Journal of Dental Research
2017, Vol. 96(5) 586–594

DOI: 10.1177/0022034516685527
journals.sagepub.com/home/jdr

Aspirin Blocks Orthodontic Relapse via Inhibition of $CD4^+$ T Lymphocytes

Y. Liu[1,2], T. Zhang[1,2], C. Zhang[1,2], S.S. Jin[1,2], R.L. Yang[1,2], X.D. Wang[1,2], N. Jiang[3], Y.H. Gan[3,4], X.X. Kou[1,2], and Y.H. Zhou[1,2]

Abstract

Immunologic response plays an important role in orthodontic tooth movement (OTM) and relapse. Nonsteroidal anti-inflammatory drugs, such as aspirin, affect immune cells and clinical orthodontic treatment. However, the mechanisms by which nonsteroidal anti-inflammatory drugs regulate immune cells to affect orthodontic relapse are unclear. In this study, male Sprague-Dawley rats were grouped as relapse and relapse + aspirin for 10 d after 14 d of OTM. Silicone impressions of the rats' maxillary dentitions were obtained to record the distance of OTM at the indicated time point. $CD4^+$ T lymphocytes in spleen were examined by flow cytometry. Serum levels of type 1 T-helper (Th1) cell–associated cytokines tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) were determined through enzyme-linked immunosorbent assay. The effects of aspirin on $CD4^+$ T and Th1 cells were also analyzed in vitro. Aspirin treatment significantly reduced the relapse rate. More interestingly, injection of CD25 neutralizing antibody basiliximab or TNF-α inhibitor etanercept can significantly reduce the relapse rate as well. Correspondingly, aspirin treatment significantly accelerated the decrease of orthodontic force–induced secretion of TNF-α and IFN-γ in serum and the expression of TNF-α and IFN-γ in periodontal ligament during relapse. Furthermore, aspirin treatment in vitro significantly repressed the differentiation of $CD4^+$ T and Th1 cells. Overall, results indicated that aspirin treatment can block orthodontic relapse by regulating Th1 cells.

Keywords: orthodontic tooth movement, immunity, bone remodeling/regeneration, cytokine, inflammation, oral medicine

Introduction

Orthodontic relapse is a major clinical challenge influencing the outcome of orthodontic treatment. Permanent retention has been advocated as the only method to ensure long-term post-treatment stability (Little et al. 1988); however, this method usually takes 10 y to obtain a stable occlusion (Kuijpers-Jagtman 2002). Efficient prevention of relapse is an immense challenge to orthodontics, but the underlying mechanism of orthodontic relapse remains largely unknown. Orthodontic tooth movement (OTM) is considered a periodontal inflammatory process characterized by osteoclastic and osteoblastic bone remodeling (Masella and Meister 2006). Recently, our group reported that orthodontic force induces systemic immune responses during OTM (Yan et al. 2015). Moreover, T cells are required for OTM depending on type 1 T-helper (Th1) cell–associated cytokines, which demonstrated a previously unrecognized mechanism of OTM. However, whether T-cell and Th1-associated cytokines are involved in relapse is unclear.

Orthodontic procedures may cause unpleasant and even painful sensations (Koritsanszky and Madlena 2011; Ashkenazi et al. 2012). Orthodontists commonly use analgesics such as aspirin, a nonsteroidal anti-inflammatory drug (NSAID), to relieve patients' pain (Polat and Karaman 2005). Unexpectedly, aspirin often represses the effect of clinical orthodontic treatment (Arias and Marquez-Orozco 2006). Animal studies have demonstrated decreased rates of tooth movement with NSAID administration (Kehoe et al. 1996). Similar to the OTM process, osteoclastic and osteoblastic bone remodeling may play an important role in relapse (Franzen et al. 2013). However, whether aspirin treatment can inhibit relapse is unknown.

Arron and Choi (2000) first proposed the concept of osteoimmunology in 2000, which described a system of interaction between bone and the immune system. Immune responses mediated by periodontal tissues under mechanical force may induce T-cell activation, thereby generating an inflammatory reaction with consequent bone resorption (Verna et al. 1999).

[1]Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China
[2]Center for Craniofacial Stem Cell Research and Regeneration, Peking University School and Hospital of Stomatology, Beijing, China
[3]Center Laboratory, School and Hospital of Stomatology, Peking University, Beijing, China
[4]Center for Temporomandibular Disorders and Orofacial Pain, Peking University School and Hospital of Stomatology, Beijing, China

A supplemental appendix to this article is available online.

Corresponding Authors:
Y. Zhou, Department of Orthodontics, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Avenue, Beijing, 100081, China.
Email: yanhengzhou@vip.163.com
X. Kou, Department of Orthodontics, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Avenue, Beijing, 100081, China.
Email: kouxiaoxing@gmail.com

Additionally, T cells can influence osteoclastogenesis by secreting various cytokines, such as interleukin 1 (IL-1), IL-6, interferon γ (IFN-γ), and IL-4 (Takayanagi et al. 2000a; Mirosavljevic et al. 2003). Cells regulating bone turnover are derived from the same precursors as inflammatory immune cells and are involved in the OTM process. Recent studies have shown that osteoimmunoregulation affected by aspirin is involved in bone metabolism and bone remodeling (Liu et al. 2011). Remodeling of the alveolar bone is one of the main causes of relapse (Yoshida et al. 1999). Therefore, we hypothesized that aspirin may prevent orthodontic relapse by inhibiting T cell–associated bone immunoregulation.

Materials and Methods

Animals

Male Sprague-Dawley rats (6 to 8 wk old) and Institute for Cancer Research mice (6 to 8 wk old) were used in this study. All the animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Peking University (LA2013-92). This study conformed with ARRIVE guidelines for preclinical animal studies.

Application of Orthodontic Devices and Relapse

Rats were anesthetized with pentobarbital sodium (40 mg/kg, body weight). Mechanical force application was performed as previously described by our group (Cao et al. 2014; He et al. 2015). Briefly, orthodontic nickel-titanium coiled springs (0.2 mm in thickness, 1 mm in diameter, 6 mm in length; Smart Technology) were ligated between the right maxillary first molars and incisors of the rats. The springs were activated to deliver an orthodontic force of 50 g, as measured by a dynamometer. After OTM for 14 d, the springs were removed to allow the first molars to relapse for 0, 0.5, 1, 2, 3, 5, 7, and 10 d. With a stereomicroscope (SWZ1000; Nikon), the relapse distance of the OTM was measured between 2 easily located points on the occlusal view of the silicone impression: the midpoint of the distal-marginal ridge of the first molar and the midpoint of the mesial-marginal ridge of the second molar. Relapse distance was calculated through the following equation: relapse distance = relapse distance at 0 d – relapse distance at n d, where n = 0.5, 1, 2, 3, 5, 7, 10. Relapse rate was obtained through the following equation: relapse rate = relapse distance / relapse distance at 0 d.

