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Original Article

Effect of age on orthodontic tooth movement in mice

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RANKL

Abstract *Background/purpose:* The number of middle-aged and elderly orthodontic patients is increasing due to changes in age composition. It is important to investigate the detailed mechanisms of bone remodeling in orthodontic tooth movement (OTM) in the elderly. However, there are few reports on the mechanism of tooth movement in the elderly. The purpose of the present study was to analyze OTM and osteoclastogenesis in aged mice and to elucidate the mechanism.
Materials and methods: It has been reported that tumor necrosis factor (TNF)-α plays an important role in osteoclast formation and OTM. First, 8-week-old and 78-week-old male C57BL/6J mice were subcutaneously injected with TNF-α into the calvaiae, and micro-CT, tartrate-resistant acid phosphatase (TRAP) staining, and real-time PCR were performed to evaluate osteoclast formation and bone resorption. Furthermore, osteoclastogenesis by TNF-α and receptor activator of nuclear factor-kappa B ligand (RANKL) using bone marrow cells was evaluated in vitro. Finally, a nickel-titanium closed-coil spring was attached, mesial movement of the maxillary left first molar was performed, and tooth movement distance and osteoclast formation were evaluated.
Results: Compared to 8-week-old mice, 78-week-old mice had decreased TNF-α-induced bone resorption, osteoclastogenesis, and TRAP and cathepsin K expression in the calvariae. In vitro osteoclast formation also decreased in 78-week-old mice. Furthermore, tooth movement distance and osteoclastogenesis were reduced.
Conclusion: OTM decreased in aged mice, which was shown to be caused by a decrease in osteoclastogenesis. Therefore, it was suggested that it is necessary to keep in mind that tooth movement may be suppressed when treating elderly patients.

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Introduction

Osteoclasts are the only cells that absorb bone in the body, and are known to differentiate from bone marrow cells. Osteoclasts play a role in maintaining homeostasis by resorbing bone. Macrophage colony-stimulating factor (M-CSF) was first discovered as a cytokine essential for osteoclastogenesis, and later receptor activator of nuclear factor-kappa B ligand (RANKL) was also found to be an essential cytokine.[1] Furthermore, it was later reported that tumor necrosis factor (TNF)-α can cause osteoclastogenesis without RANKL.[2–4]

In orthodontic tooth movement (OTM), when orthodontic forces are exerted on the teeth, osteoclasts appear on the compression side, where the periodontal ligaments are compressed, leading to the absorption of alveolar bone. Conversely, osteoblasts appear on the tension side, where the periodontal ligaments are stretched, resulting in new bone formation. RANKL[5,6] and TNF-α[7,8] are essential factors for osteoclast differentiation during OTM.

With the transition toward an aging society, the number of middle-aged and elderly individuals seeking orthodontic treatment has increased. Since bone metabolism is a major factor in orthodontic treatment, it is important to investigate the detailed mechanisms of bone remodeling in OTM in the elderly. In general, it is believed that treatment in adults takes longer than in children.[9–13] On the other hand, it has been shown that there was no significant difference of tooth displacement in orthodontic treatment between older patients and younger patients.[14] Although several experimental studies have been conducted on this relationship in rodents, the effect of aging on tooth movement remains controversial. Reports have shown much faster tooth movement in young rodents than in older animals,[15–18] while another study noted no significant difference in tooth movement rate with orthodontics.[19] Furthermore, there was no significant difference in histological changes, such as the number of osteoclasts, between young and adult rats during OTM.[20,21] Finally, it has been demonstrated that slow tooth movement in adult animals may be due to a delayed biological response.[22,23] Thus, the relationship between aging and OTM remains unclear, as well as the underlying mechanism.

The purpose of the present study was to analyze OTM and osteoclastogenesis in aged mice and to elucidate the mechanism.

Materials and methods

Experimental animals

Male C57BL6/J mice (78 weeks and 8 weeks old) were obtained from CLEA Japan (Tokyo, Japan). The mice were maintained in the Tohoku University animal facility. All animal experiment were performed in according to the guidelines of experimental animals of Tohoku University.

