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Original Article

# DR3 and its ligands take roles in periodontium remodeling during orthodontic tooth movement



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Received 31 December 2019; Final revision received 24 January 2020

Available online 19 March 2020

## KEYWORDS

Orthodontic tooth movement;  
DR3;  
PGRN;  
TL1A;  
Periodontal ligament

**Abstract** *Background/purpose:* Based on ‘pressure-tension theory’, the application of orthodontic force to the tooth site affects the remodeling of periodontal tissue, specifically, mechanical stress induces the release of cytokines. The purpose of this research paper is to examine death receptor 3/TNF-like protein 1A/progranulin (DR3/TL1A/PGRN) expression levels in periodontium tissue during orthodontic tooth movement in rats.

*Materials and methods:* Thirty-six ten-week-old male SD rats were used in this study. A total of 20 cN of orthodontic force was exerted by coil springs onto the upper right first molars in order for mesialization purposes. All rat members of their corresponding groups were euthanized based on the following time intervals: 0 day for the control group, 1 day (group two), 3 days (group three), 5 days (group four), 7 days (group five), and lastly 14 days for group six. The effects of DR3/TL1A/PGRN were observed through the use of immunohistochemical staining techniques. One-way analysis of variance followed by a LSD t-test was performed by SPSS 20 to compare the differences of the level of DR3/TL1A/PGRN amongst each specified time interval.

*Results:* The expression levels of DR3/PGRN increased significantly on day 14 compared to that of the control group, thus indicating a wide range of statistical differences. The expression levels of TL1A soared upwards on day 1, then plunged to an all-time low on day 7 before finally bouncing back to its initial value on day 14.

*Conclusion:* DR3 and its two ligands—PGRN and TL1A play indispensable roles in regulating periodontal ligament remodeling during orthodontic tooth movement.

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## Introduction

Based on 'pressure-tension theory', the application of orthodontic force to the tooth site affects the remodeling of periodontal tissue, generating tension (T) side and compression (C) side in periodontal ligament (PDL).<sup>1,2</sup> Induced by mechanical stress, signaling molecules are released in the periodontal tissue while causing a cascade of events such as angiogenesis, aseptic inflammation and osseous remodeling.<sup>3</sup> Arachidonic acid metabolites, neurotransmitters and second messengers induced by mechanical stress are released in PDL, which in turn activate the release of cytokines, including chemokines, growth factors and colony stimulating factors, then, they subsequently influence the modulator, entails osteopontin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and matrix metalloproteinases (MMPs).<sup>1</sup>

Noteworthy, RANKL/OPG/(nuclear factor kappa-B) RANK is an important system—the latter two which are also known as the members of tumor necrosis factor superfamily/tumor necrosis factor receptor superfamily (TNFSF/TNFRSF)—which regulates bone remodeling and root resorption during orthodontic tooth movement (OTM) by altering the ratio of concentration of RANKL to OPG/RANK.<sup>4</sup> High levels of OPG/RANKL in periodontal tissue was identified to have direct correlation to orthodontic force application by Melissa Grant et al., more specifically, compressive force elevates RANKL expression to induce osteoclastogenesis during the process of OTM.<sup>1</sup>

MMP9, best described as an angiogenesis promoter in other literatures, can further degrade gelatine, fibronectin, elastin and collagen. Not surprisingly, MMP9/Lipocalin-2 (NGAL) has strong influence on angiogenesis processes in PDL during OTM due to its active promoting role.<sup>3</sup>

Besides, chemokines also play a key role in mechanical loading-induced bone remodeling.<sup>5</sup> C-C chemokine receptor 1 (CCR1)'s effect on bone remodeling during OTM may partially dependent on presence of chemokine (C-C motif) ligand 3 (CCL3).<sup>5</sup> Fraser L. Collins et al. have studied the effects of DR3/TL1A signaling between osteoclast (OC)-dependent chemokine and MMP production. At the time, the mechanism by which the DR3/TL1A pathway directly enhances human OC formation and resorptive activity had just been identified.<sup>6</sup> Additionally, the osteoclastogenic chemokine CCL3 and MMP-9 were induced downstream by DR3/TL1A, meanwhile, TL1A increases OC formation and resorptive activity by increasing expression of CCL3 and MMP-9.<sup>6</sup>

TL1A (TNFSF15) has been confirmed to bind one transmembrane receptor, DR3 (TNFRSF25).<sup>6</sup> Further, DR3 competes with another TNFRSF member called Decoy Receptor 3 (DcR3) for TL1A binding, meanwhile, has two confirmed ligands—namely TL1A mentioned before and PGRN/Atsttrin—the latter one has been demonstrated to inhibit the former one's activity.<sup>7,8</sup> TL1A-DR3 axis was identified that plays significant role in many chronic inflammatory diseases, including but not limited to arthritis and inflammatory bowel diseases.<sup>8</sup> As mentioned before, OTM is a process of aseptic inflammation, we hypothesize that TL1A/DR3 may play a potential role in OTM as well. Besides, most studies that cover DR3/TL1A's role on bone

remodeling have primarily focused on axial skeleton rather than alveolar bone and the role of PGRN in DR3 pathway on modulating bone formation and resorption is scarcely mentioned. Thus, the goal of this research study is to determine whether these cytokines are a factor in regulating the periodontium remodeling during OTM.