Micro–computed Tomography Scanning

The maxillae of rats were scanned with a micro–computed tomography system (Inveon MMCT; Siemens). OTM distance was measured as previously described (Cao et al. 2014).

Cell Isolation and Flow Cytometry In Vivo

Spleen cells from rats were isolated through mechanical disruption via frosted glass slides. Circulating blood was obtained from a peritoneovenous shunt. Erythrocytes were lysed with erythrocyte lysis buffer (555899; BD Biosciences). Cell suspensions were filtered through a 40-μm nylon sieve and washed twice through centrifugation at 300 × g for 10 min. Anti-rat CD4-FITC (e-Bioscience) was incubated with the cells at 4 °C for 30 min. Cells were washed and then analyzed with a flow cytometer (BD Accuri C6; BD Biosciences).

Appendix Methods and Antibodies

Detailed methods are described in the Appendix for the following:

- Administration of aspirin, CD25 antibody, and tumor necrosis factor α (TNF-α) inhibitor
- Immunosuppressive function assay in vitro
- Histology and tartrate-resistant acid phosphatase staining
- Immunohistochemistry and immunofluorescence staining
- Enzyme-linked immunosorbent assay

Statistical Analysis

Statistical analysis was performed with SPSS 13.0. All data were presented as mean ± SD and assessed by independent 2-tailed Student's t test or 1-way analysis of variance. Statistical significance was considered at $P < 0.05$.

Results

Aspirin Inhibits Relapse after OTM

The schema in Figure 1 shows the experimental design in the relapse model, including orthodontic force application and aspirin administration; the micro–computed tomography photograph displays the maxilla from the transverse view (Fig. 1A, B). After the 10-d relapse, the distance between the first and second molars significantly decreased, from 706.7 ± 128.6 μm to 233.3 ± 46.2 μm, as compared with that at the beginning of relapse (Fig. 1C). The decrease was significantly stunted from 613.3 ± 201.3 μm to 446.7 ± 110.2 μm by aspirin treatment (Fig. 1C). To further display the effect of aspirin on relapse, continuous relapse distances and relapse rates were analyzed with silicone impressions. During relapse, the distance of OTM gradually decreased, and the aspirin-treated group showed an inhibited decreasing trend of OTM distance (Fig. 1D, E). We find that the repressive effect of aspirin on relapse distance and relapse rate was observed as early as 0.5 d of relapse (Fig. 1F, G). The relapse rate increased gradually during relapse; moreover, aspirin treatment continuously repressed the relapse rate from day 2 to day 10 of relapse (Fig. 1F, G). These results suggest that aspirin administration can block relapse following OTM in rats.

Aspirin Accelerates Decrease of Orthodontic Force–Induced Secretion of TNF-α and IFN-γ during Relapse

Spleen is an important immune organ in the body. To verify whether $CD4^+$ T lymphocytes changed after the application of orthodontic force stimuli and relapse, the percentage of $CD4^+$

