The jaws of one of the 3-month-old IL-10(–/–) mice was damaged during processing precluding any alveolar bone loss measurements.

Type I collagen C-telopeptide analysis

Type I collagen C-telopeptide serum levels were analyzed by means of a recently developed and described enzyme-linked immunosorbent assay (ELISA) (17). Samples were run in duplicate and the operator was blinded regarding type and age of animal. Sensitivity of this ELISA is < 0.1 ng/ml, with an average intra- and interassay coefficient of variation < 12% (17).

Data management and statistical analysis

Descriptive statistics are presented as mean ± standard deviation (SD). Alveolar bone loss and serum C-telopeptide data were analyzed by non-parametric tests (Mann–Whitney U and Kruskal–Wallis). Significance level for rejection of the null hypothesis was set at $\alpha = 0.05$.

Results

Alveolar bone loss

Overall, IL-10(–/–) mice had greater alveolar bone loss than IL-10(+/+) mice (Table 1), although within each of the three age groups, only the 9.5-month-old IL-10(–/–) mice had alveolar bone loss significantly different than age-matched IL-10(+/+) mice (Table 1, Fig. 1).

When all animals were analyzed together, alveolar bone loss was significantly different by age ($p < 0.0006$, Kruskal–Wallis). This difference among age groups remained significant for IL-10(–/–) mice analyzed alone

Table 1. Alveolar bone loss in interleukin-10(–/–) and interleukin-10(+/+) mice

	Alveolar bone loss (mm^2)			
Age	Interleukin-10(+/+)	Interleukin-10(–/–)	Diff. (%)	*p*-value*
All ages	0.53 ± 0.08† (0.52; 0.42–0.76; $n = 21$)‡	0.65 ± 0.22 (0.58; 0.44–1.39; $n = 23$)	23	0.025
1 month	0.50 ± 0.06 (0.52; 0.42–0.59; $n = 8$)	0.51 ± 0.06 (0.49; 0.44–0.62; $n = 8$)	2	0.753
3 months	0.51 ± 0.07 (0.49; 0.43–0.61; $n = 7$)	0.56 ± 0.08 (0.57; 0.46–0.66; $n = 6$)	10	0.317
9.5 months	0.59 ± 0.10 (0.59; 0.46–0.76; $n = 6$)	0.82 ± 0.26§ (0.81; 0.55–1.39; $n = 9$)	39	0.018

*Mann–Whitney *U*-test.
†Values (in mm^2) are mean ± SD.
‡Values in parenthesis are median, range, and number of animals.
§Significantly different than 1 month ($p < 0.003$) and 3 month ($p < 0.015$) interleukin-10(–/–) group.

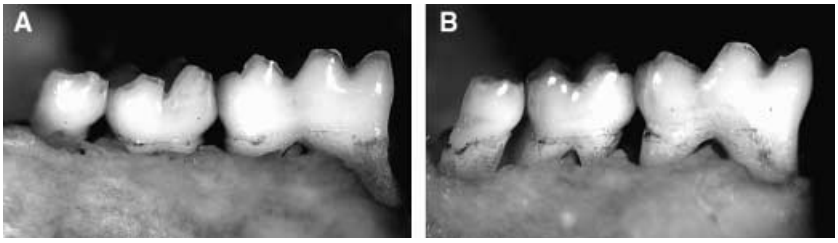

Fig. 1. Mandibular lingual aspect of representative 9.5-month-old interleukin (IL)-10(+/+) (A) and IL-10(–/–) (B) mice of the 129/SvEv strain. The much greater loss of alveolar bone in the IL-10(–/–) mouse (B) compared to the IL-10(+/+) mouse (A) is evident. The jaws depicted were chosen from mice that closely mirror the mean alveolar bone loss value of the respective group.

Table 2. Serum type I collagen C-telopeptide in interleukin-10(–/–) and interleukin-10(+/+) mice

	Serum type I collagen C-telopeptide (ng/ml)			
Age	Interleukin-10(+/+)	Interleukin-10(–/–)	Diff. (%)	*p*-value*
1 month	32.4 ± 8.6†[a] (35; 14–42; $n = 8$)‡	43.2 ± 12.0[b] (40; 32–68; $n = 8$)	33	0.093
3 months	11.6 ± 1.0[a] (12; 10–13; $n = 7$)	17.7 ± 15.4 (15; 6–51; $n = 7$)	53	0.655
9.5 months	7.5 ± 1.1[a] (7.5; 6–9; $n = 6$)	10.8 ± 3.5 (9; 8–18; $n = 9$)	44	0.011

*Mann–Whitney *U*-test.
†Values (in ng/ml) are mean ± SD.
‡Values in parenthesis are median, range, and number of animals.
[a]Significantly different than other interleukin-10(+/+) age groups ($p < 0.003$).
[b]Significantly different than other interleukin-10(–/–) age groups ($p < 0.011$).