Materials

Mouse recombinant RANKL was purchased from PeproTech (Rocky Hill, NJ, USA) and mouse recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). In addition, mouse recombinant M-CSF was produced from an M–CSF–expressing cell line (CMG14-12) as described in a former study.[24]

Osteoclast culture

Bone marrow cells were collected from the isolated femurs and tibias of 8-week-old and 78-week-old male mice for in vitro osteoclast formation experiments. The bone marrow cells were cultured in α-MEM (Sigma-Aldrich, St. Louis, MO, USA), which included 10% fetal bovine serum (FBS) with M-CSF (100 ng/mL), for 3 days. The adherent cells were bone marrow macrophages as osteoclast precursors. Osteoclast precursors (5×10^4 cells) were seeded in a 96-well plate and cultured in a medium containing M-CSF (100 ng/mL) alone, or M-CSF (100 ng/mL) along with either TNF-α (100 ng/mL) or RANKL (100 ng/mL) for 5 days. The cells were stained with tartrate-resistant acid phosphatase (TRAP). TRAP solution was prepared by mixing acetate buffer (pH 5.0), 50 mM sodium tartrate, naphthol AS-MX phosphate (Sigma Aldrich), and Fast Red Violet LB salt (Sigma Aldrich). TRAP-positive multinucleated cells (≥3 nuclei) were counted as osteoclasts.[25]

Real-time polymerase chain reaction analysis

For in vivo experiments, the total RNAs from calvariae obtained from each mouse was purified by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). After synthesizing cDNA from the total RNA, real-time PCR was carried out. The primers in this study for real-time PCR were as follows: Cathepsin K (forward: 5′-GCAGAGGTGTGTACTATGA-3,' reverse: 5′-GCAGGCGTTGTTCTTATT-3′); TRAP (forward: 5′-AACTTGCGACCATTGTTA-3,' reverse: 5′-GGGGACCTTTCGTTGATGT-3′) and GAPDH (forward: 5′-GGTGGAGCCAAAAGGGTCA-3,' reverse: 5′-GGGGGCTAAGCAGTTGGT-3′). All reactions were normalized to GAPDH as the reference gene.[26]

Preparation for histological evaluation

As per the protocol described in our previous study,[25] the 8-week-old (young) and 78-week-old (old) male mice were subcutaneously injected with phosphate-buffered saline (PBS) or TNF-α (3 μg/day) for 5 days to induce osteoclast formation. The histological preparation and osteoclast counting were described previously.[25]