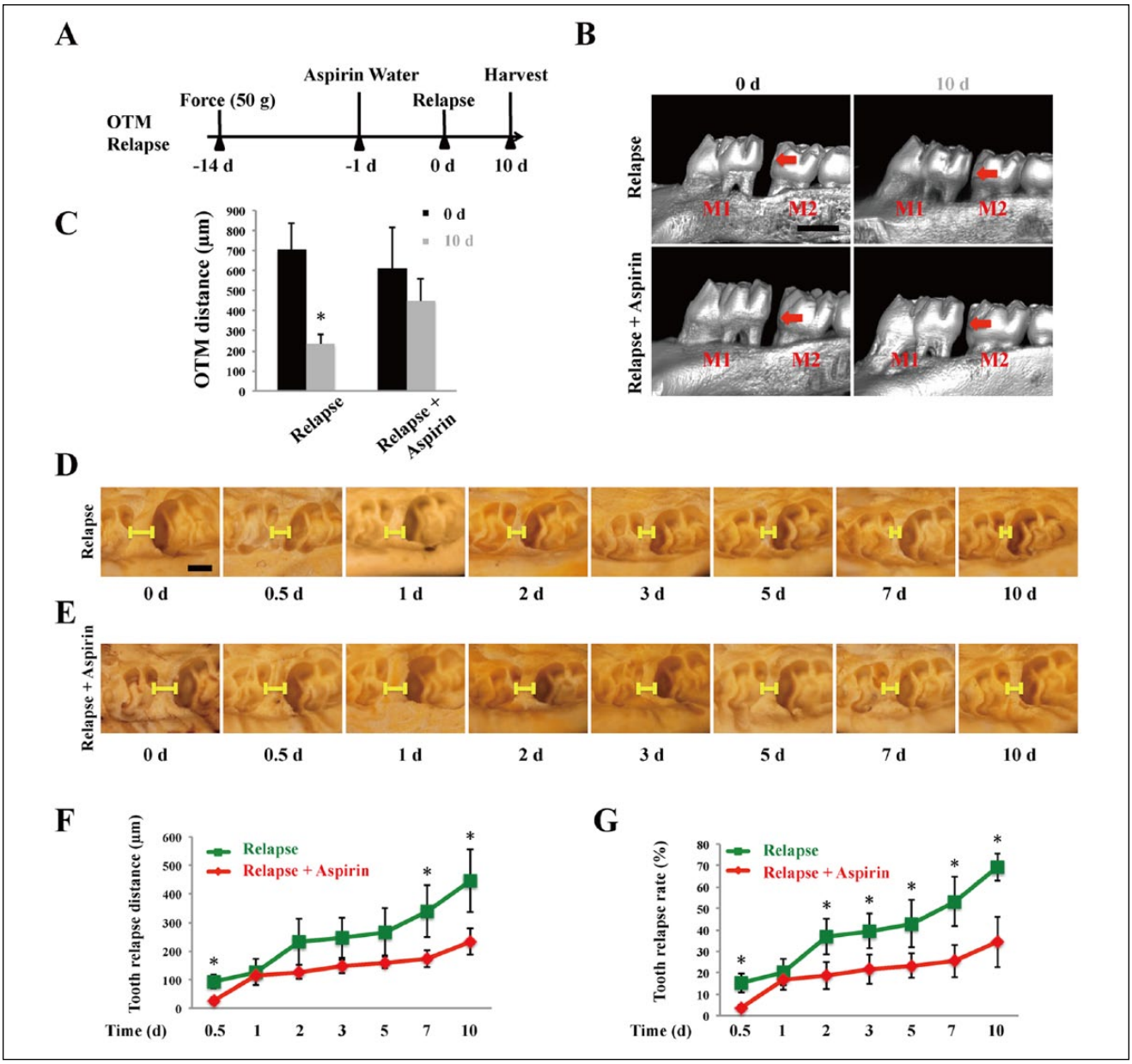

Figure 1. Aspirin inhibits relapse of orthodontic tooth movement (OTM). (**A**) Scheme for animal experiments. (**B**) Analysis of the relapse distance by micro–computed tomography scanning. The arrows indicate the relapse distance of tooth movement. M1, the first molar; M2, the second molar. Bar = 1,000 μm. (**C**) Analysis of the relapse distance. Tooth movement distance decreased with time during the relapse process. The distance between M1 and M2 in maxilla is sharply reduced from 0 d to 10 d in the relapse group vs. the relapse + aspirin group. $n = 3$. $^{*}P < 0.05$ vs. relapse at 0 d. (**D**, **E**) Analysis of continuous relapse distances by silicone impression. The distances between M1 and M2 after 0, 0.5, 1, 2, 3, 5, 7, and 10 d of relapse were recorded by silicone impression. Scale bars = 1,000 μm. (**F**) Aspirin treatment reduced tooth relapse distance. Relapse distance = relapse distance at 0 d – relapse distance at n d, where n = 0.5, 1, 2, 3, 5, 7, 10 d. $n = 3$. (**G**) Aspirin treatment reduced tooth relapse rate. Relapse rate = relapse distance / relapse distance at 0 d. $n = 3$.

T lymphocytes in spleen was determined by flow cytometry (Fig. 2A). After force application, the percentage of $CD4^+$ T lymphocytes significantly increased to 34.1%, compared with 17.2% in the control group (Fig. 2A). After 10 d of relapse, $CD4^+$ T lymphocytes declined to 24.8%, which was also higher than that of the control group (Fig. 2A). Moreover, $CD4^+$ T lymphocytes declined to 16.8% after aspirin treatment, which was lower than the force group and the relapse group (Fig. 2A). Tartrate-resistant acid phosphatase (TRAP) staining was conducted to confirm the effect of aspirin on osteoclasts during relapse. TRAP-positive multinucleated cells were barely detected in the control group, whereas an increase was detected during OTM and the relapse period after the orthodontic appliance was removed (Fig. 2B). The number of TRAP-positive multinucleated cells reduced significantly in the relapse + aspirin group (Fig. 2B, C). Furthermore, immunohistochemical

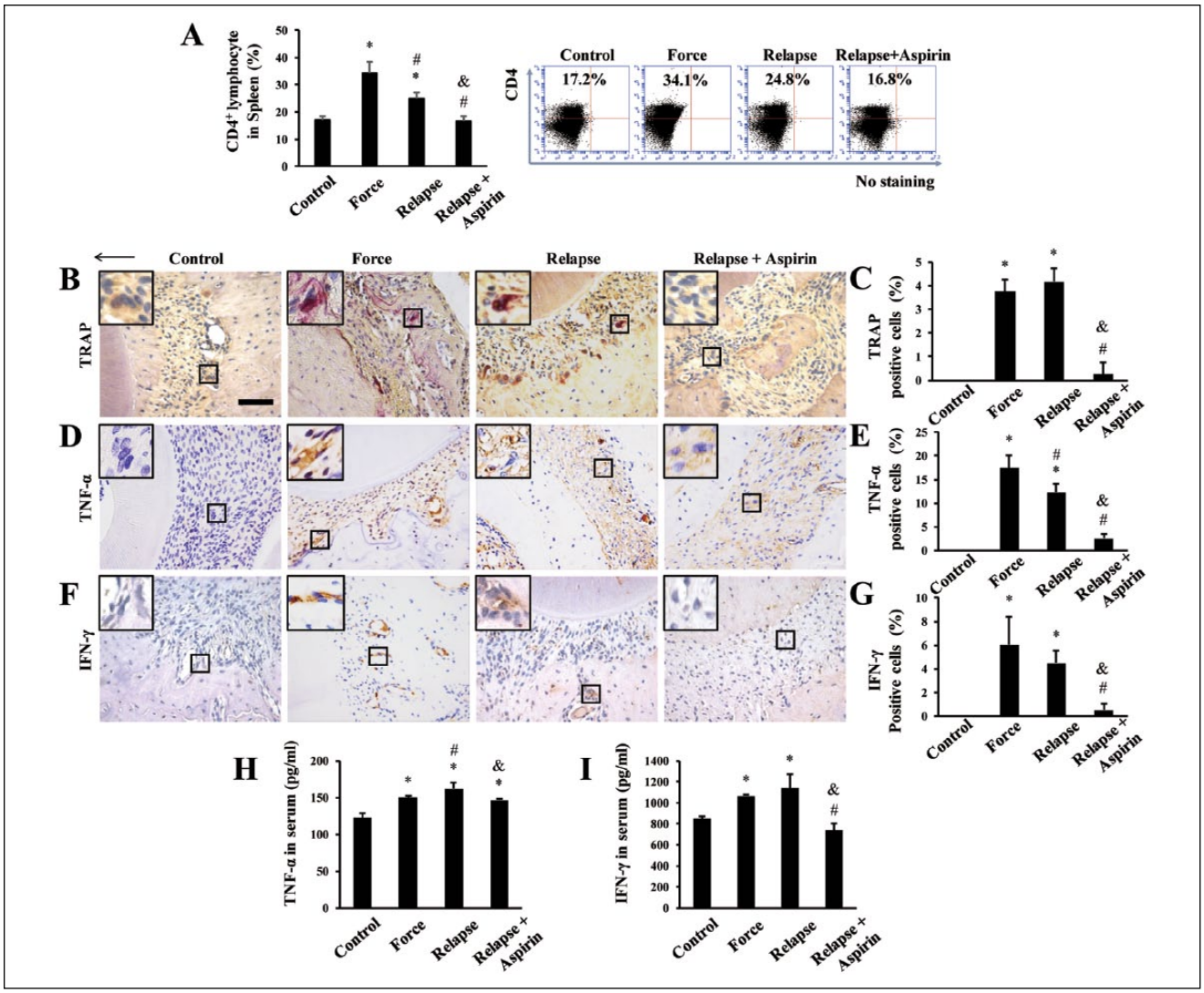

Figure 2. Aspirin represses type 1 T helper–associated cell cytokines tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) in blood and periodontal ligament during relapse. (**A**) Flow cytometry analysis of $CD4^+$ T lymphocytes in spleen. Orthodontic force–induced increase of $CD4^+$ T lymphocytes was repressed by aspirin treatment. n = 3 to 5. $^*P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. force, $^{\&}P < 0.05$ vs. relapse. (**B**) Tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts in periodontal ligament. Large boxed areas show higher-magnification views of the small boxes. Arrows represent the direction of force application. Scale bars = 50 μm. (**C**) Semiquantification of TRAP-positive multinucleated cells. Orthodontic force–induced increase of TRAP-positive cells was repressed by aspirin treatment. n = 4. $^*P < 0.01$ vs. control, $^{\#}P < 0.01$ vs. force, $^{\&}P < 0.05$ vs. relapse. (**D**, **F**) Immunohistochemical staining for TNF-α and IFN-γ of the distobuccal roots. TNF-α and IFN-γ staining was detected in the force group and the relapse group, while aspirin treatment reduced the expression of TNF-α, IFN-γ. Large boxed areas show high-magnification views of the small boxes. Scale bars = 50 μm. (**E**, **G**) Semiquantification of TNF-α- and IFN-γ-positive cells. TNF-α and IFN-γ expression increased in the force group and the relapse group but decreased in the relapse + aspirin group. n = 3 to 6. $^*P < 0.01$ vs. control, $^{\#}P < 0.01$ vs. force, $^{\&}P < 0.01$ vs. relapse. (**H**, **I**) Secretions of TNF-α and IFN-γ in serum were assessed by ELISA. Aspirin repressed serum levels of TNF-α and IFN-γ during relapse. n = 3 to 5, $^*P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. force, $^{\&}P < 0.01$ vs. relapse.