## Materials and methods

All of the experiment operations were approved by Ethical Committee of Zhongshan Hospital, Fudan University, Shanghai, China.

## Animals

Thirty-six ten-week-old male SD rats, weighing from 250 to 300g, were used in the course of this study. All the animals were provided by the Animal Experimental Center of Zhongshan Hospital. The rats were housed in a room at the temperature set to a constant  $25 \pm 2^\circ\text{C}$ . Each rat was fed the same laboratory formula and water *ad libitum* under a standard 12-h, light–dark cycle and acclimatized for 1 week before proceeding with the experiment.

## Experimental protocol

The rats were anesthetized via intraperitoneal injection (8% chloral hydrate, 0.4 ml/100g), then placed in the dorsal decubitus position with four limbs affixed to an operating table. Nickel-titanium closed-coil springs (Smart, Beijing, China) were bound between right maxillary first molar and incisors using ligature wire to produce the 20 cN of orthodontic force (Fig. 1). A dynamometer was used to ensure that the amount of force was applied precisely. Resin were bond to incisors after surface being etched to connect two incisors together to form anchorage for the appliance to



**Figure 1** The coil springs were bound between right maxillary first molar and incisors using ligature wire to produce orthodontic force.

mesialize the first molars and keep the wire from dropping off. Thirty-six male SD rats were divided into six groups. Rats in each group were euthanized after 0, 1, 3, 5, 7, 14 days respectively for histopathological analysis—rats in 0 group were taken as the control group. To avoid the center of rotation, the T side in question is defined as the distal side of molars' periodontal tissue that is in the neighborhood of gingival margin, similarly, C side is chosen from the mesial side within the same scope.

### Sample preparation

The points in time for the creating of specimen for the experiment were set to 0, 1, 3, 5, 7, 14 days after orthodontic tooth movement. Each of the six rats in each group were euthanized through cervical dislocation at different time points under general anesthesia. The upper right maxillae was excised and trimmed, including the upper right first molar with the alveolar bone and periodontal ligament around. Meanwhile, 4% neutral buffered formalin was fixed at +4 °C for 24h, and the tissue blocks were decalcified by 10% Ethylene Diamine Tetraacetic Acid (EDTA) for 6 weeks, being replaced every 3 days. Next, they were rinsed by distilled water and dehydrated with ethanol (30%, 70%, 95%, 100%) and xylene before embedding the blocks in paraffin. 5 µm sagittal sections were stained to conduct a histopathology analysis.

### Immunohistochemical staining

The paraffin sections were deparaffined and rehydrated, washed by distilled water three times and exposed to 1.5% hydrogen peroxide-methanol compound to block the activity of endogenous peroxidase before eventually washed them thoroughly using distilled water after half an hour. 0.1% trypsinase was used for epitope retrieval for an hour. Then, the sections were washed by phosphate-buffered saline (PBS) three times and blocked by goat serum (1:20) for 20 mins. The primary antibodies used were rabbit polyclonal anti-DR3 antibody (1:400, Abcam, Cambridge, UK), rabbit polyclonal anti-Granulin antibody (1:2000, Abcam, Cambridge, UK) and rabbit polyclonal anti-TL1A antibody (1:1500, Abcam, Cambridge, UK). After processing, the sections were left at temperature 37 °C for 1h, transferred in 4 °C fridge overnight, and finally washed by PBS three times before being treated with second antibody. The final phase consisted of washing the tissue sections by PBS three times and then adding chromogen (DAB) for visualization of target cells while at the same time regulating and adjusting the intensity under the microscope. Hematoxylin was used to stain cell nucleus and after drying, at last, they were mounted with neutral balsam. All sections in six groups were observed by a microscope (at 20 × magnification). To evaluate and analyse results, Image-pro plus 6.0 was used to measure the mean optical density (MOD) for semiquantitative analysis—each of the three views on T and C sides of each section was randomly selected to measure the integrated optical density (IOD) of positive cells intensity in periodontium tissue, then, divided by the measuring area.

### Tooth movement measurement

A vernier caliper—accuracy of 0.01 mm, was used to measure the distance between lingual side of incisor and convex point of mesial surface of the upper right first molar. The incisor-to-molar distance of each group at different time intervals was marked as  $D_0, D_1, D_2, D_3, D_4, D_5$ . The tooth movement distance was calculated by subtracting  $D_x$  (incisor-to-molar distance of experimental groups) from  $D_0$  (incisor-to-molar distance of control group). The measurement of each group was repeated three times, then, the mean value was calculated.