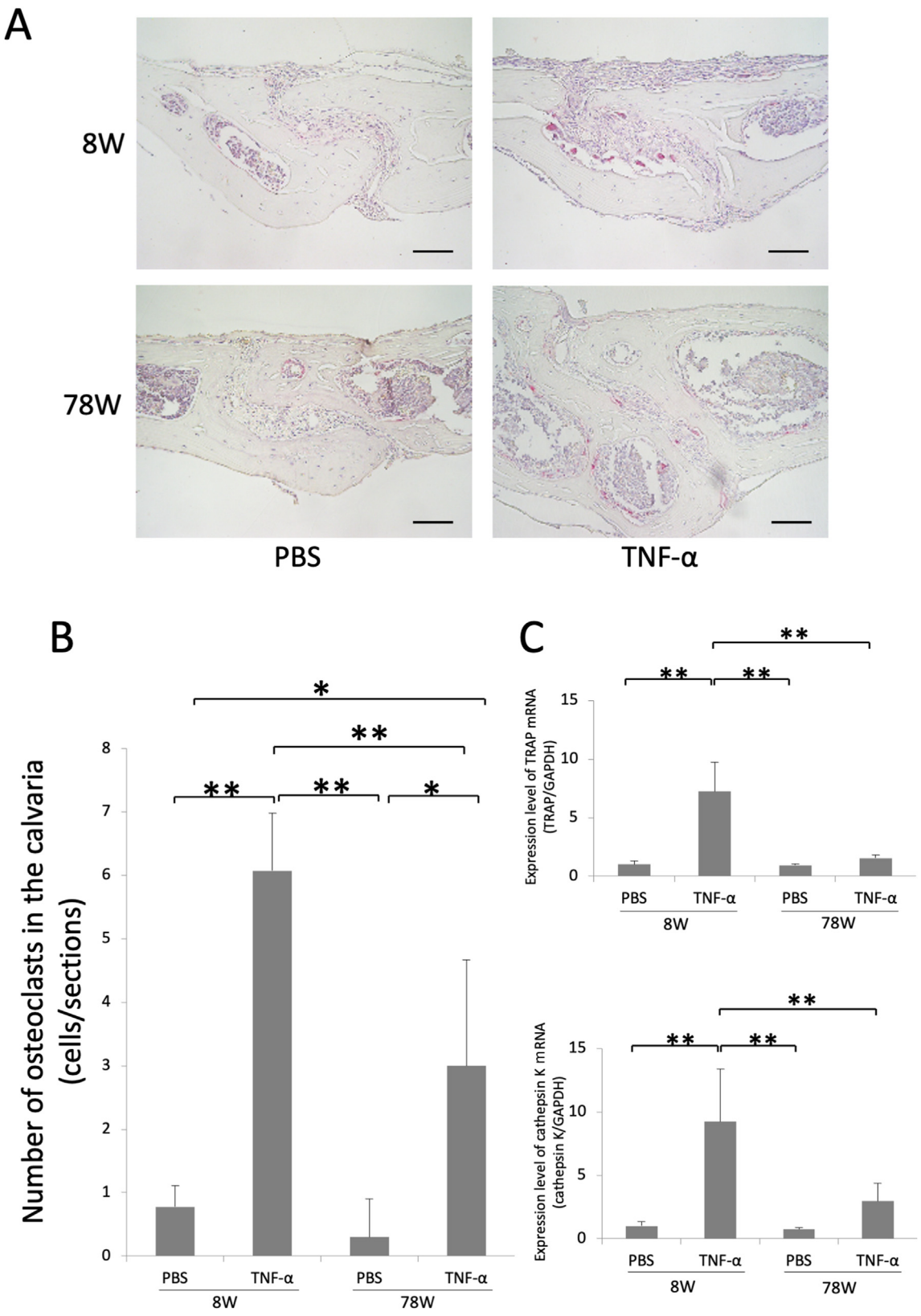

Figure 1 Tumor necrosis factor-α-induced osteoclast formation is reduced in aged mice compared to young mice in vivo. (A) Image of TRAP-stained sections of calvariae in 78-weeks-old (78W) and 8-weeks-old (8W) mice. Calvariae from both old mice and young mice were cut into 5-μm-thick sections. (B) Numbers of osteoclasts in the sagittal suture of old and young mice injected with tumor necrosis factor (TNF)-α. (C) Tartrate-resistant acid phosphatase (TRAP) and cathepsin K mRNA of calvariae in TNF-α-injected old and young mice evaluated by real-time PCR. The error bars represent the SD. The statistical significance was determined by the Tukey—Kramer test (*$P < 0.05$, **$P < 0.01$; $n = 4$). Scale bar = 50 μm. Phosphate-buffered saline (PBS).

