Cellular Physiology and Biochemistry

Cell Physiol Biochem 2022;56:270-281
DOI: 10.33594/000000532
Published online: 17 June 2022
Accepted: 23 May 2022

Original Paper

Interleukin-33 Deficiency Exacerbates Bone Loss Associated with *Porphyromonas Gingivalis*-Induced Experimental Periodontitis in Female Mice

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Key Words
Interleukin-33 • Inflammation • Periodontitis • *Porphyromonas gingivalis* • Osteoclasts

Abstract
Background/Aims: Interleukin 33 (IL-33) plays a significant role in immunity but its role in bone physiology and periodontitis needs to be further investigated. The aim of this study was to decipher the contribution of IL-33 to bone homeostasis under physiological conditions, and to alveolar bone loss associated with experimental periodontitis (EP) in IL-33 knockout (KO) mice and their wildtype (WT) littermates. ***Methods:*** The bone phenotype of IL-33 KO mice was studied in the maxilla, femur, and fifth lumbar vertebra by micro-computed tomography (micro-CT). EP was induced by a ligature soaked with the periopathogen *Porphyromonas gingivalis* (*Pg*) around a maxillary molar. Alveolar bone loss was quantified by micro-CT. The resorption parameters were assessed via toluidine blue staining on maxillary sections. *In vitro* osteoclastic differentiation assays using bone marrow cells were performed with or without lipopolysaccharide from *Pg* (LPS-*Pg*). ***Results:*** First, we showed that under physiological conditions, IL-33 deficiency increased the trabecular bone volume/total volume ratio (BV/TV) of the maxillary bone in male and female mice, but not in the femur and fifth lumbar vertebra, suggesting an osteoprotective role for IL-33 in a site-dependent manner. The severity of EP induced by *Pg*-soaked ligature was increased in IL-33 KO mice but in female mice only, through an increase in the number of osteoclasts. Moreover, osteoclastic differentiation from bone

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marrow osteoclast progenitors in IL-33-deficient female mice is enhanced in the presence of LPS-*Pg*. ***Conclusion:*** Taken together, our data demonstrate that IL-33 plays a sex-dependent osteoprotective role both under physiological conditions and in EP with *Pg*.

Introduction

Interleukin-33 (IL-33) is classified as one of the last members of the interleukin-1 (IL-1) family to be discovered [1] and as such it plays a significant role in both innate and acquired immunity. IL-33 is constitutively expressed in various tissues, particularly in the endothelial and epithelial cells of barrier tissues where it acts as an alarmin [2, 3]. Rapidly released to the extracellular space after cellular damage or injury, IL-33 alerts the immune system to danger and triggers inflammation. IL-33 acts by binding to its ST2 receptor, which is expressed on many immune and non-immune cells and which activates the NF-κB and MAPK pathways leading to inflammatory cytokine production [1, 4]. IL-33 is known to promote the orientation of type 2 immune response but exerts pleiotropic activities depending on the target cells and their microenvironment [2]. Although IL-33 is well known as a key player in inflammatory diseases (i.e., asthma, atopic dermatitis), its role in bone physiology remains uncertain and its involvement in bone diseases such as periodontitis needs further investigation.

Bone tissue is constantly renewed via bone remodeling throughout life. A critical balance between bone resorption by osteoclasts (OCs) and bone formation by osteoblasts is mandatory to achieve this physiological process (coupling). A cytokine network regulates the function of bone cells, OCs in particular, which are derived from the monocyte/macrophage hematopoietic lineage [5]. Receptor activator of nuclear factor κ-B ligand (RANKL) is the major pro-osteoclastogenic cytokine. It binds to its receptor RANK expressed by OCs and their precursors and enhances their recruitment, differentiation, fusion, and activity. However, the role of the cytokine IL-33 in bone remodeling is still debated. The overexpression of IL-33 specifically in osteoblasts of transgenic mice led to reduced osteoclastogenesis, suggesting that IL-33 is a potential regulator of OC differentiation [6]. Conversely, IL-33 overexpression in mice through systemic IL-33 adeno-associated virus (AAV) injections induced an increase in OC number in femurs and bone loss especially in the cortex [7]. The maxillary bone phenotype of IL-33 knockout (KO) male mice has not been described to date.

Under pathological conditions such as periodontitis, bone resorption outweighs bone formation leading to bone loss (uncoupling). Periodontitis is a widespread chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms [8]. It is characterized by progressive destruction of the tooth-supporting bone, i.e., the alveolar bone, which may result in severe tooth loss in patients. *Porphyromonas gingivalis* (*Pg*), an oral Gram-negative anaerobic bacterium, is considered a key periopathogen due to its ability to induce dysbiotic biofilm and inflammation [9]. Archetypal pro-inflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor (TNF)-α) produced by host cells seems crucial for the pathogenesis of periodontitis, perpetuating gingival inflammation and the subsequent alveolar bone resorption through the recruitment and activation of OCs [10]. More recently described, IL-33 has been suggested to have a pathogenic role in periodontitis. The gingival expression of IL-33 was higher in patients with periodontitis compared to healthy controls [3, 11]. This increase in IL-33 expression was also evidenced in a model of experimental periodontitis (EP) induced by *Pg*-soaked ligature and by oral gavage with *Pg* in mice. However, the role of IL-33 in alveolar bone loss associated with periodontitis remains unclear, since the induction of EP in IL-33 KO mice has not been performed to date.