### Statistical analysis

The data were described using mean ± standard error (SEM) and abnormal distributed data were handled by applying logarithm functions. Data analysis – one-way analysis of variance followed by LSD t-test – was performed by SPSS 20 to compare the differences of the level of DR3/TL1A/PGRN among each time point. A  $P$  value < 0.05 was regarded as statistically significant.

## Results

### Tooth movement distance

The tooth movement distance of each group at different time intervals are listed as bellows (Table 1).

### Immunohistochemical staining

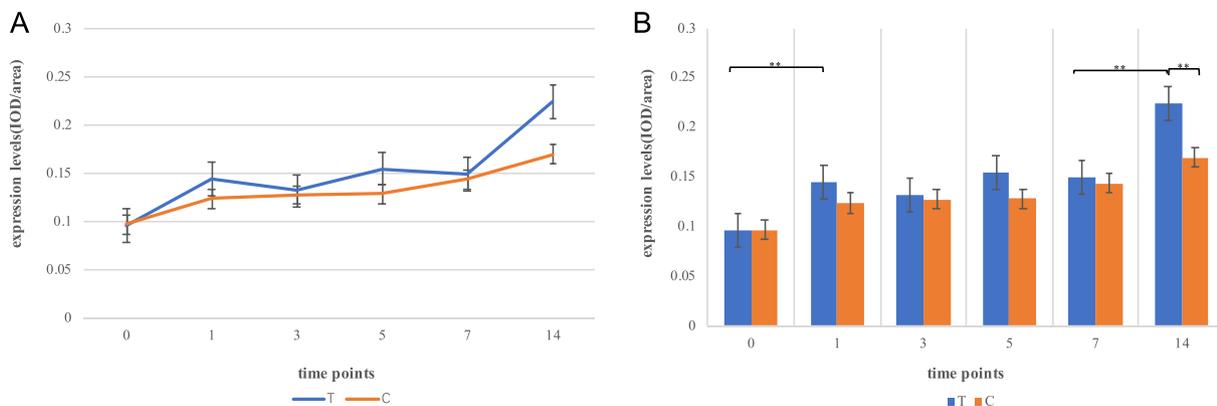
#### Periodontal ligament

The graph (Fig. 2) given indicates that the expression levels of DR3 on T side climb to a high on day 1, which shows significant differences compared to the control group, followed by a plateau (no significant difference was observed among day 3, 5, 7), reaching a peak on day 14 ( $P < 0.05$ ). It is evident from Figs. 3 and 4 that DR3 expression on periodontium tissue changes from each time period, which positive area on day 14 is much darker than that on control group on both T and C sites.

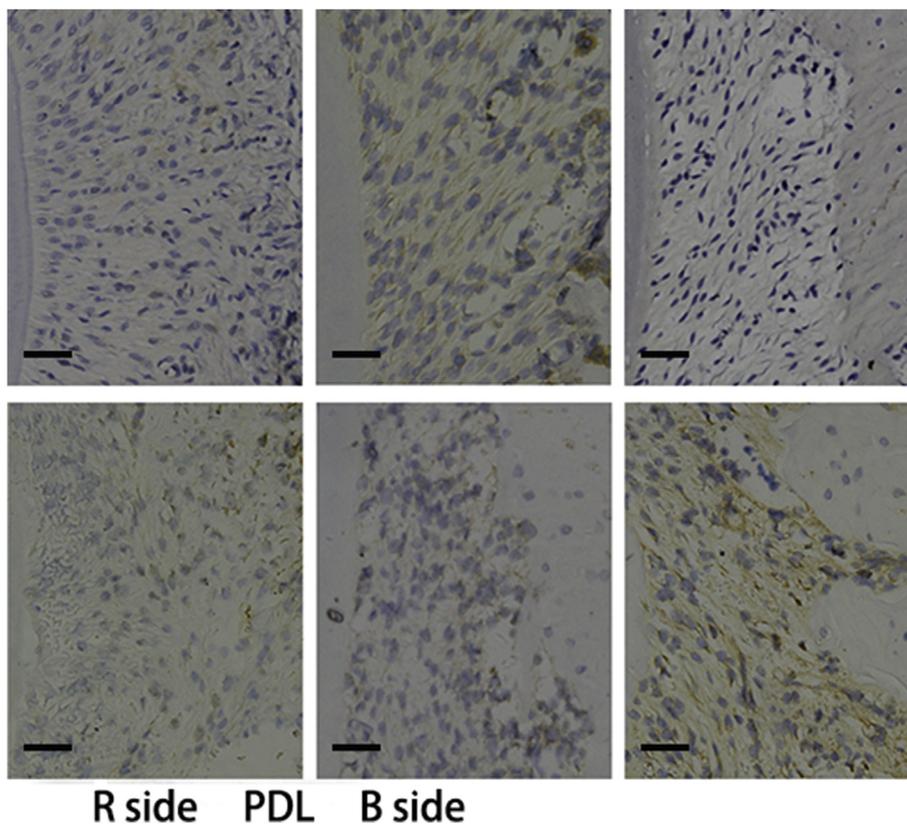
Ignoring the negligible values of the control group, the expression levels of PGRN rapidly increased on day 1 ( $P < 0.05$ ) (Fig. 5). Day 3–7 saw a fluctuating period of ups and downs. On day 14, then, the figures proceed to rise back to their maximum value ( $P < 0.05$ ). Immunohistochemical staining results showed that positive area on day