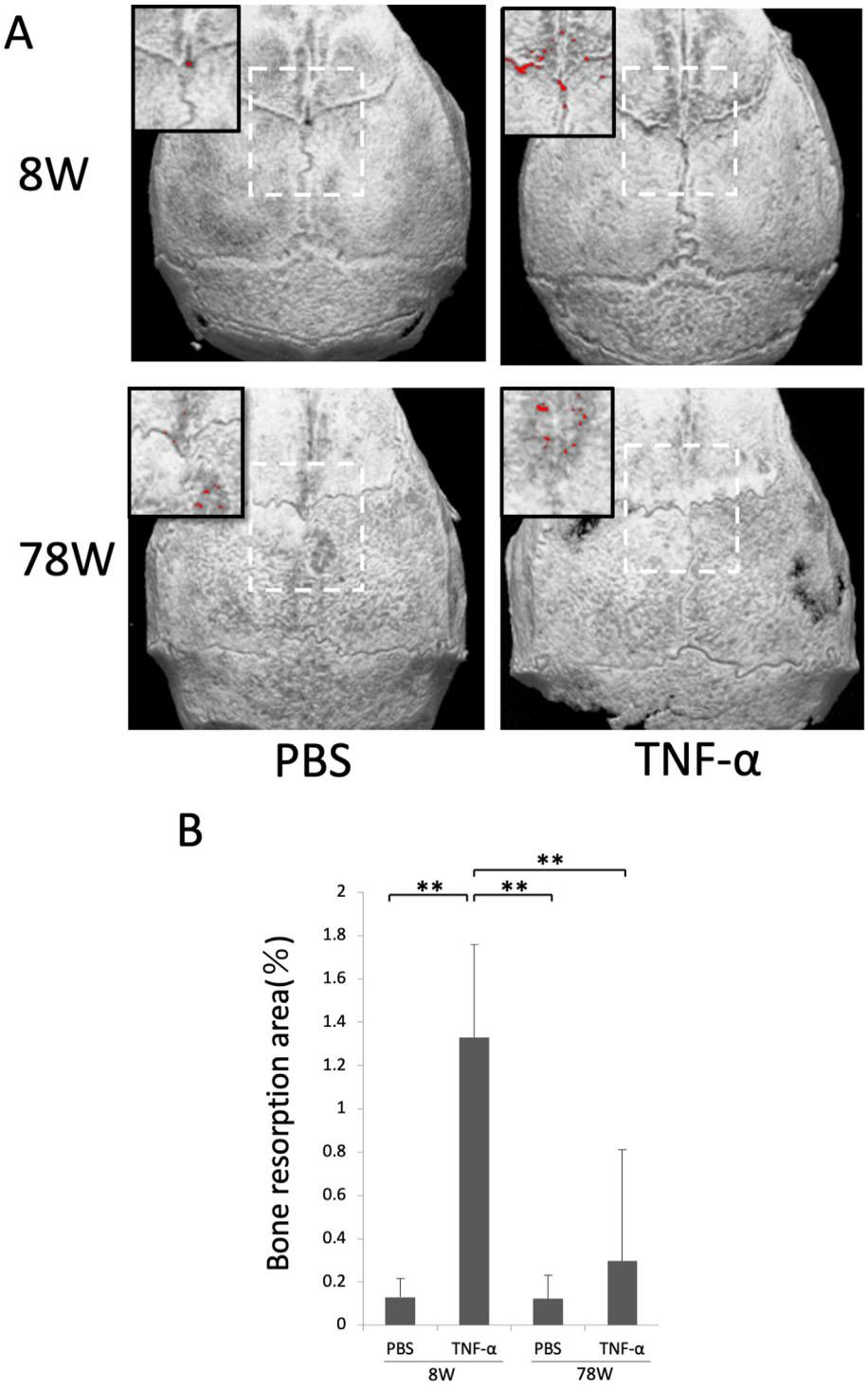

Figure 2 Tumor necrosis factor-α-induced bone resorption is reduced in aged mice compared to young mice in vivo. (A) Three-dimensional reconstructed images of calvariae in 78-weeks-old (78W) and 8- weeks-old (8W) mice. After being injected with tumor necrosis factor (TNF)-α for five days, calvariae were resected and scanned by micro-CT. (B) The ratio of bone resorption area to total calvarial bone area in old mice and young mice. The error bars represent the SD. The statistical significance was determined by the Tukey—Kramer test (**$P < 0.01$; $n = 4$). Phosphate-buffered saline (PBS).

Evaluation of bone resorption area

The mouse calvariae were scanned using a micro-CT scanner (ScanXmate-E090; Comscan, Kanagawa, Japan) to assess the amount of bone resorption. Image analysis software (TRI/3D-BON64; RATOC System Engineering, Tokyo, Japan) was used to construct 3D images of the mouse calvariae, and then ImageJ software (NIH, Bethesda, MD, USA) was then used to evaluate the bone resorption.[25]

