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# Age-related changes of CD4$^+$ T cell migration and cytokine expression in germ-free and SPF mice periodontium

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ABSTRACT

*Objective:* Increasing age is a potential risk factor for periodontal tissue breakdown, which may be affected by commensal flora. The aim of this study evaluated age-related changes in CD4$^+$ T cells, C-C chemokine ligand 5 (CCL5), interleukin (IL)-17A, and receptor activator of nuclear factor-kappa B ligand (RANKL) expression using germ-free (GF) and conventionally reared (SPF) mice.

*Design:* GF and SPF mice at 8 (n = 6/group) and 22 weeks old (n = 6/group) were used. Immunohistochemical analyses were performed to determine the effects of aging on protein expression in periodontal tissues. Age-related changes in alveolar bone were quantified using micro-CT analysis.

*Results:* SPF mice, but not GF mice, showed an age-related increase in alveolar bone loss ($P < 0.01$). SPF mice at 22 weeks of age increased expression of CD4$^+$ T cells, CCL5, IL-17A, and RANKL compared to those at 8 weeks of age in connective tissue and alveolar bone surface ($P < 0.01$). Furthermore, there was increased CD4$^+$ T cells, which were co-expressed with IL-17A and RANKL in SPF mice at 22 weeks of age. On the other hand, the GF mice did not show any significant differences in CD4$^+$ T cells, CCL5, IL-17A and RANKL expression between the two age groups.

*Conclusions:* SPF mice induced an age-related increase in CD4$^+$ T cells co- expressed with IL-17A and RANKL, with occurring alveolar bone loss. In contrast, GF mice did not show age-related changes in CD4$^+$ T cell migration and cytokine expression.

## 1. Introduction

Aging is a complex phenomenon in which a progressive deterioration of function occurs in a variety of organs, including bone (Razi et al., 2015). As a function of aging, bone displays distinct changes in structural and functional characteristics that lead to distinct pathologies (Rauchner, Kholsa, & Hofbauer, 2011; Zhang et al., 2008). In a young adult skeleton, a steady state of bone is maintained through the tightly coupled processes of bone resorption and formation in bone metabolism (Boskey & Coleman, 2010). However, during the process of aging, the amounts of bone decreases due to the tipping of this delicate balance towards enhanced resorption coupled with decreased bone formation (Carrington, 2005). The incidence of occurring alveolar bone loss also increases with age, and the highest rate is found among the elderly (Oz & Puleo, 2011). Increasing age could potentially be a risk factor for occurring alveolar bone loss (Liang, Hosur, Domon, & Hajishengallis, 2010).

The contribution of commensal flora to the structure and function of periodontal tissue has been investigated (Hajishengallis et al., 2011; Irie et al., 2015). Our previous report showed that alveolar bone loss was greater in conventionally reared – specific pathogen free (SPF) mice when compared to germ-free (GF) mice at a young age (Irie, Novince, &

*Abbreviations:* CCL5, C-C chemokine ligand 5; IL, interleukin; RANKL, receptor activator of nuclear factor-kappa B ligand; GF, germ free; SPF, specific pathogen free; CT, computed tomography; MRS, de Man Rogosa and sharpe; BV, bone volume; TV, total volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; CEJ, cement-enamel junction; ABC, alveolar bone crest; M, molar; PCR, polymerase-chain reaction; AMV, avian myeloblastosis virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRAP, tartrate-resistant acid phosphatase

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Darveau, 2014). Therefore, it is suggested that commensal flora could stimulate age-related alveolar bone loss. However, our previous study used only one stage (12 weeks of age) of mice. Then, it is further required to confirm age-related bone loss using the GF and SPF mice with different two ages.

In addition, the mechanism of age-related alveolar bone loss by commensal flora needs to be elucidated. Recent reports have demonstrated that gut microbiota influence the development of the host immune system, and this condition make the major regulator of bone mass (Sjögren et al., 2012). GF mice have a reduced number of $CD4^+$ T cells in the spleen and femur, suggesting that gut microbiota are capable of shaping systemic immunity (Mazmanian et al., 2005). Furthermore, the absence of gut microbiota leads to an increased bone mass associated with a reduced number of osteoclasts in trabecular bone (Oz & Puleo, 2011). This increase in bone mass may occur because the absence of gut microbiota causes a decrease in the number of $CD4^+$ T cells recirculating in the blood and spleen, which results in a decrease in $CD4^+$ T cells in bone marrow and a subsequent decrease in osteoclastogenesis (Ohlsson & Sjögren, 2015). Therefore, it is suggested that $CD4^+$ T cells play a key role in bone loss stimulated by commensal flora.

Commensal flora can also stimulate the migration of $CD4^+$ T cells, with increasing (IL)-17A and receptor activator of nuclear factor-kappa B ligand (RANKL) expression in the normal periodontium (Hajishengallis et al., 2011; Irie et al., 2014). Increases in IL-17A and RANKL expression are involved in alveolar bone loss (Carrington, 2005). These indicate that commensal flora could induce alveolar bone loss, with increasing $CD4^+$ T cell, IL-17, and RANKL expression. However, it remains unclear how age-related increases in $CD4^+$ T cell and cytokines expression differ between mice with and without oral commensal flora. In addition, although it is known the migration of $CD4^+$ T cells is regulated by the expression of chemokines (e.g. Chemokine (C-C motif) ligand 5 [CCL5]) (Walcher et al., 2010), the effects of commensal flora on CCL5 expression is also unknown.