**Table 1** Tooth movement distance of each group.

Time (days)	Tooth Movement Distance (mm)
0	0.00 ± 0.000
1	0.045 ± 0.008
3	0.058 ± 0.009
5	0.087 ± 0.021
7	0.119 ± 0.030
14	0.232 ± 0.041



**Figure 2** The expression levels of DR3 on both T and C sites at each time interval and statistical differences between the sites for each time interval ( $P < 0.05$ ).



**Figure 3** Changes of DR3 expression on periodontium tissue in compression side for days 0,1,3,5,7,14. B alveolar bone; PDL periodontal ligament; R root; bar = 50  $\mu$ m.

14 is the darkest among the six groups on both T and C sites (Figs. 6 and 7).

According to Fig. 8, the expression level of TL1A on T side on day 1 climbed rapidly to somewhere in the vicinity of 0.29, followed by a decline trend. Levels on both sides plunged to a low on day 7, and eventually recovered on day 14. Precisely, TL1A expression on T side showed a significant difference between every two neighboring time points, with the exception of the period between day 3 to day 5, where levels on C side went through a stable period to day 5. It is evident that based on the results from Figs. 9 and 10, the positive area from day 3 to day 7

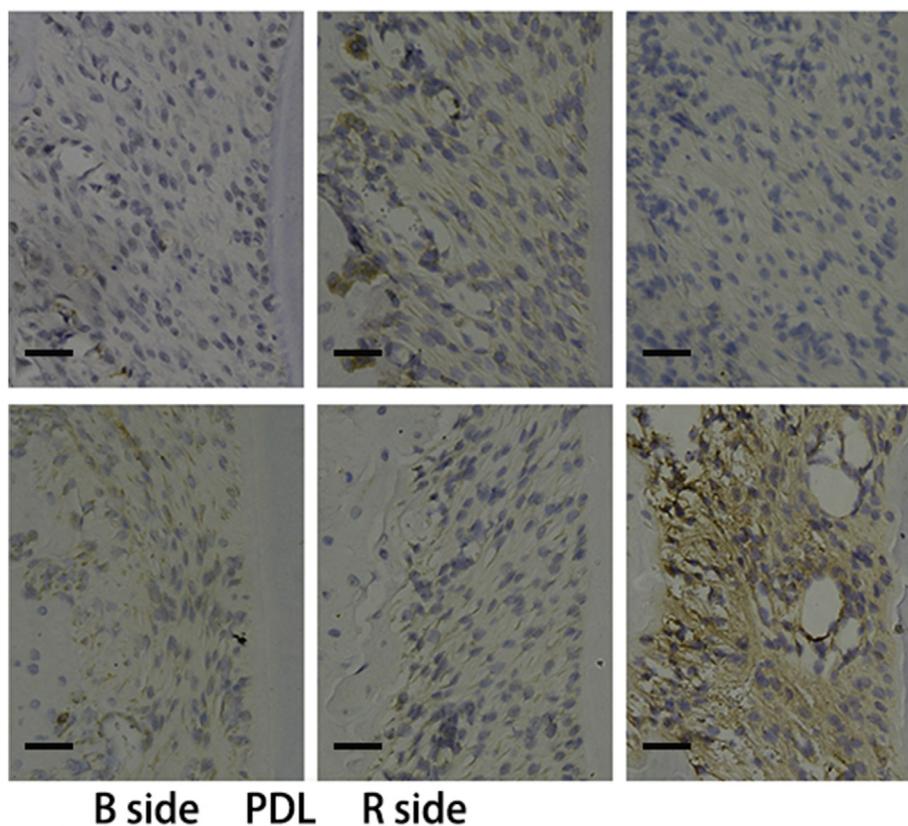
is much lighter when compared to that of the other three groups.