(Table 1), but not for IL-10(+/+) mice ($p = 0.2$).

Serum type I collagen C-telopeptide levels

There was a significant age-dependent decrease in C-telopeptide levels in all mice (Table 2). Although C-telopeptide levels were consistently higher in IL-10(–/–) mice, compared to age-matched IL-10(+/+) controls, the difference between the two types of animals reached statistical significance only in the 9.5-month-old group (Table 2).

Discussion

The results of this study indicate that IL-10(–/–) mice exhibit much greater periodontal alveolar bone loss compared to age-, sex-, and strain-matched IL-10(+/+) mice maintained under identical conditions. However, within each of the three age groups examined, the difference between IL-10(–/–) and IL-10(+/+) mice was significant only for the 9.5-month-old animals. These results are consistent with our previous report on 7-month-old 129/SvEv and C57BL/6 J IL-10(–/–) and IL-10(+/+) mice (15). The present results indicate that the accelerated alveolar bone loss observed in IL-10(–/–) mice is a late onset condition that does not precede the appearance of the inflammatory bowel disease-like colitis that develops in the IL-10(–/–) mice (4). Although by the age of 3 months 100% of the 129/SvEv IL-10(–/–) mice are affected by colitis, the time of onset may vary (4). The ability of IL-10 to suppress pro-inflammatory cytokine synthesis (3, 4, 8, 9) may contribute to the alveolar bone loss acceleration in IL-10(–/–) mice, as the same pro-inflammatory cytokines have been implicated in alveolar bone resorption (13, 14).

To our knowledge, the present study is the first to report that IL-10(–/–) mice, compared to age-matched IL-10(+/+) controls, manifest significantly elevated serum levels of type I collagen C-telopeptide, a systemic marker of bone resorption (17–19). This finding and the accelerated alveolar bone loss are consistent with the reported inhibitory effect of IL-10 on osteoclast formation (20) and on infection-stimulated periapical bone resorption (21). These observations suggest that lack of IL-10 may have a direct effect on bone homeostasis. However, this does not exclude the possibility that present results reflect a colitis-induced systemic osteopenia, a highly prevalent finding in adults as well as children diagnosed with inflammatory bowel disease (22–28), and in rat models of experimentally induced colitis (29, 30).

The levels of type I collagen C-telopeptide in serum from 3-month-old male 129/SvEv mice reported here are comparable to levels previously reported in 11-week-old female C57BL/6 N mice (17). The significant variation in C-telopeptide levels observed in the 3-month-old IL-10(–/–) mice may reflect the variation in time of onset of the gastrointestinal involvement in these animals (4). The present results indicate that C-telopeptide levels decrease significantly with age in both normal and IL-10(–/–) mice, particularly between the ages of 1 month and 3 months. This finding is consistent with the reported significant decrease of serum osteocalcin levels in mice between the ages of 3 and 12 weeks (31).

The late onset of accelerated alveolar bone loss in IL-10(–/–) mice is consistent with findings in HLA-B27 transgenic rats (32, 33), another animal model of heightened inflammatory responses and inflammatory bowel disease-like chronic colitis. The alveolar bone loss findings in the two animal models may reflect the described association of aggressive periodontal bone loss with inflammatory bowel disease cases (34, 35).

The presence of normal gut flora is a prerequisite for development of the inflammatory bowel disease-like colitis in IL-10(–/–) mice (10, 11). Whether the accelerated alveolar bone loss in IL-10(–/–) mice is dependent on the presence of commensal flora remains

to be established. In this context, it should be noted that the oral environment of these animals was never manipulated. The susceptibility of IL-10(–/–) mice to alveolar bone loss suggests that this animal model could become an excellent model to study the virulence of periodontal pathogens and the significance of IL-10 in the regulation of host responses to such pathogens, as suggested by human data (36).

In summary, the results of this study indicate that IL-10(–/–) mice are susceptible to late-onset spontaneous alveolar bone loss and exhibit significantly elevated serum levels of type I collagen C-telopeptide, a systemic marker of bone resorption. These results suggest that IL-10 may be a significant host factor in the pathogenesis of alveolar bone loss.

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