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# Orthodontic Force Induces Systemic Inflammatory Monocyte Responses

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**Abstract**

Periodontal inflammation and alveolar bone remodeling during orthodontic tooth movement are considered regional reactions. However, how systemic immune responses are involved in this regional reaction remains unclear. In this study, we explored the systemic effects of orthodontic force by focusing on the mononuclear phagocyte system. Flow cytometric analysis showed that the percentage of inflammatory monocytes, in peripheral blood and in the monocyte reservoir spleen, decreased on days 1 and 3 and then recovered on day 7 after force application. Along with the systemic decrease of inflammatory monocyte percentage, the number of tartrate-resistant acid phosphatase–positive osteoclasts increased in the compression side of the periodontal tissue during orthodontic tooth movement. Systemic transfusion of enhanced green fluorescent protein–labeled inflammatory monocytes showed recruitment of these monocytes to the orthodontic force compression side of periodontal tissues. These monocytes were colocalized with tartrate-resistant acid phosphatase–positive osteoclasts. In vivo and in vitro experiments showed that orthodontic force could upregulate the expression of pivotal monocyte chemokine monocyte chemotactic protein 1 in periodontal tissues or cultured periodontal ligament cells, which may contribute to monocyte recruitment to regional sites. These data suggest that orthodontic force induces systemic immune responses related to inflammatory monocytes and that systemic inflammatory monocytes can be recruited to periodontal tissues by orthodontic force stimulus.

**Keywords:** osteoclasts, mononuclear phagocyte system, chemokine CCL2, tooth movement, immune system, inflammation

## Introduction

Orthodontic tooth movement (OTM) is considered a periodontal inflammatory process characterized by osteoclastic and osteoblastic bone remodeling, in which the teeth move to a new periodontal position formed by bone resorption in the compression side and bone formation in the tension side (Meikle 2006). In recent decades, bone homeostasis and inflammation, as well as related diseases, have been proved to be regulated by the immune system. T cells, specific tissue cells, and important cytokines modulate the differentiation and activity of osteoclasts in cases of disease, such as rheumatoid arthritis and osteoporosis (Arron and Choi 2000; McInnes and Schett 2007; Takayanagi 2007; Zaidi 2007; Lorenzo et al. 2008). However, studies on immune regulations in OTM alveolar inflammation mainly focused on regional reactions (Krishnan and Davidovitch 2006). The systemic responses of the immune system to orthodontic force stimuli and these responses' effects on OTM are still unclear.

During OTM, osteoclasts play an important role and are believed to be regulated by the immune system during the process (Xie et al. 2008; Brooks et al. 2011). Monocytes are myeloid-derived cells originating in the bone marrow and residing in the peripheral blood and spleen, and they are considered osteoclasts precursors (Kurihara et al. 1990; Matsuzaki et al. 1998; Kotani et al. 2013). These cells are critical in the inflammatory responses to various stimuli. At least 2 major mice monocyte subsets are classified in the literature (Gordon and Taylor 2005). The inflammatory monocyte subset is characterized by high expression of Ly6C, CCR2, and CD11b (Geissmann et al. 2003). Inflammatory monocytes can be recruited to aseptic inflamed tissues from the blood and spleen in cases of atherosclerosis, cerebral ischemia, and myocardial infarction (Dimitrijevic et al. 2007; Tacke et al. 2007; Swirski

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A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

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et al. 2009). OTM represents an aseptic inflammation. Although it could be deduced that inflammatory monocytes may be involved in OTM through osteoclast differentiation, this hypothesis is untested.

Orthodontic force is required for tooth movement. Many studies found that under mechanical force stimulus, periodontal ligament cells (PDLCs) can produce immunoregulatory cytokines, such as tumor necrosis factor α and interferon γ (Kook et al. 2011; He et al. 2015). Other studies have detected monocyte chemotactic protein 1 (MCP-1) upregulation in periodontal tissues during OTM (Madureira et al. 2012; Taddei, Andrade, et al. 2012). MCP-1 is reported as the pivotal chemokine in inflammatory monocyte recruitment (Kuziel et al. 1997; Lu et al. 1998). Hence, it needs to be explored whether MCP-1 upregulation in PDLCs could be induced by orthodontic force and contribute to the recruitment of inflammatory monocytes to the periodontal region during OTM.

This study aimed to examine the changes of systemic inflammatory monocytes after orthodontic force stimuli to explore whether orthodontic force can induce systemic immune responses.