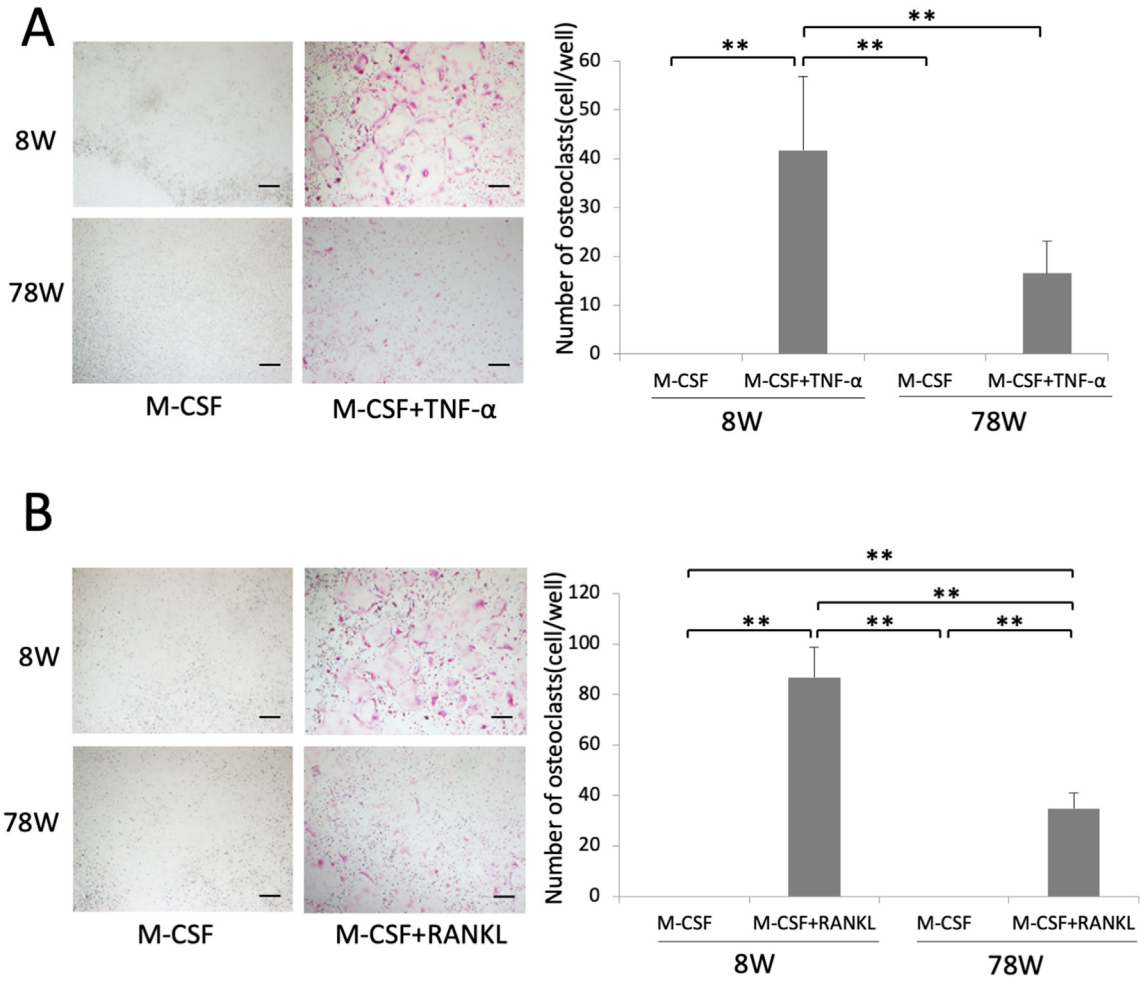

Figure 3 Tumor necrosis factor-α- and receptor activator of nuclear factor-kappa B ligand-induced osteoclast formation is reduced in aged mice compared to young mice in vitro. Images and quantification of (A) Tumor necrosis factor (TNF)-α-induced and (B) receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoclast formation in 78- weeks-old (78W) and 8-weeks-old (8W) mice. Osteoclast precursor cells were cultivated with macrophage colony-stimulating factor (M-CSF), or M-CSF along with TNF-α or RANKL followed by TRAP staining. The error bars represent the SD. The statistical significance was determined by the Tukey—Kramer test (**P < 0.01; n = 4). Scale bar = 200 μm.

Experimental orthodontic tooth movement in mice

Mice were anesthetized, and a nickel-titanium (Ni—Ti) closed coil spring (Takara TOMY, Fukushima, Japan) was wedged into the left maxillary first molar and the maxillary alveolar bone below the incisor. The left maxillary first molar was then moved proximally with a 10 g force for 12 days.[8,27]

Statistical analyses

The data collected are presented as the mean ± SD. Student's t-test was used for comparisons between the two groups. The Tukey—Kramer test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Osteoclast formation induced by tumor necrosis factor-α in old mice in vivo

Administration of TNF-α significantly increased the number of osteoclasts in sutures of tissue sections in both old and young mice. But, the number of osteoclasts was less in the old mice compared with young mice (Fig. 1A and B). Moreover, according to the result of real-time PCR,