The objectives of this study were to decipher the contribution of IL-33 (i) to bone homeostasis under physiological conditions and (ii) to alveolar bone loss associated with EP in IL-33 KO mice and their wildtype (WT) littermates.

Materials and Methods

WT and IL-33 KO mice

This study was approved by the Ethics Committee for Animal Experiment of Pays de la Loire (CEEA 2017.06) and conformed to the European Directive 2010/63/EU. To avoid genetic drift, IL-33 KO mice of the n generation were collected from Professor Bernhard Ryffel (Molecular Immunology, University of Orleans and CNRS, France). The breeder respected the rules of crossbreeding. We then generated heterozygotes which we crossed together, and we used the mice of this n+2 generation for all our experiments. The genetic status of IL-33 KO and WT mice with pure C57BL/6 genetic background (Charles River) was confirmed by polymerase chain reaction (PCR) (data not shown). The mice were housed in a specific pathogen-free facility under light- (12-h light/dark cycle), temperature- (22–25 °C), and humidity-controlled (50–60%) conditions. All animals were fed a regular diet and had access to water ad libitum.

Mice kept under physiological conditions

WT (6 males and 5 female) and IL-33 KO mice (6 males and 5 females) were sacrificed at 15–16 weeks of age by cervical dislocation for baseline measurements. Maxillae, femurs, and vertebrae were removed for micro-computed tomography (micro-CT) analyses. Femoral and tibial bone marrow cells were used for OC differentiation.

Mouse model of EP

We used a well-established ligature-induced EP model, as previously described (Fig. 1A) [3]. Briefly, after a washout period of antibiotics (sulfamethoxazole-trimethoprim) to reduce differences in the background oral flora of mice [3], EP was induced by placement of a silk ligature optionally soaked with *Pg* (ATCC 33277*)* in the gingival crevice around the maxillary left first molar (Fig. 1B). Then, 11- and 12-week-old mice were randomly assigned to three different groups (at least six mice for each genotype):

- Sham group: mice without ligature placement received a slight incision into the sulcular epithelium to mimic the ligature placement (7 WT: 3 males and 4 females, 7 KO: 3 males and 4 females)
- Lig group: an unsoaked ligature was placed (7 WT: 4 males and 3 females, 9 KO: 4 males and 5 females)
- *Pg*-Lig group: a *Pg*-soaked ligature was inserted (6 WT: 3 males and 3 females, 8 KO: 4 males and 4 females)

At 28 days, the animals were sacrificed by cardiac exsanguination under intraperitoneal anesthesia with xylazine and ketamine (10 and 80 mg/kg, respectively), and maxillae were removed for further analysis (micro-CT analyses, histological examinations).

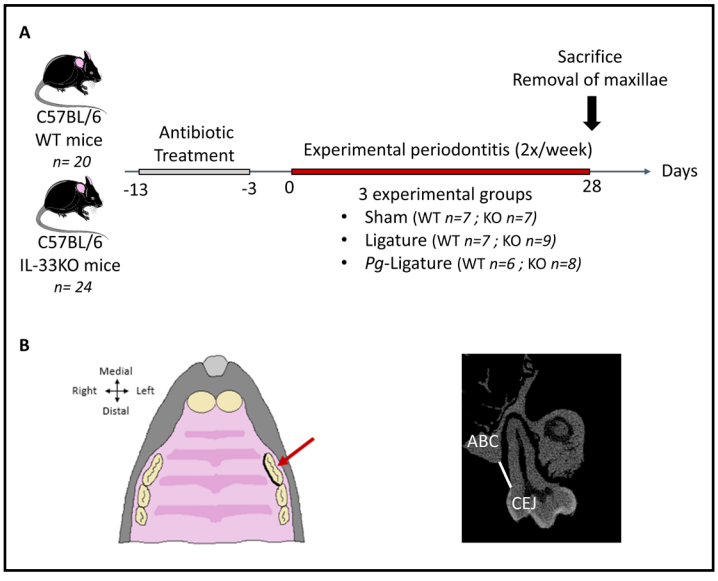

Fig. 1. Experimental protocol of ligature-induced periodontitis. Study design of experimental periodontitis (A). Schematic illustration of the placement of the ligature: the silk ligature was inserted and fixed in the gingival crevice around the first maxillary left molar on the palatine side (red arrow) (B). The distance between the palatal cementum–enamel junction (CEJ) of the maxillary left molar and the alveolar bone crest (ABC) was measured in the coronal plane (white) (C).

Bacterial and strain culture

Pg (ATCC 33277) was cultured at 37 °C on Schaedler agar plated with sheep blood (BD), in an oxygen-free atmosphere. After 10 days of culture, *Pg* colonies were selected and resuspended in brain–heart broth at 10^9 CFU/mL for ligature placement or at 10^5 CFU/mL for *Pg* injection. A 6/0 silk thread was placed in the suspension 24 h before the EP procedure.