## Materials and Methods

### Animals

Male C57BL/6, Institute for Cancer Research (ICR), and ICR enhanced green fluorescent protein (EGFP) transgenic mice (Weitong Lihua Experimental Animal Center, Beijing, China) were used in this study. These mice were 6 to 8 wk of age when used in the experiments. All experimental procedures were reviewed and approved by the Animal Care and Use Committee of Peking University (LA2013-92).

### Orthodontic Force Application

The mice were anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneally). Orthodontic forces were delivered by coiled springs (wire size, 0.2 mm; diameter, 1 mm; length, 1 mm; Smart Technology, Beijing, China) tied between the incisors and first molars with resin (3M ESPE, USA), as previously described (Cao et al. 2014). Each coiled spring delivered approximately 30 g of force, measured by a dynamometer: the previous study showed that this force magnitude range was optimal for mice orthodontic treatment (Taddei, Moura, et al. 2012). Both maxillary quadrants were used in this study to mimic clinical orthodontic treatment (Appendix Fig.). The OTM distance was measured between 2 easily located points (the midpoint of the distal-marginal ridge of the first molar and the midpoint of the mesial-marginal ridge of the second molar) using a stereo microscope (SWZ1000, Nikon, Japan).

### Orthodontic Treatment Grouping

The mice were randomly divided into 4 groups, including 1 sham group and 3 force groups. Orthodontic force was applied to the 3 force groups for 1 d, 3 d, and 7 d. The sham group received sham operations of anesthesia and occlusal resin bonding and comprised mice from 1 d, 3 d, and 7 d of sham operations. At sacrifice, mice with additional inflammation because of fighting or scratching were excluded from the study. The final numbers of the different groups of mice and their treatments are shown in the Appendix Table.

### Cell Isolation

Spleen cells were isolated by mechanical disruption with 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 by centrifugation at 300 × g for 10 min. Cells were used immediately for flow cytometric analysis.

### Flow Cytometry

Isolated cells were incubated on ice for 30 min, using the following fluorescently conjugated antibodies for cell surface markers: CD11b (PE, 557397, BD Biosciences) and Ly6C (APC, 560595, BD Biosciences). Isotype controls used were Rat IgG2b, κ Isotype Control (PE, 553989, BD Biosciences) and Rat IgM, κ Isotype Control (APC, 551486, BD Biosciences). Analysis of stained cells was performed with a BD Accuri C6 flow cytometer (BD Biosciences). Isotype controls were used to set appropriate gates. For all samples, approximately 50,000 cells were analyzed to generate scatter plots. Inflammatory monocytes in mice were labeled as $CD11b^{+}Ly6C^{high}$ (Sunderkotter et al. 2004; Gordon and Taylor 2005). In peripheral blood gating, pregating of $CD11b^{+}SSC^{low}$ was set in CD11b vs. side scatter (SSC) field to exclude $SSC^{high}$ $CD11b^{+}$ granulocytes; $Ly6C^{high}$ gate was then set to isolate inflammatory monocytes from $SSC^{low}$ monocytes. In spleen inflammatory monocyte gating, a previous method was used (Dunay et al. 2010). In brief, $CD11b^{+}Ly6C^{high}$ gate was set in the Ly6C vs. the CD11b field.

### Tartrate-resistant Acid Phosphatase Staining

Maxillae were collected, fixed in 4% paraformaldehyde, demineralized in 15% ethylenediaminetetraacetic acid, and embedded in paraffin. Consecutive horizontal sections (4 μm) were obtained from the middle third of the distal root of each molar. Sections from similar positions of the roots were used for histologic analysis. Tartrate-resistant acid phosphatase (TRAP) staining (387A-1KT, Sigma-Aldrich, St. Louis, MO, USA) was used to label activated osteoclasts.

### Immunohistochemistry

Immunohistochemistry was performed as previously described (Kou et al. 2015). Briefly, maxillae and spleens were removed en bloc and fixed in 4% paraformaldehyde. Maxillae were demineralized in 15% EDTA. After being embedded in paraffin, the specimens were sectioned (4 μm) at the middle third of

the distal root of the first molars in the maxillae and at subcapsular regions from middle sagittal section of the spleen. Sections were subjected to antigen retrieval and blocked with 5% bovine serum albumin for 30 min at room temperature. Then the sections were incubated overnight at 4 °C with antibodies against mouse CD11b (1:400, Ab64347, Abcam) for spleen subcapsular monocyte staining and MCP-1 (1:200, Ab7202, Abcam) for periodontal staining. After extensive washing with phosphate-buffered saline, the sections were incubated with horseradish peroxidase–conjugated secondary antibodies and visualized using diaminobenzidine (Zhongshan Golden Bridge Biotechnology, Beijing, China). Sections at the same histologic levels were used for semiquantitative analysis.