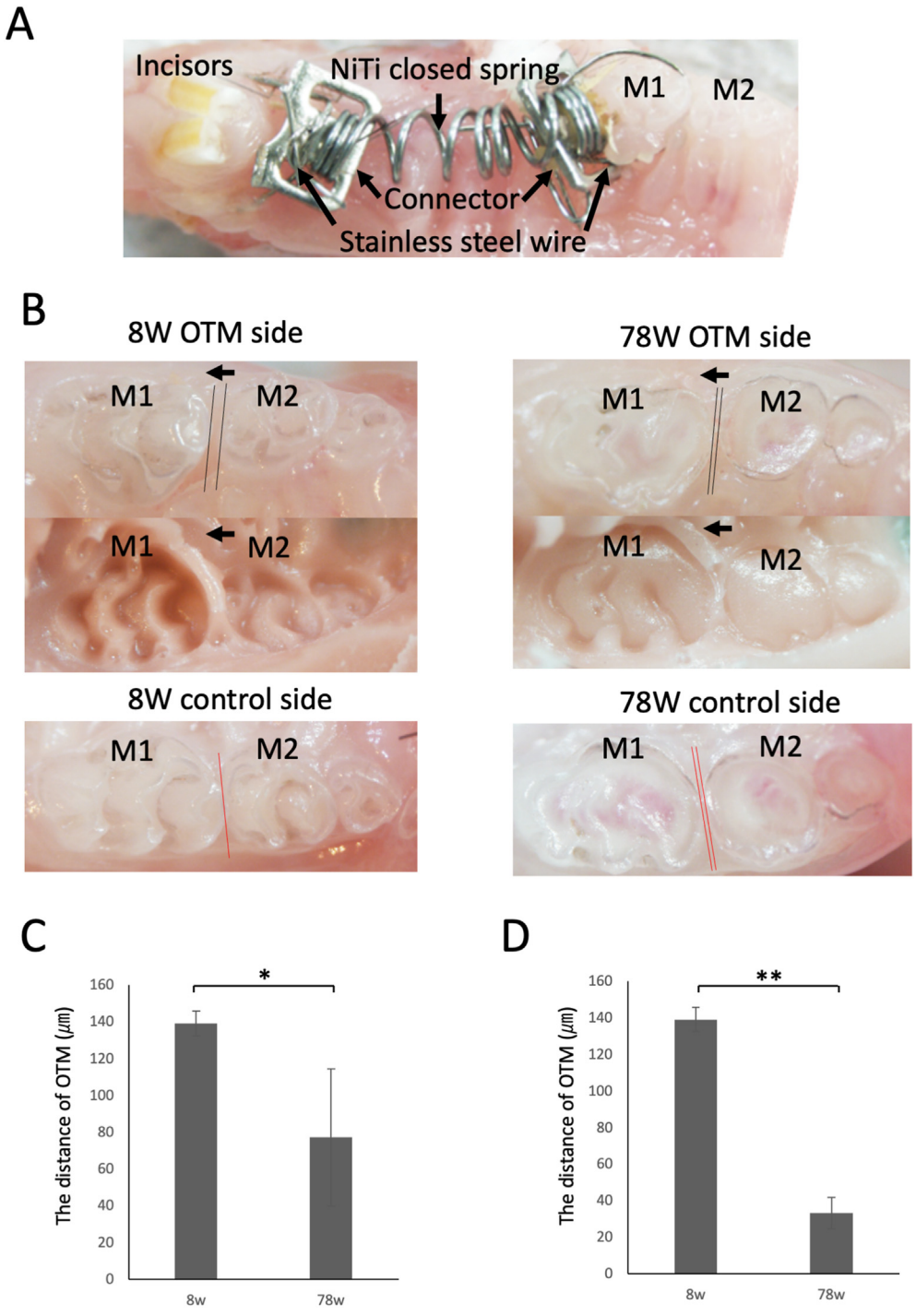

Figure 4 The distance of tooth movement in orthodontic tooth movement is reduced in aged mice compared to young mice. (A) Image of the appliance for orthodontic tooth movement in mice. M1, first molar; M2, second molar. Nickel-titanium (Ni–Ti). (B) Images of teeth after 12 days of experimental loading in 78-weeks-old (78W) and 8 weeks old (8W) mice, as well as in the unloaded control sides. (C) Distance of tooth movement in old and young mice after 12 days of orthodontic tooth movement (OTM). (D) Actual distance of tooth movement in old and young mice after 12 days of OTM. The error bars represent the SD. The statistical significance was determined by the Tukey–Kramer test (*$P < 0.05$, **$P < 0.01$; $n = 4$).