**Alveolar bone tissue**

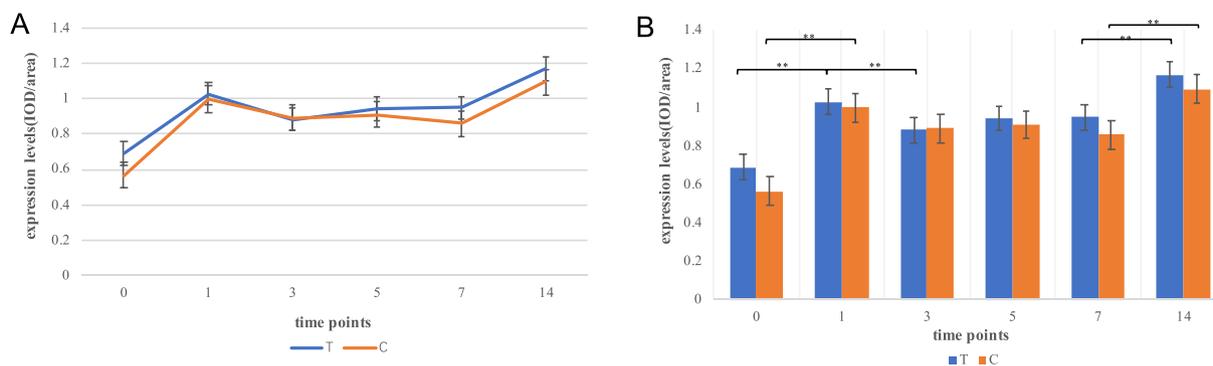
No yellow granular positive reactant in osteocytes was observed on both T and C sides after orthodontic force applied compared with control group.

**Discussion**

Current concepts of OTM reveal that the mechanical force could change five micro-environments during the whole



**Figure 4** Changes of DR3 expression on periodontium tissue in tension side for days 0,1,3,5,7,14. B alveolar bone; PDL periodontal ligament; R root; bar = 50  $\mu$ m.

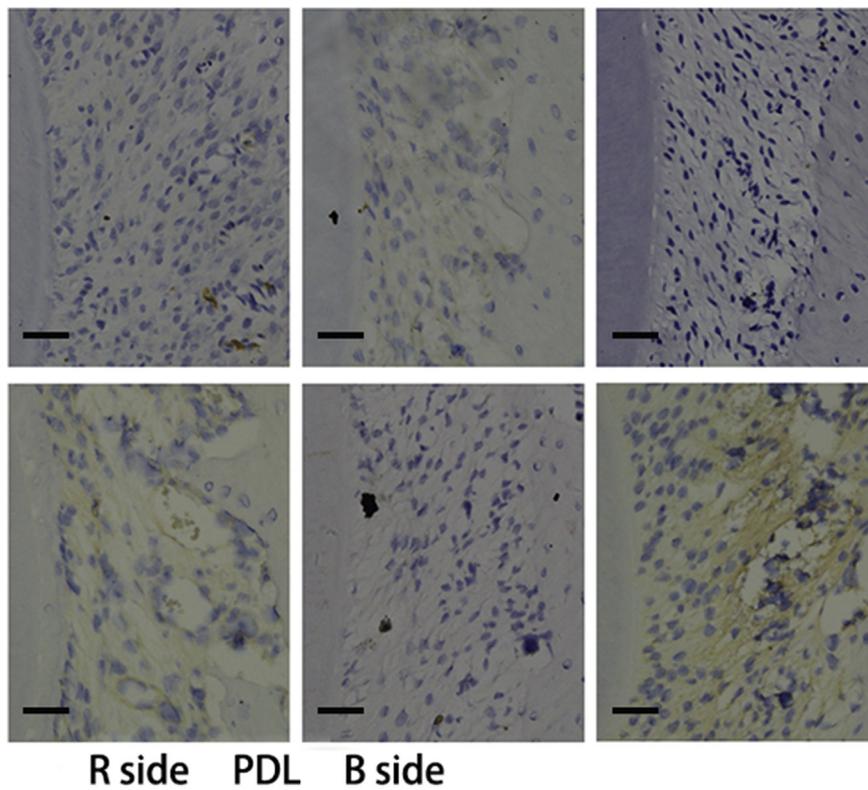


**Figure 5** The expression levels of PGRN on both T and C sites at each time interval and statistical differences between the sites for each time interval ( $P < 0.05$ ).