staining showed that the expression of Th1-associated proinflammatory cytokines TNF-α and IFN-γ were elevated on the stretching side of periodontal tissues after force application compared with the control group (Fig. 2D–G). We used immunofluorescence staining to show that TNF-α was co-localized with $CD4^+$ T cells around periodontal tissue after orthodontic force (Appendix Fig. 1). After 10 d of relapse, the expression of TNF-α showed a declining trend as compared with the force group but maintained higher amounts than the control group (Fig. 2D, E). Although no statistically significant difference was detected, the IFN-γ expression showed a declining trend after the 10-d relapse (Fig. 2F, G). More important, aspirin treatments significantly accelerated the decline of TNF-α and IFN-γ expression on the stretching side of periodontal tissues during relapse (Fig. 2D–G). To confirm the effect of aspirin on Th1 lymphocytes during relapse, TNF-α and IFN-γ secretions in the rats were detected with ELISA (enzyme-linked immunosorbent assay). The serum levels of TNF-α and IFN-γ increased

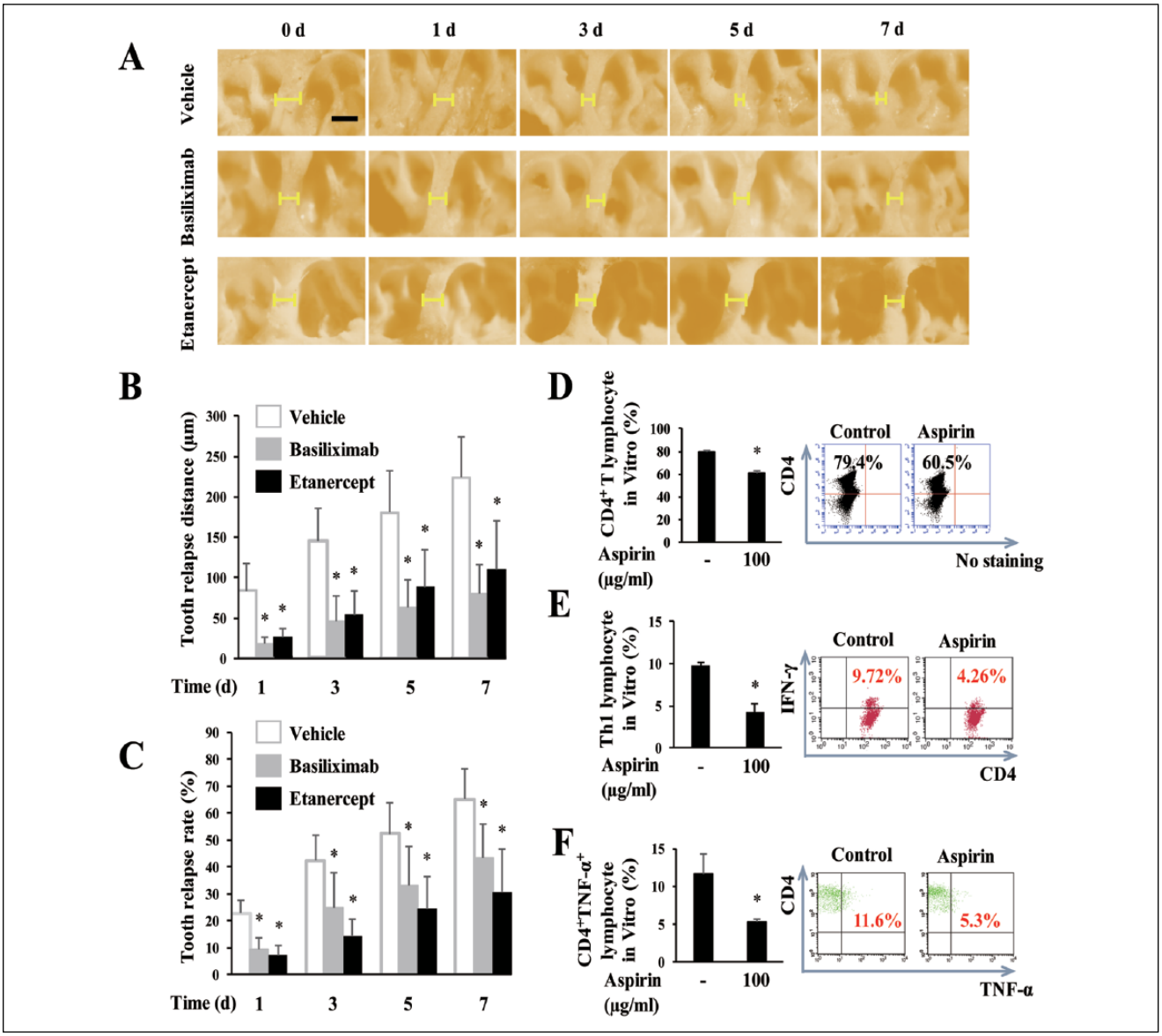

Figure 3. T cells are involved in the relapse process, and aspirin inhibits $CD4^+$ T lymphocytes and type 1 T-helper (Th1) cells in vitro. (**A**) Analysis of continuous relapse distances in vehicle group, basiliximab group, and etanercept group by silicone impression. The distances between the first molar and second molar after 0, 1, 3, 5, and 7 d of relapse were recorded by silicone impression. Scale bars = 1,000 μm. (**B**) Basiliximab treatment and etanercept treatment reduced tooth relapse distance as compared with vehicle treatment. Relapse distance = relapse distance at 0 d – relapse distance at *n* d, where *n* = 1, 3, 5, 7. *n* = 4 to 5. $^*P < 0.05$ vs. vehicle. (**C**) Basiliximab treatment or etanercept treatment reduced tooth relapse rate as compared with vehicle treatment. Relapse rate = relapse distance / relapse distance at 0 d. *n* = 4 to 5. $^*P < 0.05$ vs. vehicle. (**D**) Analysis of $CD4^+$ T lymphocytes in vitro. Flow cytometry showed that the ratio of $CD4^+$ T lymphocytes significantly decreased after 100 μg/mL of aspirin treatment for 48 h in vitro. (**E**, **F**) Analysis of Th1 cells in vitro. Aspirin treatment with 100 μg/mL can also decrease the Th1 cell level after 48 h in vitro. *n* = 3, $^*P < 0.01$ vs. aspirin (–).

during OTM and the relapse period as compared with the control group (Fig. 2H, I). However, aspirin treatment significantly reversed the secretion of TNF-α and IFN-γ during relapse (Fig. 2H, I). These data suggest that not only can aspirin repress the systemic increase of $CD4^+$ T lymphocytes and secretion of Th1-associated proinflammatory cytokines, but it can also localize the expression of TNF-α and IFN-γ induced by orthodontic force, which may contribute to the repressive effect of aspirin on relapse.