Micro-computed tomography (micro-CT)

For *ex vivo* analysis, the bone samples were scanned by micro-CT (Skyscan 1272; Skyscan). Scans were acquired for each bone: maxilla (80 kV, 1 mm aluminum filter, 0.35° rotation angle, 12-µm resolution), lumbar vertebra (70 kV, 0.5-mm aluminum filter, 0.6° rotation angle, 12-µm resolution), and distal femur (60 kV, 0.25-mm aluminum filter, 0.6° rotation angle, 8.5-µm resolution).The NRecon software (Skyscan) was used for the 3D reconstruction: maxilla (smoothing=0, ring artifact=4, beam hardening=20%), lumbar vertebra (smoothing=0, ring artifact=4, beam hardening=10%) and distal femur (smoothing=0, ring artifact=4, beam hardening = 20%). Calibration was carried out with a known density of calcium hydroxyapatite phantoms (Bruker, Belgium).

The bone phenotype of 15- to 16-week-old mice (baseline) was analyzed on the distal femur (excluding the growth plate), the fifth lumbar vertebra, and the alveolar bone surrounding the first molar for the maxilla (at least five mice per group). The total bone mineral density (BMD: g/cm^2), trabecular bone volume/total volume ratio (BV/TV: %), bone volume (BV: mm^3), trabecular thickness (Tb.Th: µm), trabecular separation (Tb.Sp: µm), and trabecular number (Tb.N: $1/mm^2$) were assessed. The distance between the palatal cementum–enamel junction (CEJ) of the maxillary left first molar and the alveolar bone crest (ABC) was measured in the coronal plane to assess alveolar bone height (Fig. 1C). Ten consecutives sections were analyzed per animal. All measurements were performed by two independent operators.

Histological analysis

After fixation in 4% paraformaldehyde at 4 °C for 1 day, maxillae were decalcified in EDTA 0.5 M at 4 °C for 3 weeks. After dehydration, the samples were embedded in paraffin. Histological staining was performed on 4-µm-thick sections in the frontal plane. Masson-Goldner trichrome and Toluidine blue (pH: 3.8) staining were used on two sections for each sample. Automated whole-slide imaging was performed using the NanoZoomer 2.0 (Hamamatsu). For each section, resorption parameters were recorded with the ImageJ software including the number of trabecular OCs (OCL) in contact with bone (N.Oc/BS: 1/µm), the OC surface (Oc.S/BS: %), and the average length of the zone of contact per OC (Oc.S/N.Oc: µm), which is a sensitive indicator of OC activity [12]. All the measurements were performed by two independent operators.

OC differentiation

Bone marrow cells were aseptically collected from the femur and tibia, depleted of red blood cells in a lysate buffer (Sigma Aldrich), and plated in minimum essential medium α–containing 10% fetal bovine serum in 8-well Labtek slides (4.10^5 cells/well) for tartrate-resistant acid phosphatase (TRAP) staining. Cells were resuspended and cultured in α-MEM with 10% FCS (Hyclone, GE healthcare), 1% penicillin–streptomycin, and 1% glutamine at 37 °C in 5% CO_2. On day one, the media were changed with the same medium in the presence of 25 ng/mL recombinant mouse M-CSF (PeproTech) and with an additional 100 ng/mL recombinant mouse RANK-L (PeproTech) to induced OC differentiation. For LPS-*Pg* Standard (SD) stimulation, 5 µg/mL was added to the media on day 5 and on every second day thereafter up to the end of OC differentiation. Cells were incubated for 14 days at 37 °C in 5% CO_2 and the medium was changed every other day. At the end of the culture period, cells were fixed with 4% paraformaldehyde in PBS and stained for TRAP using the Leukocyte Acid Phosphatase kit (Sigma-Aldrich) to identify TRAP-positive cells. Multinucleated cells with three nuclei or more were considered as OCs and the ratio of TRAP-positive multinucleated cells compared to the total TRAP-positive cells was calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Software (v.6). All data are presented as mean ± standard derivation (SD). The differences between groups were analyzed in a two by two using an unpaired Student *t* test or two-way analysis of variance (number of groups greater than 2) followed by the Bonferroni post hoc test if the differences were significant. Statistical significance was set at $p < 0.05$.