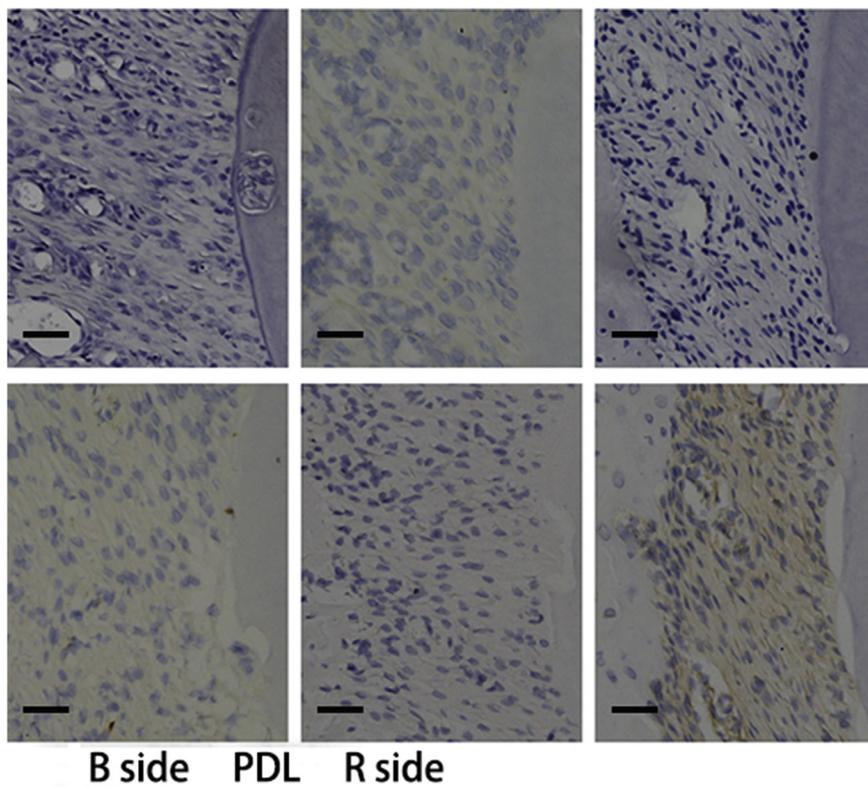
process: extracellular matrix, cell membrane, cytoskeleton, nuclear protein matrix and genome.<sup>2</sup>

Members of TNFSF/TNFRSF like LIGHT (TNFSF14), B lymphocyte stimulator (TNFSF13B), a proliferation-inducing ligand (TNFSF13A), TL1A (TNFSF15) and DR3 (TNFRSF25) were proved to play essential roles in bone pathology with respect to musculoskeletal disease. Furthermore, other ones such as OPG, RANK (TNFSF11) participate in regulating the bone remodeling during OTM which were previously confirmed.<sup>6</sup> Nevertheless, the previous literature only digs into the function of DR3, TL1A and PGRN on long bone in systemic bone loss diseases. Therefore, this paper is one of the first studies to actually explore the role of DR3/PGRN/TL1A on periodontium remodeling during OTM.

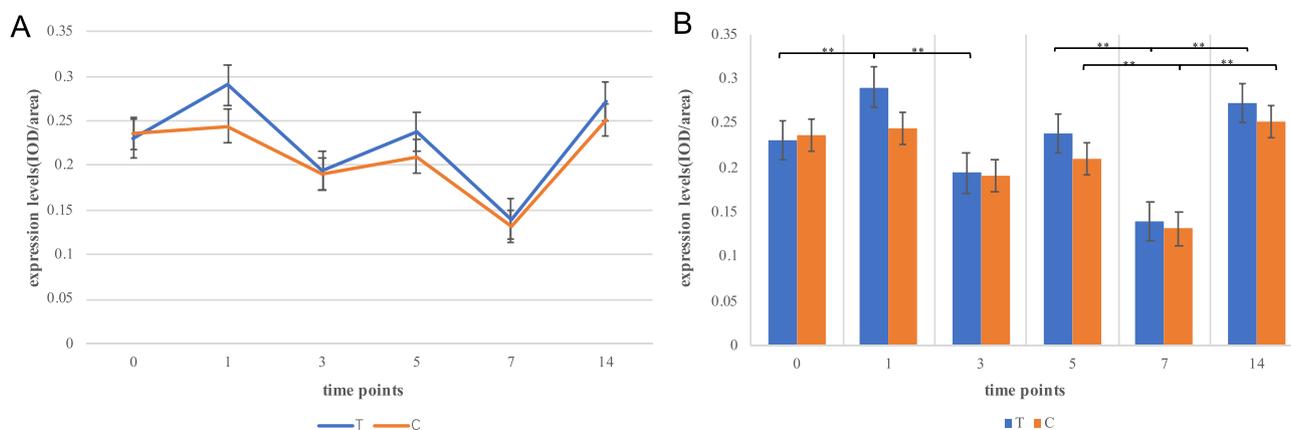
The term 'osteimmunology' was put forward by Arron et al., in 2000, specially, to depict the mutual effects between bone and immune system.<sup>9</sup> OTM could be treated as a result of alveolar bone remodeling accompanied by the periodontal inflammatory process.<sup>10</sup> Thus, more attention was paid to the relationship between lymphocyte and OTM recently, which was proved that CD4<sup>+</sup> T cells affect OTM through Th1-associated cytokines, referring to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ )—the former one acts as a proinflammatory chemokine by positively regulating osteoclastogenesis and MMP expression, notably, MMP has an effect on the remodeling of PDL through targeting on extracellular matrix (ECM).<sup>11,12</sup> More specifically, periodontal ligament fibroblasts could secrete



**Figure 6** Changes of PGRN expression on periodontium tissue in compression side for days 0,1,3,5,7,14. B alveolar bone; PDL periodontal ligament; R root; bar = 50  $\mu$ m.



**Figure 7** Changes of PGRN expression on periodontium tissue in tension side on days 0,1,3,5,7,14. B alveolar bone; PDL periodontal ligament; R root; bar = 50  $\mu$ m.