T Cells Are Involved in the Relapse Process, and Aspirin Inhibits $CD4^+$ T Lymphocytes and Th1 Cells In Vitro

We used CD25 neutralizing antibody basiliximab injection to repress the activation of T lymphocytes in SD rats, and we found that the relapse distance and relapse rate significantly decreased after treatment with CD25 neutralizing antibody when compared with phosphate-buffered saline treatment in

the vehicle group (Fig. 3A–C), which further confirmed the effect of T cells during relapse. The efficiency of basiliximab on repression of T-cell activation in rats was confirmed by FACS analysis (fluorescence-activated cell sorting), which showed that the percentage of $CD25^+$ T lymphocytes in peripheral blood and spleen and $CD3^+CD4^+$ T lymphocytes in blood from rats was repressed by basiliximab injection (Appendix Fig. 2A–C). In addition, we found that blockage of TNF-α by etanercept injection in rats during relapse significantly repressed the relapse distance and relapse rate (Fig. 3A–C), which suggests that Th1-associated production of TNF-α is involved in the relapse process. Subsequently, we performed an in vitro test to determine whether aspirin regulated $CD4^+$ T lymphocytes, using T cells separated from the spleen of Institute for Cancer Research mice with and without 100 μg/mL of aspirin. The percentage of $CD4^+$ T lymphocytes was significantly repressed from 79.4% to 60.5% after 48 h treatment of aspirin (Fig. 3D). Aspirin treatment also repressed the ratio of $CD4^+IFN\text{-}\gamma^+$ T cells from 9.72% to 4.26% (Fig. 3E) and $CD4^+TNF\text{-}\alpha^+$ T cells from 11.6% to 5.3% (Fig. 3F). These data suggest that aspirin inhibits $CD4^+$ T lymphocytes and Th1 cells in vitro.

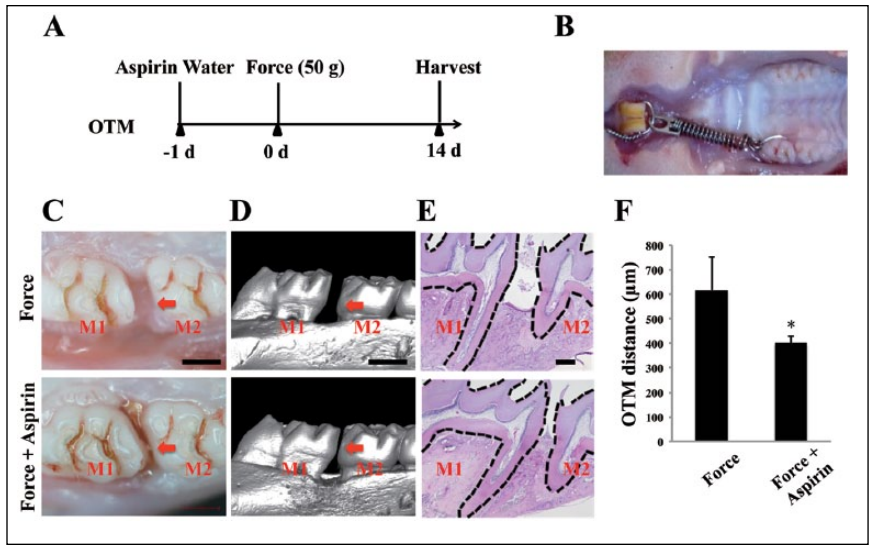

Figure 4. Aspirin treatment inhibited orthodontic tooth movement (OTM) in rat. (**A**) Scheme for the experimental design, orthodontic force application, and aspirin administration. (**B**) Photograph for rat maxillae with the orthodontic appliance. (**C**) Analysis of the OTM distance by stereoscope. Arrows indicate OTM distance. M1, the first molar; M2, the second molar. Bar = 1,000 μm. (**D**) Analysis of the OTM distance by micro–computed tomography scanning. The distance between M1 and M2 in maxilla is reduced in the force + aspirin group as compared with the force group. Bar = 1,000 μm. (**E**) Hematoxylin and eosin staining showed the integrity of the periodontal tissue around the 2 molars after tooth movement. Bar = 100 μm. (**F**) Analysis of the OTM distance. OTM distance decreased in the force + aspirin group vs. the force group after 14 d of force application. $n = 6$. $^*P < 0.05$ vs. force.

Aspirin Inhibits OTM

Following orthodontic force application for 14 d, the tooth movement distance increased (Fig. 4A, B). Moreover, aspirin treatment significantly repressed the force-induced increase of tooth movement distance (Fig. 4C, D). Hematoxylin and eosin staining showed the integrity of the periodontal tissue around the 2 molars after OTM (Fig. 4E). The OTM distance in the force-applied side resulted in 618.3 ± 132.1 μm in the force group (Fig. 4F), whereas no movement was detected in the opposite side (data not shown). By contrast, OTM distance in the force + aspirin group significantly decreased (400 ± 28.3 μm) compared with that of the control group (Fig. 4F), suggesting that orthodontic force–induced tooth movement was partially suppressed by aspirin.

Aspirin Inhibits $CD4^+$ T Lymphocytes and Th1 Cytokines during OTM

To evaluate the effect of aspirin on $CD4^+$ T lymphocytes in the periphery, we first observed the levels of these cells in peripheral blood 14 d after aspirin treatment. The percentage of $CD4^+$ T lymphocytes in peripheral blood significantly increased from 31.9% at the beginning of OTM (control group) to 41.3% on day 14 of OTM (force group) but decreased to 31.7% after the treatment with aspirin (Fig. 5A). Interestingly, the ratio of $CD4^+$ T lymphocytes in spleen also increased significantly after orthodontic force application, a trend that can be reversed by aspirin treatment (Fig. 5B). TNF-α- and IFN-γ-positive cells were barely detected surrounding the midbuccal roots in the control group in rats (Fig. 5C–F). However, strong staining of TNF-α and IFN-γ was detected after orthodontic force application (Fig. 5C–F). Moreover, aspirin can reverse the increasing trend of TNF-α- and IFN-γ-positive cells in the process (Fig. 5C–F). To further confirm the effect of aspirin on Th1-associated cytokines during OTM, the serum levels of TNF-α and IFN-γ were detected. The serum levels of TNF-α and IFN-γ of the force group significantly increased versus those in the control groups (Fig. 5G, H). Aspirin treatment significantly repressed force-induced secretion of TNF-α and IFN-γ, as assessed by ELISA (Fig. 5G, H). Aspirin can repress the systemic immune response regarding the reduction of $CD4^+$ T lymphocytes in peripheral blood and spleen, as well as the secretion of TNF-α and IFN-γ in peripheral blood (Fig. 5 A, B, G, H). Aspirin can also repress the local expression of TNF-α and IFN-γ surrounding the midbuccal roots during OTM (Fig. C–F). These results indicate that aspirin may repress OTM through repression of $CD4^+$ T lymphocytes and Th1-associated cytokines.