Results

IL-33 deficiency affected bone phenotype in a site- and sex-dependent manner under physiological conditions

First, to assess the potential role of IL-33 on bone phenotype under physiological conditions, the BV/TV of the maxilla, femur, and vertebra of 15- to 16-week-old WT and IL-33 KO mice were determined. The IL-33 KO mice were not different in weight from WT mice [7]. In the maxillary bone, male and female IL-33 KO mice had a higher BV/TV compared to their WT littermates (Fig. 2A and 2B). This increase in bone mass can be explained by the Tb.Th, which was significantly higher in the maxilla in both male and female IL-33 KO mice (Supplementary Table S1), but also by a decreasing trend in Tb.Sp that was not significant (for all supplementary material see www.cellphysiolbiochem.com). Mature bone and lamellar bone formation with typical extracellular matrix embedded osteocytes were observed in both WT and IL-33KO males and females (Supplementary Fig. S1). In the femur, no significant difference in BV/TV or in the trabecular parameters was found between WT and IL-33 KO mice (Fig. 2C and 2D). Lastly, although the increase in BV/TV observed in the fifth lumbar vertebra was not significant (Fig. 2E and F), Tb.Th was significantly higher in IL-33 KO animals. Some differences in BMD were recorded between WT and IL-33 KO male mice, except in the maxilla (Supplementary Table S1).

IL-33 deficiency did not interfere with alveolar bone measurement (CEJ/ABC) under physiological conditions

Using 2D micro-CT, the CEJ/ABC distance was assessed to evaluate the height of the alveolar bone in the palatal region of the first maxillary molar. No significant difference was observed in CEJ/ABC distance between 15- to 16-week-old IL-33 KO and WT male and female mice under physiological conditions.

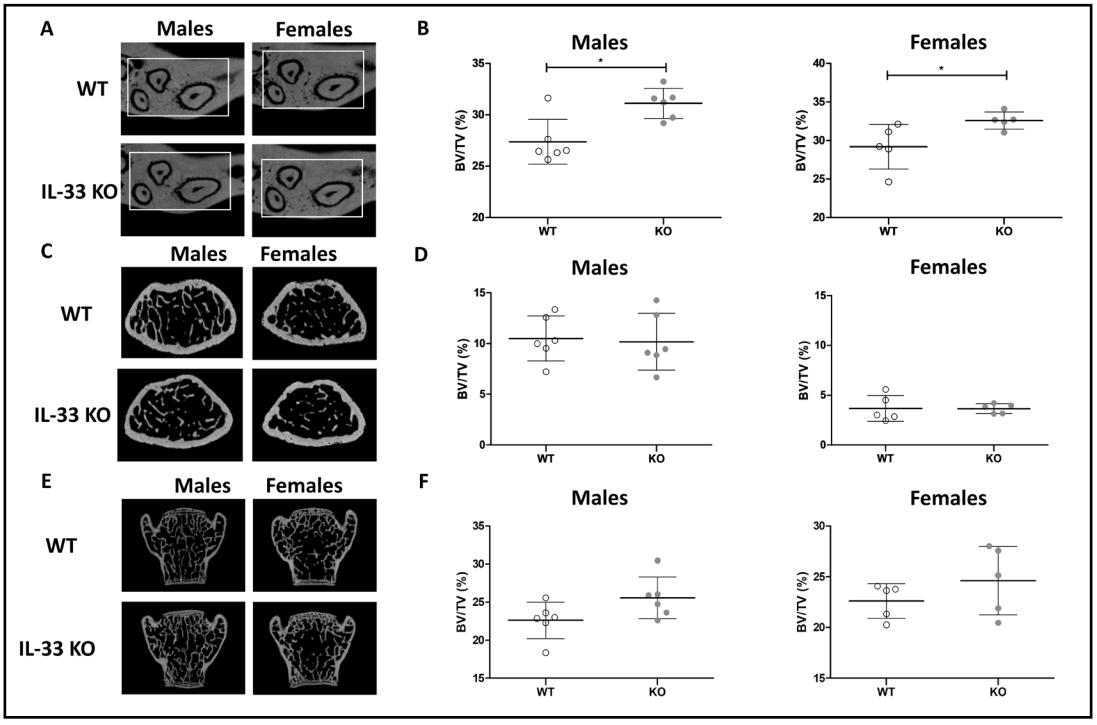

Fig. 2. Bone phenotype of 15- to 16-week-old WT and IL-33 KO mice. Micro-computed tomography (micro-CT) analyses were performed of the maxillary bone (A) and (B), femur (C) and (D), and fifth lumbar vertebra (E) and (F). Representative micro-CT sections and the percentage of trabecular bone/total volume (BV/TV) are presented. Data are shown as mean ± SD; n ≥ 5 per group. *p <0.05. (Student t test).

IL-33 deficiency increased the severity of EP induced by Pg ligature only in females

We then assessed whether IL-33 deficiency could be involved in alveolar bone loss induced by EP. Around the untreated maxillary right first molar no significant difference in the CEJ/ABC distance was observed between the 3 experimental groups (sham, Lig, and *Pg*-Lig) in males or in females, for both genotypes (data not show). As expected, in WT males (Fig. 3A and 3B) and females (Fig. 3C and 3D), ligature placement for 4 weeks around the maxillary left first molar (Lig and *Pg*-Lig groups) induced a significant increase in the CEJ/ABC distance indicating significant alveolar bone loss compared to the respective sham groups. In female and male IL-33 KO mice, ligature placement also induced a significant increase in the CEJ/ABC distance compared with animals in the sham group. No significant difference was evidenced between the Lig and *Pg*-Lig groups, in males or in females, for both genotypes, indicating that soaking the ligature with *Pg* had no additional effect on the alveolar bone loss than ligature alone.