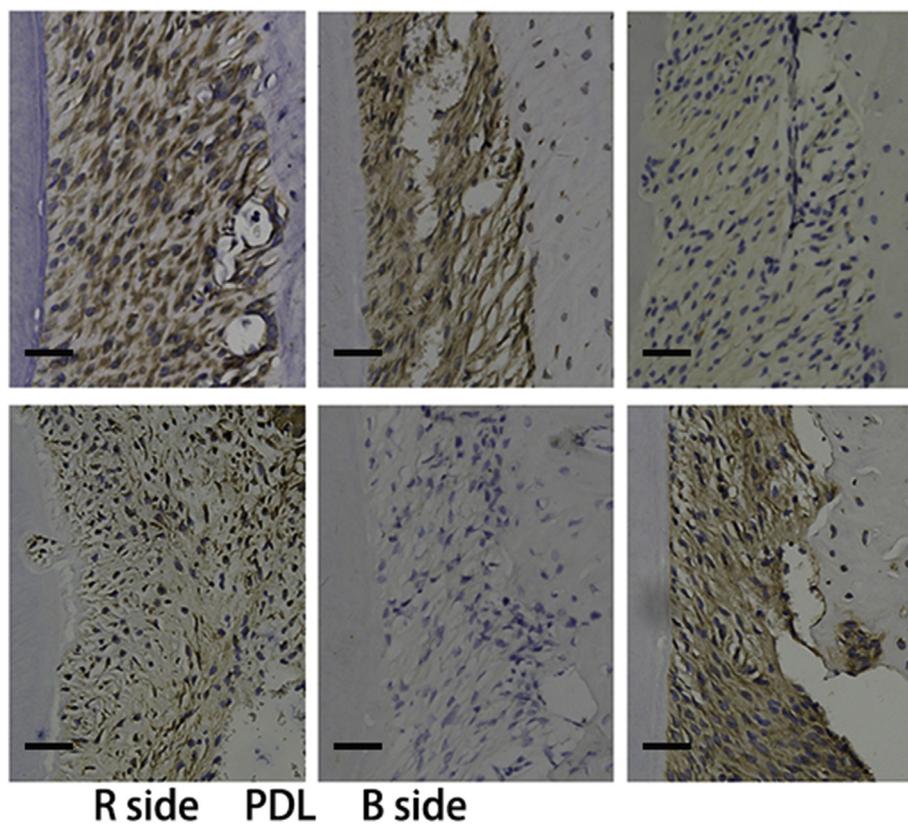


**Figure 8** The expression levels of TL1A on both T and C sites at each time interval and statistical differences between the sites for each time interval ( $P < 0.05$ ).

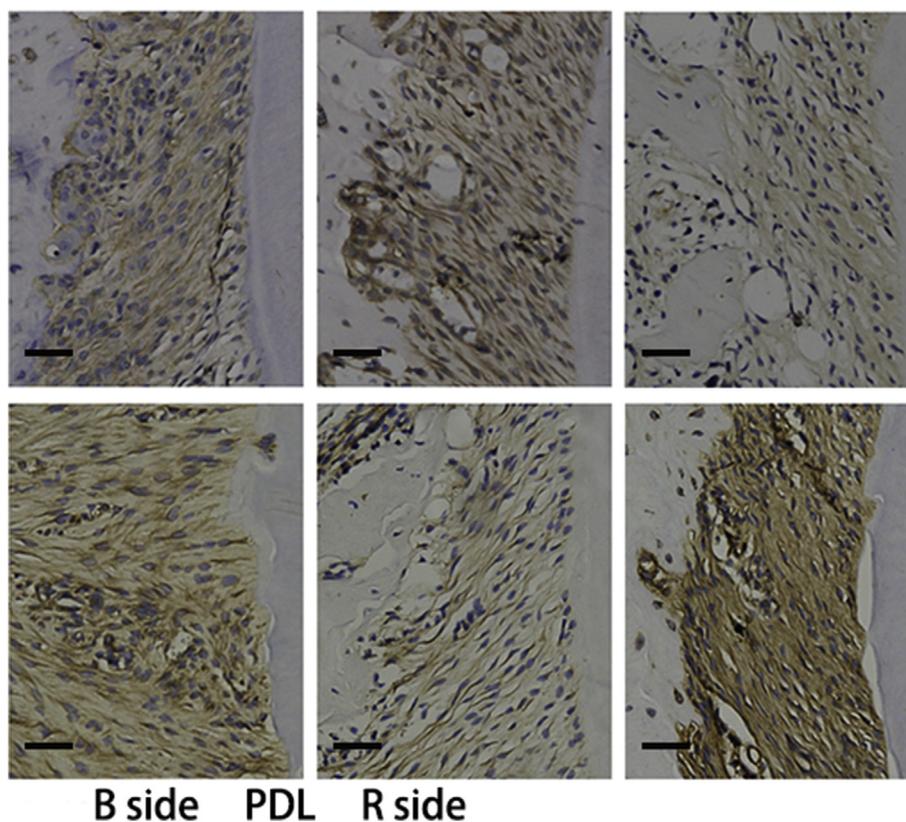
higher levels of  $\text{TNF-}\alpha$  at C side to activate  $\text{CD4}^+$  T cell, thus, promoting RANKL expression to facilitate bone resorption.<sup>13</sup> Intriguingly, DR3/TL1A system was happened to be found to work as costimulation on T cells and enhances proliferation of regulatory cells (Tregs).<sup>8</sup>