Discussion

We explored the repressive effects of aspirin on relapse and their underlying mechanisms in rats. First, aspirin significantly reduced

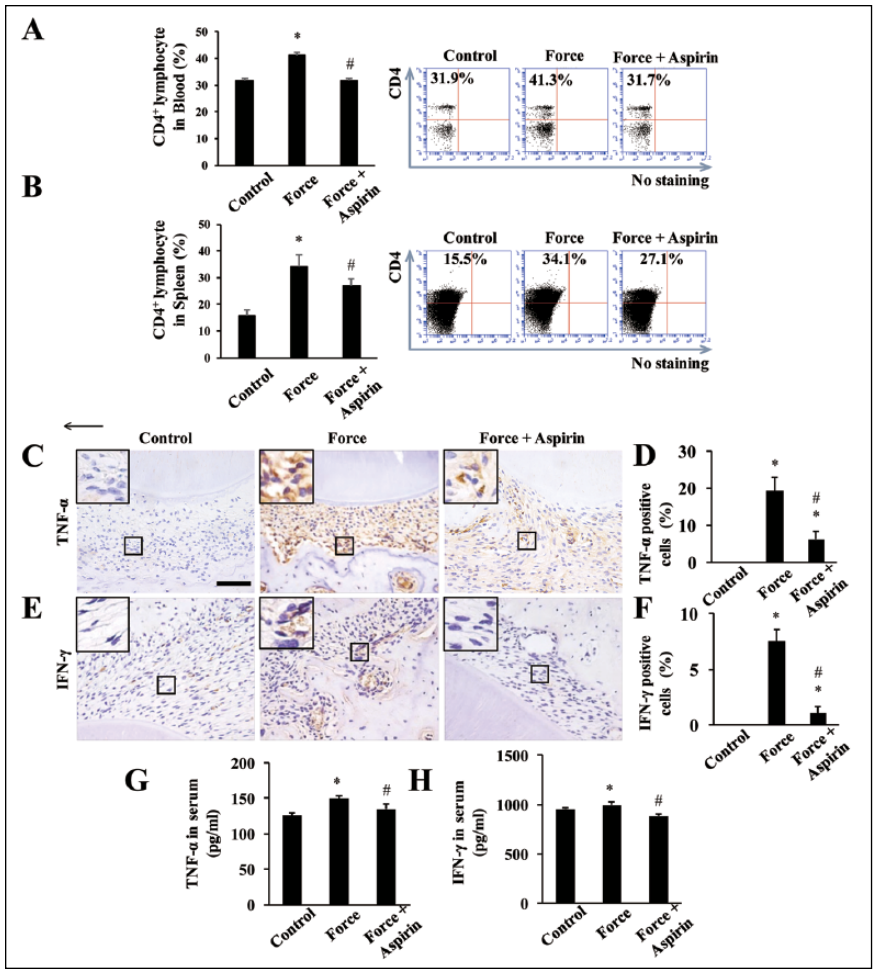

Figure 5. Aspirin inhibited Th1 cytokine tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) in blood, spleen, and periodontal ligament. (**A**, **B**) Flow cytometry analysis of $CD4^+$ T lymphocytes in blood and spleen. The percentages of $CD4^+$ T lymphocytes in peripheral blood and spleen significantly increased after 14 d of orthodontic tooth movement (OTM) in vivo but decreased after the treatment with aspirin, as compared with the force group. n = 3 to 5. $^*P < 0.05$ vs. control, $^\#P < 0.01$ vs. force. (**C**, **E**) Immunohistochemical staining for TNF-α and IFN-γ of the midbuccal roots. TNF-α and IFN-γ were significantly enhanced after force vs. control, but aspirin reduced increasing levels of TNF-α and IFN-γ in the periodontal ligament. Large boxed areas show high-magnification views of the small boxes areas. Arrows represent the direction of force application. Scale bars = 50 μm. n = 4 to 6. (**D**, **F**) Semiquantification of TNF-α- and IFN-γ-positive cells. TNF-α and IFN-γ expression increased in the force group but decreased in the force + aspirin group. $^*P < 0.01$ vs. control, $^\#P < 0.05$ vs. force. (**G**, **H**) Secretions of TNF-α and IFN-γ in serum were assessed by ELISA. Aspirin repressed serum levels of TNF-α and IFN-γ during OTM. n = 3 to 5. $^*P < 0.05$ vs. control, $^\#P < 0.05$ vs. force.

the relapse distance and relapse rate and, correspondingly, accelerated the decrease of TNF-α and IFN-γ, systemically in serum and locally in periodontal ligament (PDL), during the relapse process. In addition, blockage of T-lymphocyte activation and Th1-associated cytokine TNF-α repressed relapse distance and relapse rate, which suggests that T cells and associated cytokines contribute to relapse. Second, OTM upregulated Th1 cytokines TNF-α and IFN-γ in the blood, and local PDL tissue was reduced by aspirin treatment. Third, OTM-induced increase of $CD4^+$ T-lymphocyte cell ratio in spleen was detected but decreased in the relapse procedure. The $CD4^+$ T-lymphocyte cell ratio decreased sharply in aspirin treatment during the same process. Finally, aspirin significantly decreased the percentage and number of $CD4^+$ T-lymphocyte cells in vitro. We first provided the mechanism of OTM and orthodontic relapse suppressed by aspirin from an immunologic aspect and observed that aspirin can inhibit OTM and relapse.