In males, for each experimental group (sham, Lig, and *Pg*-Lig), no significant difference was found between the CEJ/ABC distance measured in WT and IL-33 KO mice (Fig. 3B). Interestingly, in female mice, the CEJ/ABC distance was significantly higher (+20%) in alveolar bone loss in *Pg*-Lig IL-33 KO mice versus WT mice, but it was not significantly different between IL-33 KO and WT mice in the Lig groups and sham groups (Fig. 3D).

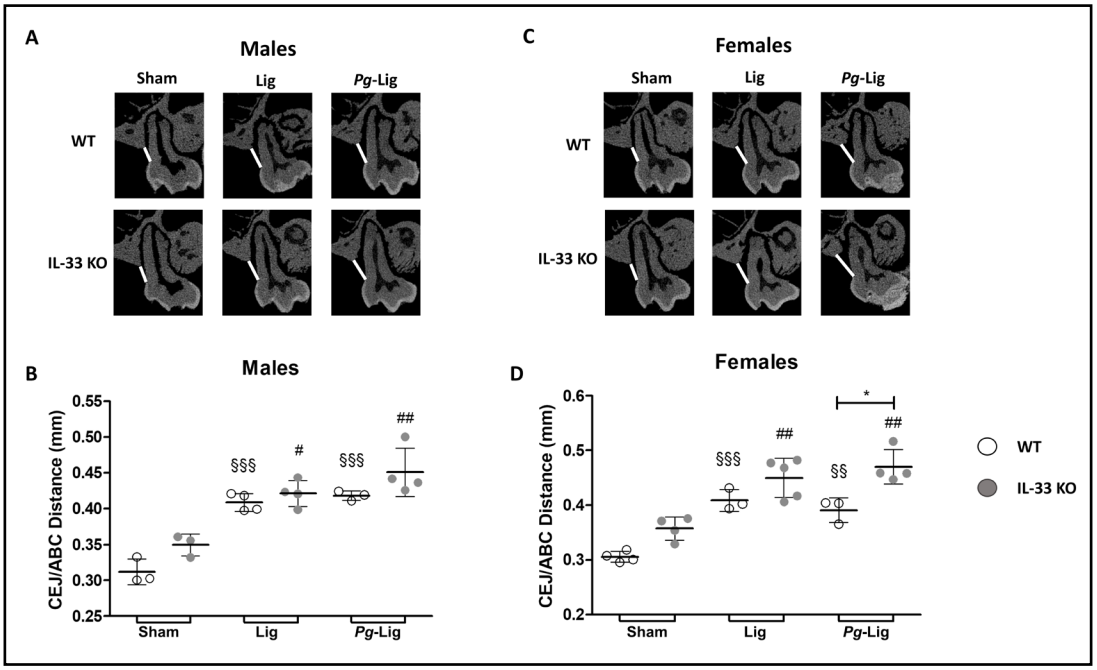

Fig. 3. Effect of IL-33 deficiency on alveolar bone loss in a ligature-induced murine model of EP. Four weeks after ligature placement or sham operation, alveolar bone CEJ/ABC distance was assessed using 2D micro-CT in male (A) and (B) and female (C) and (D) mice. Data are shown as mean ± SD; n = 3–5 per experimental groups. WT-*Pg*-Lig vs. Representative micro-CT sections in the coronal plane are presented in male (A) and female (C) with a white line to indicate the distance between the palatal cementum–enamel junction (CEJ) of the maxillary left molar and the alveolar bone crest (ABC). IL-33 KO-*Pg*-Lig: *p<0.05; WT-Lig and WT *Pg*-Lig vs. WT-sham: §§*p*<0.01 §§§*p*<0.001; IL-33 KO-Lig and IL-33 KO-*Pg*-Lig vs. IL-33 KO-sham: #*p*<0.05, ##*p*<0.01. (One-way analysis of variance with Bonferroni multiple comparison post hoc test).

In the Pg-Lig groups, IL-33 deficiency increased the number of OCs and the resorption surface in females

To explain the higher bone resorption observed in the *Pg*-Lig female IL-33 KO mice, osteoclastic parameters were analyzed on the histological maxillary sections stained with toluidine blue (Fig. 4). In the *Pg*-Lig group, the number of OCs (Fig. 4B) and the resorption surface (Fig. 4C) were significantly higher (34.8%) in IL-33 KO female mice, whereas no difference was recorded regarding the mean length of the contact zone between OC and alveolar bone (Fig. 4D).

IL-33 deficiency enhanced OC differentiation in vitro in presence of LPS-Pg

To analyze the *in vitro* effect of IL-33 KO on osteoclastogenesis, bone marrow progenitors were harvested from WT and IL-33 KO female mice. Differentiation of WT and KO bone marrow progenitors was similar, and the percentage of TRAP-positive cells with more than three nuclei was not significantly different between the two genotypes (Fig. 5). The addition of LPS-*Pg* SD in the culture medium enhanced osteoclastogenesis significantly in both genotypes, with a slight but significant 2.08-fold increase in WT cells and 2.78-fold increase in IL-33 KO cells.