Comparisons between GF and SPF mice are acceptable to see through differences in histopathological changes with and without commensal flora (Mazmanian, Liu, Tzianabos, & Kasper, 2005). In the present work, we hypothesized that age-related changes in $CD4^+$ T cells migration and chemokine and cytokine expression in GF mice might be lower than those in SPF mice. Age-related bone loss is often seen from mature age (e.g. 22 weeks of age). Therefore, the aim of this study was to compare age-related changes in $CD4^+$ T cells, CCL5, IL-17A, and RANKL expression in GF and SPF mice at 8 and 22 weeks of age (from a young to a mature age).

## 2. Materials and methods

### 2.1. Mice

Animal experiments were performed in the Central Institute of Experimental Animals (CIEA; Kanagawa, Japan). SPF and GF IQI/Jic mice were housed air in a Trexler-type flexible film isolator in a standard germ-free state and were screened on a weekly basis for germ-free status by sterile feces sampling and culturing on MRS agar plates under aerobic and anaerobic conditions. Mice were housed in an air-conditioned room (temperature 24 ± 1 °C) with a controlled light/dark cycle (light on between 6:30 AM and 7:00 PM). Sterile food and water were available *ad libitum*. Both GF and SPF mice were sacrificed at 8 and 22 weeks of age (n = 6 for each group) (Chen et al., 2013). The rearing of the mice and the animal experiments were conducted according to the institutional rules following approval from the Animal Experiment Committee of Central Institute for Experimental Animals in Japan.

### 2.2. Sampling

For histological analysis, the left maxillary regions were resected *en bloc* from each mouse and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 day. Periodontal biopsy samples of the right maxillary molar regions were homogenized in a frozen cell crusher (Microtec Co., Chiba, Japan) and were used for real-time polymerase chain-reaction (PCR). Thereafter, the right maxillary regions were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) to measure the structural changes of alveolar bone. To assess the systemic effect on bone, femur and plasma from each group of mice were also harvested.

### 2.3. Micro-computed tomography of imaging and analysis

#### 2.3.1. Bone mineral density in femur

The bone mineral density of femur was determined ex vivo by scanning with a micro-computed tomography (μCT) device (R_mCT: Rigaku, Tokyo, Japan) with the following settings: 1) slice thickness, 50 μm; 2) voltage, 90 kV; and 3) electrical current, 150 mA. Three-dimensional images were obtained using a bone analysis system (TRI/3D-BON: Ratoc, Tokyo, Japan) to evaluate the changes in bone and the structural parameters. The structural parameters of trabecular bone were percentage of bone volume/total volume (BV/TV: %), trabecular thickness (Tb.Th: μm), trabecular number (Tb.N: 1/mm) and trabecular separation (Tb.Sp: μm) (Bouxsein et al., 2010).

### 2.4. Analysis of structural changes in alveolar bone

The right maxilla of each mouse was scanned using a μCT device with the following settings: 1) slice thickness, 50 μm; 2) voltage, 90 kV; and 3) electrical current, 150 mA. Three-dimensional images were obtained using a bone analysis system. The distance between the cement-enamel junction (CEJ) and the alveolar bone crest (ABC) was measured at five points for each molar (first molar [M1] to the third molar [M3] of the maxillae) and the distances of 5 points was summed as alveolar bone loss (Fig. 1a) (Koide et al., 2013). The furcation area of the first molar root was taken for analysis of the BV/TV, Tb.Th, Tb.N and Tb.Sp.

### 2.5. Histopathological analysis

The left maxillary jaws were dissected without disturbing the periodontal soft tissues. Specimens were embedded in paraffin, in a buccal-lingual orientation. Serial frontal sections (4-μm thick) from the mesial buccal root of the first molar to the distal buccal root of the third molar were cut parallel to the long axis of the teeth (sagittal). The paraffin-embedded sections were stained with hematoxylin and eosin or other stains, as described below.

We performed immunofluorescence labeling of the area around the alveolar bone surface to evaluate the expression of CD4, IL-17A, CCL5 and receptor activator of nuclear factor-kappa B ligand (RANKL) using rat-anti-CD4 monoclonal; sc-13573 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:300), rabbit-anti-IL-17A polyclonal; 13082-1-AP (Proteintech, Rosemont, IL, USA) (1:300), rabbit-anti-CCL5 polyclonal; bs-1324R (Bioss, Woburn, MA, USA) (1:200) and goat-anti-RANKL polyclonal; sc-7628 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:300) respectively, as the primary antibodies. No primary antibody controls were performed for each antibody of interest. The secondary antibody used was either anti-rat IgG (Alexa Fluor 488 Conjugate), anti-rabbit IgG (Alexa Fluor 594 Conjugate) or anti-goat IgG (Alexa Fluor 647 Conjugate) (Invitrogen, Carlsbad, CA, USA). Images were captured using all-in-one fluorescent microscope BZ-X700 (KEYENCE, Osaka, Japan). The CD4 and polymorphonuclear leucocytes in the connective tissue subjacent to the junctional epithelium were counted in two standard areas (0.05 mm × 0.1 mm each) at a magnification of ×400 (Endo et al., 2013).