Whether the relapse after OTM is a process of immunoregulation remains unclear. The major concern regarding the use of NSAIDs to manage orthodontic pain is that these drugs may interfere with tooth movement by inhibiting cyclooxygenase activity, leading to prostaglandin production. In the cranial inflammation pathway, aspirin can suppress the production of prostaglandin E2 (PGE2), which is an osteoclastogenesis promotion factor by acting on the osteoclast's cell signal pathway. However, in immune regulation, PGE2 induced activation of cyclic adenosine monophosphate and produced an inhibitory effect on T-cell activation and proliferation (Okano et al. 2006; Bryn et al. 2008; Kalinski 2012), especially inhibiting Th1 lymphokine IL-2 and IFN-γ production (Betz and Fox 1991; Anastassiou et al. 1992). Although we cannot exclude the role of PGE2-mediated osteoclast production during relapse in the inflammation pathway, our study found that aspirin (which could affect osteoimmunoregulation) was able to significantly reduce systemic and local levels of TNF-α and IFN-γ with inhibition of $CD4^+$ lymphocyte and Th1 cells and then diminish the number of osteoclasts in relapse bone remodeling. This speculation is further supported by the observations that aspirin significantly improved renal allograft survival and allograft function in humans (Grotz et al. 2004).

Many recent studies have established that, under inflammatory conditions, T cells produce cytokines to increase osteoclast activity and promote bone loss (Caetano-Lopes et al. 2009; Takayanagi 2013). Proinflammatory cytokines, such as TNF-α, potentiate bone loss either by inducing the expression of receptor activator of nuclear factor-κ B ligand (RANKL) in osteoblasts or by increasing osteoclast generation (Gillespie 2007). The changes in cytokines, such as TNF-α and IFN-γ in the blood serum and PDL, were associated with relapse and aspirin treatment. Aspirin can block Th1 cell development (Mazzeo et al. 1998) and reduce TNF-α- and IFN-γ-producing cells in implanted tissue-engineered bones (Liu et al. 2011). In

2013, Wang et al. (2013) discovered that aspirin can block TNF-α and IFN-γ in ovariectomized mice, thereby rescuing them from mesenchymal stem cell deficiency and tumorigenesis. Indeed, we demonstrated that systemic administration of aspirin could decrease concentrations of TNF-α and IFN-γ in blood serum and PDL. Our findings suggest that aspirin may inhibit $CD4^+$ T lymphocytes, especially the Th1 lymphocyte ratio and cytokines, thus providing novel insights into the underlying mechanism of relapse.

Despite the secretion of Th1-associated cytokines, the increased number of T cells may produce functional RANKL, which is essential for osteoclast differentiation and activation (Udagawa et al. 1999; Takayanagi et al. 2002). Previous study reported that after mechanical force application, PDL fibroblasts secreted higher levels of TNF-α at the PDL compression side than at the tension side, and this imbalance promoted the osteoclastogenesis activation through TNF-α-mediated RANKL expression in $CD4^+$ T cells (Kook et al. 2011). This idea is consistent with our result that aspirin treatment repressed the expression of TNF-α associated with the reduced number of TRAP-positive osteoclasts. Further studies are needed to explore in detail the molecular mechanism underlying the aspirin-Th1 axis during the bone-remodeling process of relapse. However, our results indicated that $CD4^+$ T lymphocytes are involved in the repressive function of aspirin on relapse and at least partially depended on Th1-associated cytokine TNF-α, thus providing novel insights into the underlying mechanism of relapse.

Previous study reported that IFN-γ inhibits osteoclastogenesis during bone remodeling by activating the ubiquitin proteasome pathway within the osteoclasts, specifically resulting in degradation of the adapter protein TRAF6 and blocking RANKL-induced osteoclast differentiation (Takayanagi et al. 2000b; Kohara et al. 2011; Cheng et al. 2012; Xiong et al. 2016). However, the effects of IFN-γ are still controversial. Systemic administration of IFN-γ stimulates bone resorption, and blockage of IFN-γ production reduces the increase of bone resorption and T-cell activation in mice (Gao et al. 2007), suggesting that IFN-γ is a bone destruction cytokine in vivo. Taken together, these data showed that IFN-γ directly inhibited osteoclast formation through maturing osteoclast precursors, while it indirectly promoted osteoclastogenesis by stimulation of T-cell activation.

Various systemically and locally administrated pharmacologic agents have been reported to reduce or prevent the amount of relapse in animal models, such as bisphosphonate (Kim et al. 1999), osteoprotegerin (Zhao et al. 2012), simvastatin (Han et al. 2010), relaxin (Hirate et al. 2012), and low-level laser therapy (Franzen et al. 2015). However, most studies focused on the effect of tooth movement through the regulation of osteoclasts. Our study revealed that aspirin could inhibit relapse and OTM through $CD4^+$ T lymphocytes. Interestingly, aspirin-suppressed relapse may be one of the effective methods to prevent tooth relapse through inhibition of $CD4^+$ T lymphocytes.

Author Contributions

Y. Liu, X.X. Kou, Y.H. Zhou, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; T. Zhang, C. Zhang, S.S. Jin, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; R.L. Yang, contributed to conception and design, drafted and critically revised the manuscript; X.D. Wang, N. Jiang, Y.H. Gan, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This work was supported by the International Science and Technology Cooperation Program of China (No. 2015DFB30040, Y.Z.), the National Science Foundations of China (No. 81300897, X.K.; No. 81571815, Y.L.; No. 81300850, X.W.; No. 81470717, Y.Z.), the Beijing Municipal Natural Science Foundation (No. 7152156, Y.L.), and the China Scholarship Council (No. 201506010245, Y.L.). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

Anastassiou ED, Paliogianni F, Balow JP, Yamada H, Boumpas DT. 1992. Prostaglandin E2 and other cyclic AMP-elevating agents modulate IL-2 and IL-2R alpha gene expression at multiple levels. J Immunol. 148(9):2845–2852.

Arias OR, Marquez-Orozco MC. 2006. Aspirin, acetaminophen, and ibuprofen: their effects on orthodontic tooth movement. Am J Orthod Dentofacial Orthop. 130(3):364–370.

Arron JR, Choi Y. 2000. Osteoimmunology: bone versus immune system. Nature. 408(6812):535–536.

Ashkenazi M, Berlin-Broner Y, Levin L. 2012. Pain prevention and management during orthodontic treatment as perceived by patients. Orthodontics (Chic). 13(1):e76–e81.

Betz M, Fox BS. 1991. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol. 146(1):108–113.

Bryn T, Yaqub S, Mahic M, Henjum K, Aandahl EM, Tasken K. 2008. LPS-activated monocytes suppress T-cell immune responses and induce FOXP3+ T cells through a COX-2-PGE2-dependent mechanism. Int Immunol. 20(2):235–245.

Caetano-Lopes J, Canhão H, Fonseca JE. 2009. Osteoimmunology: the hidden immune regulation of bone. Autoimmun Rev. 8(3):250–255.

Cao H, Kou X, Yang R, Liu D, Wang X, Song Y, Feng L, He D, Gan Y, Zhou Y. 2014. Force-induced Adrb2 in periodontal ligament cells promotes tooth movement. J Dent Res. 93(11):1163–1169.

Cheng J, Liu J, Shi Z, Jules J, Xu D, Luo S, Wei S, Feng X. 2012. Molecular mechanisms of the biphasic effects of interferon-gamma on osteoclastogenesis. J Interferon Cytokine Res. 32(1):34–45.