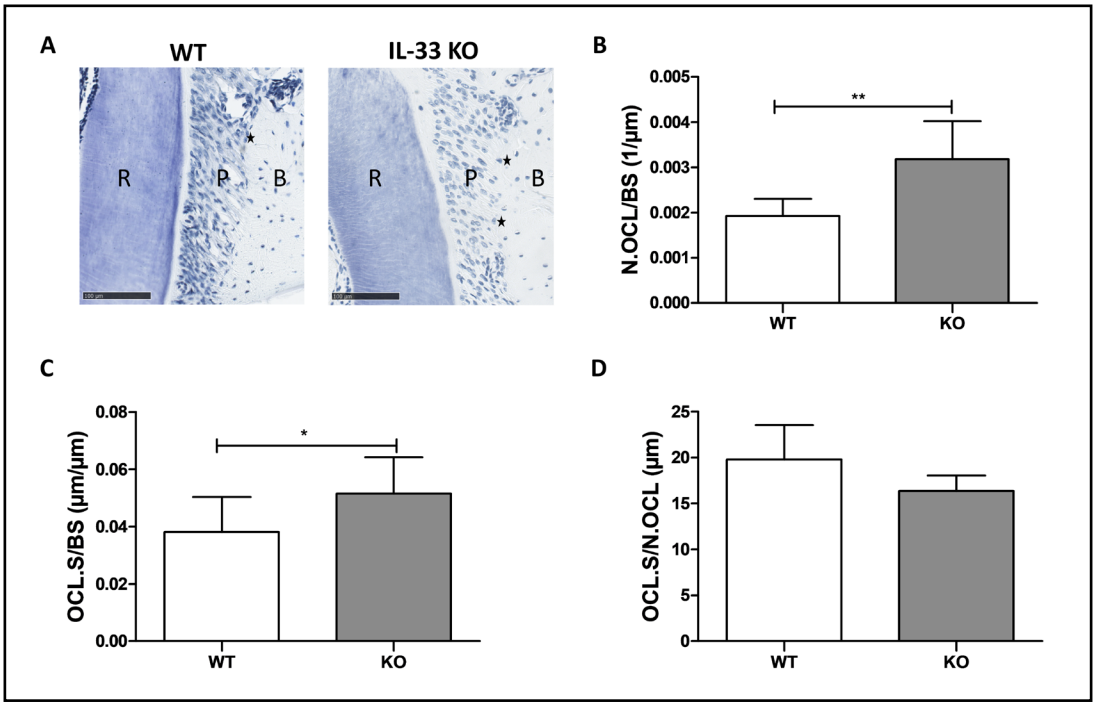

Fig. 4. Effect of IL-33 deficiency on osteoclastic parameters measured in maxilary bone of *Pg*-Lig females. The osteoclastic parameters were assessed on maxillary sections after toluidine blue staining. Representative histological section, R: root of the tooth, P: periodontal ligament, , B: Bone, and ✶: osteoclast, scale bar = 100 µm. The number of OCs (N.OCL) (B), the OC surface (OCL.S/BS) (C), and the average length of the zone of contact per OC (OCL.S/N.OCL) (D) are presented. Data are shown as mean ± SD n≥3; *p<0.05, **p<0.01. (Student t test).