On the contrary to the cytokines aforementioned, transforming growth factor- $\beta$  (TGF- $\beta$ ) takes a role as an anti-inflammatory factor to inhibit osteoclastogenesis and ECM synthesis.<sup>11,14</sup> According to Garlet TP, TGF- $\beta$  is the only cytokine that is elevated on both the C and T sides in the study.<sup>11</sup>

This research study is relevant as the expression levels of DR3/PGRN in question during the experimental period have similar patterns—general upward trends on both C and T side were observed, which both rapidly climbed on day 1 and reached their peaks on day 14. It is interesting to note that the expression level of TGF- $\beta$  and DR3/PGRN had a parallel trend during OTM. Besides, there is synergistic effect between PGRN and TGF- $\beta$  in inducing Tregs.<sup>15</sup> In addition to DR3, PGRN has higher binding affinity to TNFR2 than to TNFR1.<sup>15</sup> Thus, PGRN plays its anti-inflammatory role basically through two following pathways: a. By



**Figure 9** Changes of TL1A expression on periodontium tissue in compression side on day 0,1,3,5,7,14. B alveolar bone; PDL periodontal ligament; R root; bar = 50  $\mu\text{m}$ .



**Figure 10** Changes of TL1A expression on periodontium tissue in tension side on day 0,1,3,5,7,14. B alveolar bone; PDL periodontal ligament; R root; bar = 50  $\mu$ m.

activating PGRN/TNFR2 pathway b. By inhibiting DR3/TL1A and TNF/TNFR1 pathways.<sup>15</sup> Turning to TGF- $\beta$ , those related studies demonstrate that it is indeed an indicator of inhibition of bone resorption. T.A. Brady et al. found that TGF- $\beta$  induced by mechanical stress might be mitogenic to PDL cells while down-regulating its osteoblast-like features, meanwhile, reducing the bone resorption induced by IL-6.<sup>16</sup> Perhaps, DR3/PGRN might also take part in inhibiting bone resorption activity during OTM, which seems contrary to the conclusion that PGRN is a potent osteoclastogenic factor in the RANK/RANKL axis by Jaemin Oh et al, while later studies have arrived on different conclusions.<sup>17</sup>

In contrast, the expression levels of TL1A skyrocketed to their maximum level on day 1 and fell dramatically on day 3, dropping to a minimum on day 7 before finally rebounding back to their normal level at the initial stage of OTM on day 14.

K. Yokoya et al. mentioned that day 1–7 witnessed a steady increase in the number of osteoclasts and pre-osteoclastic cells in the compression side after force was applied, then, it dropped dramatically by day 14, which was contrary to the trend of TL1A's expression in this study.<sup>18</sup> Thus, it is reasonable to surmise that TL1A could inhibit the activity of OC during OTM. However, TL1A was proved to increase OC formation and resorptive activity in previous literature, more specifically, the experiment has confirmed that TL1A was related to pathologic OC activity associated with inflammatory arthritis-induced focal bone erosion and

secondary osteoporosis.<sup>6</sup> Further, TL1A might directly effect on fibroblast-like synoviocytes (FLS) of rheumatoid arthritis (RA) patients as a pro-inflammatory cytokine through increasing IL-6 expression, evidently, TL1A was identified to bind with TNFR2 rather than DR3 on FLS to influence RA FLS.<sup>19</sup> Surprisingly, not only are OCs regulated by DR3/TL1A axis, this pathway also modulates osteoblast function. To be specific, the recent researches demonstrate that DR3/TL1A axis works as a homeostatic system in bone physiology.<sup>15</sup> Back to this study, the expression levels of TL1A and DR3/PGRN show opposite trends, which might due to the antagonistic effect between TL1A and PGRN mentioned in previous literature. Additionally, TL1A's inhibition on OC and increase of DR3/PGRN during OTM might work together to form a protective mechanism to reduce inflammation caused by mechanical force and bone resorption, namely, those three cytokines might interact to maintain the homeostasis in PDL remodeling during OTM.

Overall, DR3 and its two ligands—PGRN and TL1A play indispensable roles in periodontium remodeling during OTM. Moreover, additional research is needed to explore the mechanism how DR3/PGRN/TL1A regulate the remodeling.

### Declaration of Competing Interest

None declared.

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