Franzen TJ, Brudvik P, Vandevska-Radunovic V. 2013. Periodontal tissue reaction during orthodontic relapse in rat molars. Eur J Orthod. 35(2):152–159.

Franzen TJ, Zahra SE, El-Kadi A, Vandevska-Radunovic V. 2015. The influence of low-level laser on orthodontic relapse in rats. Eur J Orthod. 37(1):111–117.

Gao Y, Grassi F, Ryan MR, Terauchi M, Page K, Yang X, Weitzmann MN, Pacifici R. 2007. IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. J Clin Invest. 117(1):122–132.

Gillespie MT. 2007. Impact of cytokines and T lymphocytes upon osteoclast differentiation and function. Arthritis Res Ther. 9(2):103.

Grotz W, Siebig S, Olschewski M, Strey CW, Peter K. 2004. Low-dose aspirin therapy is associated with improved allograft function and prolonged allograft survival after kidney transplantation. Transplantation. 77(12):1848–1853.

Han G, Chen Y, Hou J, Liu C, Chen C, Zhuang J, Meng W. 2010. Effects of simvastatin on relapse and remodeling of periodontal tissues after tooth movement in rats [editor's comment]. Am J Orthod Dentofacial Orthop. 138(5):550, e1–e7.

He D, Kou X, Luo Q, Yang R, Liu D, Wang X, Song Y, Cao H, Zeng M, Gan Y, et al. 2015. Enhanced M1/M2 macrophage ratio promotes orthodontic root resorption. J Dent Res. 94(1):129–139.

Hirate Y, Yamaguchi M, Kasai K. 2012. Effects of relaxin on relapse and periodontal tissue remodeling after experimental tooth movement in rats. Connect Tissue Res. 53(3):207–219.

Kalinski P. 2012. Regulation of immune responses by prostaglandin E2. J Immunol. 188(1):21–28.

Kehoe MJ, Cohen SM, Zarrinnia K, Cowan A. 1996. The effect of acetaminophen, ibuprofen, and misoprostol on prostaglandin E(2) synthesis and the degree and rate of orthodontic tooth movement. Angle Orthodontist. 66(5):339–349.

Kim TW, Yoshida Y, Yokoya K, Sasaki T. 1999. An ultrastructural study of the effects of bisphosphonate administration on osteoclastic bone resorption during relapse of experimentally moved mt molars. Am J Orthod Dentofacial Orthop. 115(6):645–653.

Kohara H, Kitaura H, Fujimura Y, Yoshimatsu M, Morita Y, Eguchi T, Masuyama R, Yoshida N. 2011. IFN-gamma directly inhibits TNF-alpha-induced osteoclastogenesis in vitro and in vivo and induces apoptosis mediated by Fas/Fas ligand interactions. Immunol Lett. 137(1–2):53–61.

Kook SH, Jang YS, Lee JC. 2011. Human periodontal ligament fibroblasts stimulate osteoclastogenesis in response to compression force through TNF-alpha-mediated activation of CD4+ T cells. J Cell Biochem. 112(10):2891–2901.

Koritsanszky N, Madlena M. 2011. Pain and discomfort in orthodontic treatments: literature review. Fogorvosi Sz. 104(4):117–121.

Kuijpers-Jagtman AM. 2002. Repair and revision 8. Relapse of lower incisors: retreatment? Ned Tijdschr Tandheelkd. 109(2):42–46.

Little RM, Riedel RA, Artun J. 1988. AN evaluation of changes in mandibular anterior alignment from 10 to 20 years postretention. Am J Orthod Dentofacial Orthop. 93(5):423–428.

Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, Yang R, Chen W, Wang S, Shi S. 2011. Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-gamma and TNF-alpha. Nat Med. 17(12):1594–1601.

Masella RS, Meister M. 2006. Current concepts in the biology of orthodontic tooth movement. Am J Orthod Dentofacial Orthop. 129(4):458–468.

Mazzeo D, Panina-Bordignon P, Recalde H, Sinigaglia F, D'Ambrosio D. 1998. Decreased IL-12 production and Th1 cell development by acetyl salicylic acid-mediated inhibition of NF-kappa B. Eur J Immunol. 28(10):3205–3213.

Mirosavljevic D, Quinn JMW, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. 2003. T-cells mediate an inhibitory effect of interleukin-4 on osteoclastogenesis. J Bone Miner Res. 18(6):984–993.

Okano M, Sugata Y, Fujiwara T, Matsumoto R, Nishibori M, Shimizu K, Maeda M, Kimura Y, Kariya S, Hattori H, et al. 2006. E prostanoid 2 (EP2)/EP4-mediated suppression of antigen-specific human T-cell responses by prostaglandin E2. Immunology. 118(3):343–352.

Polat O, Karaman AI. 2005. Pain control during fixed orthodontic appliance therapy. Angle Orthod. 75(2):214–219.

Takayanagi H. 2013. Osteocytes and osteoclasts: crosstalk in osteoimmunology. Ann Rheum Dis. 72 Suppl 3:A16.

Takayanagi H, Kim S, Taniguchi T. 2002. Signaling crosstalk between RANKL and interferons in osteoclast differentiation. Arthritis Res. 4 Suppl 3:S227–S232.

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, et al. 2000a. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature. 408(6812):600–605.

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, et al. 2000b. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature. 408(6812):600–605.

Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, Nakagawa N, Yasuda H, Goto M, Tsuda E, et al. 1999. Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand. Bone. 25(5):517–523.

Verna C, Zaffe D, Siciliani G. 1999. Histomorphometric study of bone reactions during orthodontic tooth movement in rats. Bone. 24(4):371–379.

Wang L, Zhao Y, Liu Y, Akiyama K, Chen C, Qu C, Jin Y, Shi S. 2013. IFN- and TNF-synergistically induce mesenchymal stem cell impairment and tumorigenesis via NFB signaling. Stem Cells. 31(7):1383–1395.

Xiong Q, Zhang L, Ge W, Tang P. 2016. The roles of interferons in osteoclasts and osteoclastogenesis. Joint Bone Spine. 83(3):276–281.

Yan Y, Liu F, Kou X, Liu D, Yang R, Wang X, Song Y, He D, Gan Y, Zhou Y. 2015. T cells are required for orthodontic tooth movement. J Dent Res. 94(10):1463–1470.

Yoshida Y, Sasaki T, Yokoya K, Hiraide T, Shibasaki Y. 1999. Cellular roles in relapse processes of experimentally-moved rat molars. J Electron Microsc (Tokyo). 48(2):147–157.

Zhao N, Lin J, Kanzaki H, Ni J, Chen Z, Liang W, Liu Y. 2012. Local osteoprotegerin gene transfer inhibits relapse of orthodontic tooth movement. Am J Orthod Dentofacial Orthop. 141(1):30–40.