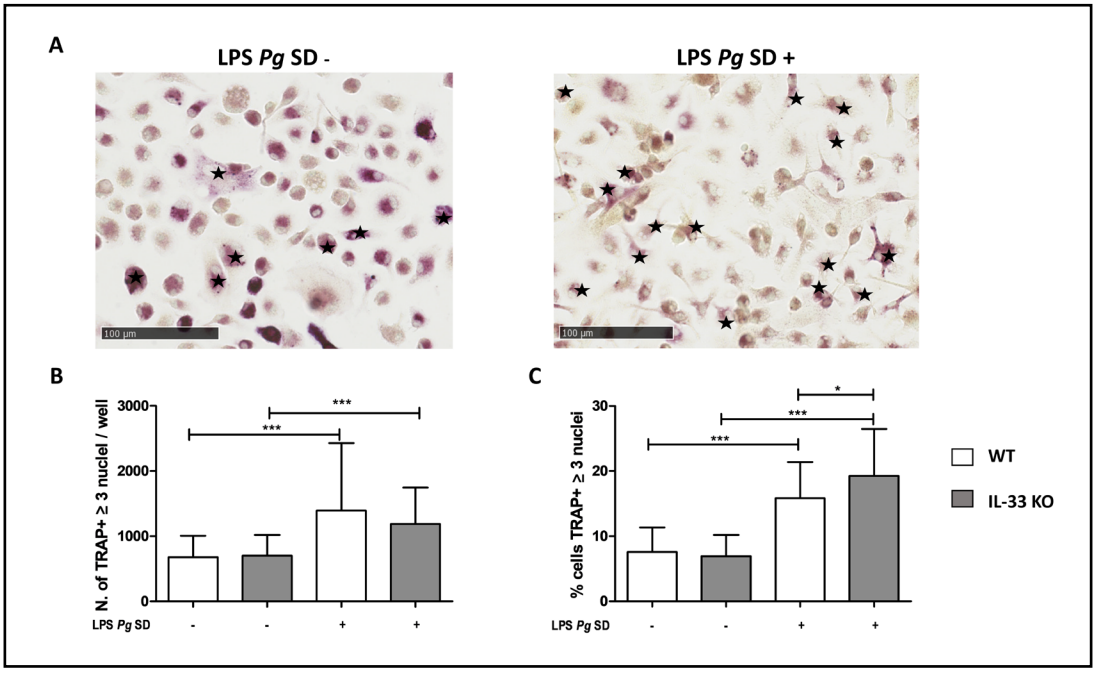

Fig. 5. Effect of IL-33 deficiency on in vitro osteoclastic differentiation with or without LPS-Pg standard stimulation. Osteoclastic bone marrow progenitors were harvested from WT and IL-33 KO female mice. (A) Representative microscopic view, TRAP+ cells are stained in violet and ✶indicates osteoclast. (B) Number of TRAP+ cells with 3 or more nuclei per well. (C) Percentage of TRAP+ cell with 3 or more nuclei over the number of TRAP+ cells. Data are shown as mean ± SD of four independent experiments (biological replicates); *p<0.05, ***p<0.001. (One-way analysis of variance with Bonferroni multiple comparison post hoc test).

Discussion

For the age studied and under physiological conditions, IL-33 was involved in the bone phenotype of mice in a site- and sex-dependent manner. Nevertheless, no difference was found for the alveolar bone measurement regarding the CEJ/ABC between IL-33 KO and WT mice. The ligature model of EP used in this study induced significant alveolar bone loss in WT and IL-33 KO mice. IL-33 deficiency increased the severity of EP induced by ligature soaked with *Pg*, a key periopathogen, but only in female mice mainly through an increase in the OC number and the resorption surface. These findings were corroborated by *in vitro* osteoclastic differentiation assays in the presence of LPS-*Pg*, showing that TRAP-positive multinucleated cells were more numerous when OC progenitors originated from female mice.

The putative role of IL-33 in bone phenotype at steady-state is unclear. Here, we showed for the first time that IL-33 deficiency increased alveolar bone mass in the maxilla in 15- to 16-week-old male and female mice, suggesting an osteoprotective role for IL-33. Conversely, Macari et al. reported reduced BV/TV in the maxilla of younger 8-week-old C57BL/6 IL-33 KO female mice [13]. However, it should be underlined that skeletal maturity in C57BL/6 mice is not reached at 8 weeks of age and that investigating adult bone phenotype should be performed in older mice [14]. There are also few points of comparison in the literature [7, 13] regarding other bone sites (femur, fifth lumbar vertebra). Overall, these results support the site- and sex-dependent involvement of IL-33 in the bone phenotype under physiological conditions [7]. The different embryological origins (maxilla from viscerocranium, femur from lateral lamina mesoblast, and vertebra from para-axial mesoblast) could explain this maxilla site-dependent involvement of IL-33, while the sex-dependent involvement of IL-33 may be related to estrogen [13].

We found evidence that IL-33 deficiency is not sufficient to block the induction of alveolar bone loss induced by the apposition of a ligature (soaked or not with *Pg*). Malcolm et al. showed that mice lacking ST2 (ST2KO) did not exhibit significant alveolar bone loss induced by oral gavage with *Pg* [11]. Thus, it seems that unlike IL-33, its receptor ST2 is required to induce murine EP. This discrepancy could be mainly attributed to differences in the genetic background of mice (C57Bl/6 vs. BALB/c), in the EP mice models (ligature vs. gavage), and also in the *Pg* strains (ATCC33277 vs. W83) used. In addition, the existence of another unknown ligand for ST2 should be considered, as this has already been proposed to explain discrepant observations in IL-33 and ST2KO mice in a model of K/BxN serum transfer-induced arthritis [15].

In this study, we did not record a cumulative effect of *Pg* on the induction of alveolar bone loss (no difference between ligature soaked with *Pg* and *Pg* free ligature) conversely to a previous work [3]. As previously reported [16], the genetic background of the mice could explain this difference as C57BL/6 mice were used in this study and CD1 Swiss mice in the previous work.

In the present study we found that in female mice, IL-33 deficiency increased the severity of alveolar bone loss induced by *Pg* ligature whereas it had no effect on *Pg*-free ligature. *Pg* is one of the key periopathogens involved in the onset and progression of periodontitis both in humans and in mouse models [17, 18]. *In vitro, Pg* enhances the expression of IL-33 in oral epithelial cells, the first cell population in contact with periopathogens, which could explain the impact of IL-33 deficiency only in the presence of *Pg* [3, 19]. Consistently with our micro-CT alveolar bone measurements, our *in vivo* histological analyses of the maxilla showed a higher number of OCs and an increased resorption surface in IL-33 KO than in WT females in the presence of *Pg*. Thus, our results indicate an osteoprotective role for IL-33 in female mice in the context of bacterial infection, since in its absence the alveolar bone loss is exacerbated. This role may be linked to estrogen, as already mentioned by Macari and colleagues [13]. Interestingly, an osteoprotective role for IL-33 has been evidenced in a mouse model of rheumatoid arthritis [20], a disease sharing immunopathological similarities with periodontitis.