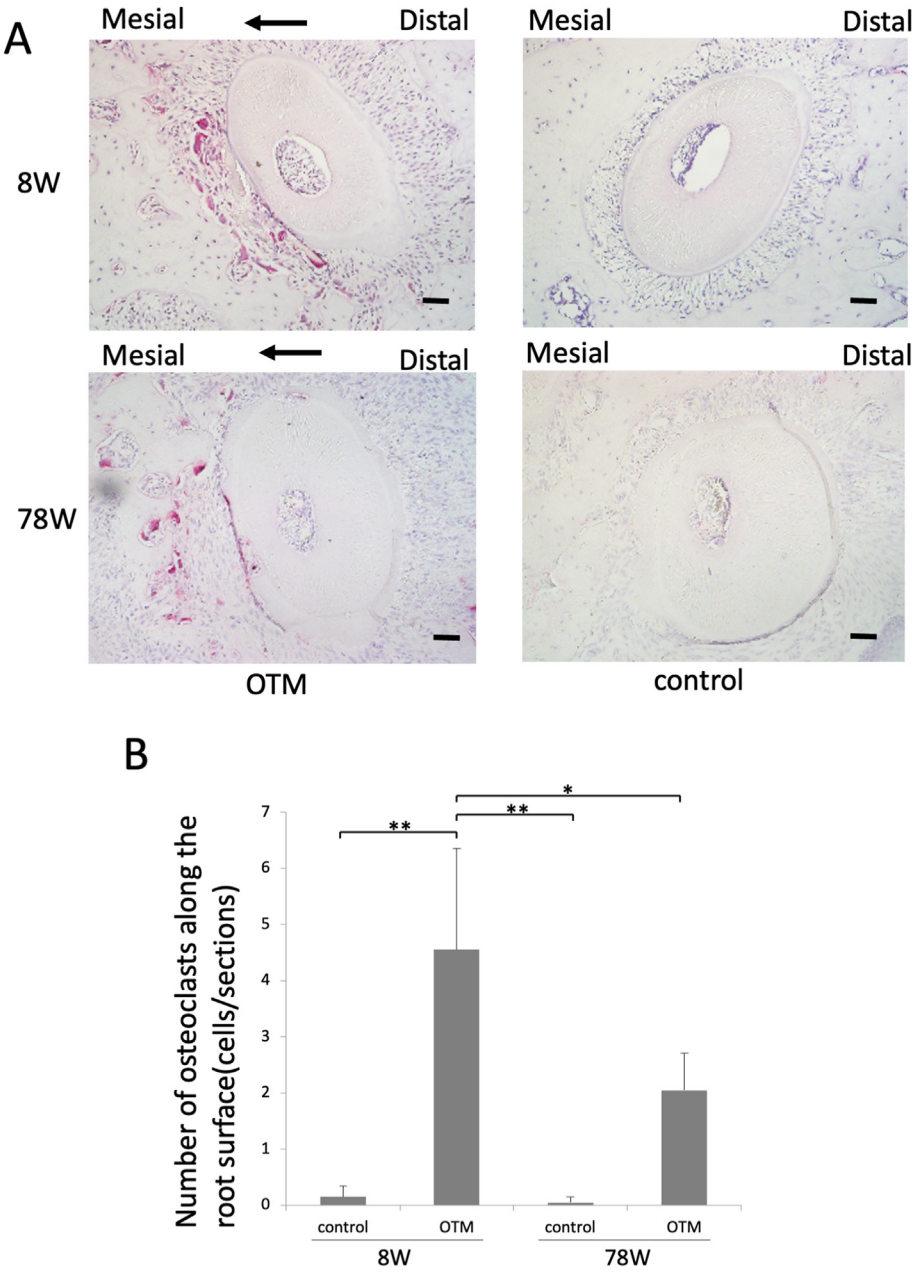

Figure 5 The osteoclast formation at the pressure side in orthodontic tooth movement is reduced in aged mice compared to young mice. (A) Image of TRAP staining of horizontal sections of the alveolar bone surface of the distobuccal root of the left maxillary first molar after 12 days of orthodontic tooth movement (OTM) in 78-weeks-old (78W) and 8-weeks-old (8W) mice. (B) Evaluation of the number of osteoclasts in 78W and 8W mice. The error bars represent the SD. The statistical significance was determined by the Tukey–Kramer test (*$P < 0.05$, **$P < 0.01$; $n = 4$). Scale bar = 50 μm.

expression levels of TRAP mRNA and cathepsin K mRNA were significantly lower in old mice compared with young mice (Fig. 1C).

Bone resorption induced by tumor necrosis factor-α in old mice in vivo

The calvariae of all mouse groups were scanned with micro-CT to analyze bone resorption. The ratio of bone resorption area to total area in old mice was much lower than in young mice (Fig. 2A and B).

Osteoclast formation induced by receptor activator of nuclear factor-kappa B ligand- and tumor necrosis factor-α in old mice in vitro

To analyze whether age affects osteoclast precursors for osteoclast formation, we investigated the osteoclast

formation induced by RANKL and TNF-α in old and young mice in vitro. The number of RANKL- and TNF-α-induced osteoclasts of old mice was significantly lower than that of young mice (Fig. 3A and B).

Tooth movement in old mice

The Ni–Ti closed coil spring was attached with a 0.1 mm diameter stainless steel wire between the anterior alveolar bone and the maxillary left first molar (Fig. 4A). The distance of tooth movement in the old mice was 77.25 ± 37.3 μm, which was significantly lower than that in the young mice (139.2 ± 6.6 μm) after 12 days of OTM (Fig. 4B and C).

Aged mice had more tooth abrasion on their molars than younger mice, resulting in a gap between the first and second molars, even in the absence of tooth movement (Fig. 1B). To accurately represent the true extent of tooth movement, the space between the first and second molars on the control side was subtracted from the distance on the tooth movement side. In old mice, this calculation yielded a tooth movement distance of 33.25 ± 8.6 μm, while in young mice, the distance was 139.2 ± 6.6 μm.

Number of osteoclasts at the alveolar bone of pressure side on orthodontic tooth movement in old mice

TRAP staining was performed on the sections of the distobuccal root of the upper left first molar after OTM for 12 days. A significantly lower number of osteoclasts along the alveolar bone (2.05 ± 0.66 cells/section) was observed in old mice compared with that in young mice (4.55 ± 1.79 cells/section) (Fig. 5A and B).

Discussion

In this study, we used 78-week-old mice as the 'old' group of mice. The average lifespan of laboratory mice is about 24 weeks,[28] while that of a person in developed countries is about 80 years.[29] Based on these reports, it is accepted that 40 days of a human's lifespan is equivalent to one day in the lifespan of a mouse.[30] Thus, the mice used in this study (78 weeks old) were equivalent to 60-year-old humans. However, the same report also outlined a distinction in age calculation between mouse puberty and mouse adulthood. According to the report, 3.65 days for adolescent mice correspond to one year for humans.[30] The young mice used in this study were 8 weeks old, which corresponds to approximately 15 years of age in humans.

Several reports showed that TNF-α could directly generate osteoclast from osteoclast precursors in vitro.[2–4] We have shown that TNF-α injections into supracalvarial region could induce TNF-α induced osteoclastogenesis.[31,32] In this study, when TNF-α was injected, the number of osteoclasts in the old mice was less than that in young mice. Furthermore, the ratio of bone resorption area to total area in the old mice was much lower than those in the young mice. These results suggested that older mice are less reactive to TNF-α in osteoclastogenesis in vivo.

Therefore, we investigated whether age affects osteoclast precursors directly. We analyzed both TNF-α- and RANKL-induced osteoclastogenesis in old and young mice. Bone marrow macrophages, which are osteoclast precursors, from the young and old mice were cultured in a medium including M-CSF and RANKL or TNF-α. The number of old mice was significantly lower than that of young mice. The results suggested that bone marrow cells from older mice are less reactive to RANKL and TNF-α in osteoclast formation in vitro. Moreover, this may be one of the reasons why old mice formed less osteoclasts in vivo.

A number of studies have shown that TNF-α expression was induced in periodontal tissue and gingival sulcus by orthodontic force, these results suggested TNF-α has critical role in OTM.[33–39] We previously established a mouse OTM model to elucidate the mechanism of OTM. This model, referred to as the TNF receptor-deficient mouse model, involved mice lacking both TNF receptor 1 and 2, enabling us to investigate the role of TNF-α.[7] Several studies using TNF receptor-deficient mice have shown a decrease in both the distance of tooth movement and the number of osteoclasts when compared to wild-type mice. These studies concluded that TNF-α plays a crucial role in osteoclast formation and bone resorption during OTM.[7,8,27] In the present study, we analyzed tooth movement and osteoclast formation in old and young mice. The tooth movement distance and number of osteoclasts was much higher in younger mice than that in old mice. Furthermore, we showed that osteoclast precursors derived from bone marrow cells from older mice are less reactive to RANKL and TNF-α in osteoclast formation in vitro. This may be one of the reasons why old mice showed less tooth movement and osteoclast formation during OTM.

In this study, OTM and osteoclast formation were found to decrease in aged mice. Therefore, it is necessary to consider the potential suppression of tooth movement in the treatment of older patients.

Declaration of competing interest

The authors declare no conflicts of interest.

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