The resorption of bone tissue is a unique ability of OCs, which are formed by the differentiation and fusion of precursor cells derived from the monocyte/macrophage lineage [5]. IL-33 deficiency did not affect *in vitro* differentiation of OC precursors from the bone marrow of mice without the context of bacterial infection, as the number of TRAP-positive multinucleated cells generated was similar between OC progenitors from IL-33 KO and from WT mice. This is in agreement with the only study in the literature that performed a comparable assay with bone marrow cells from arthritic IL-33 KO and WT mice [21]. Other studies have focused on the effect of IL-33 on various OC progenitors both in humans (peripheral blood $CD14^+$ monocytes, bone marrow $CD11b^+$ cells, cord blood mononuclear cells) and in mice (total bone marrow cells, bone marrow $CD11b^+$cells, spleen cells, RAW 264.7) and showed conflicting results [20, 22–26]. Taken together, the findings from these studies indicated that IL-33 exhibits a weak osteoclastogenic activity.

To mimic the bacterial environment of periodontitis *in vitro*, we stimulated bone marrow cells with lipopolysaccharide (LPS) from *Pg*. This represents a major virulence factor of *Pg* involved in the pathogenesis of periodontitis mainly through TLR 4 stimulation on host cells [27]. We showed that LPS-*Pg* enhanced the number of TRAP-positive multinucleated cells obtained from RANKL-primed bone marrow cells in IL-33 KO and WT female mice. In accordance with this result, AlQranei et al. reported that LPS-*Pg* stimulated OC differentiation from a RANKL-primed murine macrophage cell line (RAW 264.7) through the secretion of the pro-osteoclastogenic cytokine TNF-α via TLR4 signaling [28]. In this process, RANKL was only required to shift macrophages toward an osteoclastic-like phenotype, which then enables these cells to promote LPS-*Pg*-induced osteoclastogenesis. As we used whole bone marrow cells in our experiments, a complementary explanation for the LPS-*Pg* pro-osteoclastogenic effect could be an indirect effect via stimulation of stromal cells to secrete pro-inflammatory cytokines such as IL-1 or IL-6 [29].

Several mechanisms by which IL-33 inhibits pathological bone loss and osteoclastogenesis have been described in the literature. IL-33 has been shown to protect transgenic mice overexpressing human TNF-α from inflammatory bone loss by skewing the induction of OC precursors toward alternatively activated macrophages and dendritic cells and by stimulating the systemic secretion of anti-osteoclastogenic cytokines (GM-CSF, IL-4, and IFN-γ) [20]. Recently, it was also reported that IL-33 inhibited TNF-α-induced bone resorption and osteoclastogenesis in a mouse calvarial model through inhibition of the phosphorylation of IκB in OC precursors [30].

Therefore, further experiments are needed to decipher the potential role of IL-33 in periodontitis. They should describe the macro-characteristics of periodontitis such as the level of attachment and the periodontal pocket depth using for example histomorphometric assessment. Inflammatory cells and mediators should also be assessed in IL-33 KO mice as compared to WT mice, with particular attention to the main cytokines involved in bone resorption such as RANKL. The use of IL-33/ST2 double-KO mice may finally help clarify its involvement in the pathogenesis of EP.

Conclusion

In conclusion, under physiological conditions, IL-33 deficiency increased BV/TV in maxillary bone in male and female mice, but not in the femur and fifth lumbar vertebra, suggesting an osteoprotective role for IL-33 in a site-dependent manner. In EP, IL-33 deficiency increased the severity of the alveolar bone loss induced by *Pg*-ligature in female mice via an increase in the number of OCs, suggesting a sex-dependent role under pathological conditions. Moreover, *in vitro*, IL-33 deficiency enhanced osteoclastic differentiation from bone marrow OC progenitors in female mice in the presence of LPS-*Pg*. Therefore, the potential role of IL-33 in the pathogenesis of periodontitis remains elusive and warrants more investigation using IL-33/ST2KO mice, for example.

Acknowledgements

The authors thank Bernhard Ryffel and Marc Le Bert for providing IL-33 KO mice as well as Caroline Vignes (SC3M Facility), Sarah Beck, Aida Gutierrez-Corrales, and José-Ramón Corcuera-Flores for technical assistance.

Author Contributions

AL, VG, PL and AC contributed to the conception or design of the work. AL, VG, BH, OL, JC, PL and AC contributed to the investigation. AL, VG, PL and AC drafted the manuscript. BH, OL, JV, JC, JG critically revised the manuscript; All authors gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

Funding

The study was supported by Région des pays de la Loire (grant 2012-05775-05776) and by the Nantes School of Dentistry.

Statement of Ethics

Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

Disclosure Statement

The authors have no conflicts of interest to declare.

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