### *Sorting and Transfusion of EGFP-labeled Inflammatory Monocytes*

Spleen leukocytes from ICR EGFP mice were washed in phosphate-buffered saline supplemented with 2% heat-inhibited fetal bovine serum. Cells were then transferred to a 5-mL polystyrene tube (Falcon, Franklin Lakes, NJ, USA) and applied through FACSAria II (BD Biosciences). All FITC-positive $CD11b^{+}Ly6C^{high}$ cells were collected as EGFP-labeled systemic inflammatory monocytes. The spleen inflammatory monocytes percentage was <1%; therefore, a relatively low transfusion concentration was selected. These systemic inflammatory cells were immediately transfused into ICR mice through the tail vein, according to methods described previously (Xu et al. 2013). In brief, $1 \times 10^5$ of systemic inflammatory monocytes were suspended in 100 μL of phosphate-buffered saline and transfused intravenously into ICR mice immediately before the sham operation or orthodontic treatment ($n = 3$, each group). All mice were euthanized at day 7, and maxillae were collected to track EGFP-labeled inflammatory cells.

### *Static Compression on PDLCs*

The protocol used to obtain human tissue samples was approved by the Ethical Guidelines of Peking University (PKUSSIRB-201311103) and performed with proper informed consent. Human PDLCs were isolated from the periodontium of normal bicuspids as described in previous research (Seo et al. 2004). Mechanical force was also applied on primary cultured PDLCs after 4 passages as previously described (Mitsui et al. 2005; Cao et al. 2014; He et al. 2015). In brief, a layer of glass cover and additional metal weights on top were placed over an 80% confluent cell layer in 6-well plates. PDLCs were treated under continuous compressive force (2 $g/cm^2$) for different durations, ranging from 0 to 6 h.

### *Quantitative Real-time Polymerase Chain Reaction*

Total RNA was isolated from cell lysate with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription and real-time polymerase chain reaction (PCR) was performed as previously described (Kou et al. 2011). The commercially synthesized primers (designed by Primer Premier 5.0 software) used in this study were as follows:

Human GAPDH sense/antisense: 5′-ATGGGGAAGGTGAAGGTCG-3′/5′-GGGGTCATTGATGGCAACAATA-3′
Human MCP-1 sense/antisense: 5′-CAGCCAGATGCAATCAATGCC-3′/5′-TGGAATCCTGAACCCACTTCT-3′

The efficiency of the newly designed primers was confirmed by sequencing the conventional PCR products.

### *Statistical Analyses*

Statistical analyses were performed using SPSS 13.0. One-way analysis of variance was used to compare the differences among groups, followed by least significant difference posttest in analyzing blood and spleen inflammatory monocytes percentage and in vitro and in vivo MCP-1 upregulation after static compression in PDLCs. All data are expressed as mean ± SEM. A value of $P < 0.05$ was considered statistically significant.

## Results

### *Peripheral Blood Inflammatory Monocyte Percentage Showed Dynamic Changes from Early Decline to Later Recovery*

After orthodontic force application, tooth movement distance was gradually increased from day 1 to day 7 (Fig. 1A). Meanwhile, peripheral blood flow cytometry was performed on both force and sham groups to test inflammatory monocyte percentage in circulating leukocytes (Fig. 1B). Sham operations on days 1, 3, and 7 showed no statistical differences (data not shown). Inflammatory monocyte percentage in the sham group was 2.45% ± 0.32%; in the force groups, it underwent dynamic changes from early decline to later recovery (Fig. 1B). On day 1 of force application, the percentage significantly decreased to 0.31% ± 0.03% when compared with the sham group ($P < 0.05$); on day 3, the percentage remained low at 0.53% ± 0.20%, which was also statistically lower compared vs. the sham group ($P < 0.05$); in longer treatment of 7 d, the percentage recovered to 1.65% ± 0.37% and showed no statistical difference when compared with the sham group (Fig. 1B).

### *Spleen Blood Inflammatory Monocyte Percentage Showed Dynamic Changes from Early Decline to Later Recovery*

The spleen is an important component of the mononuclear phagocyte system, and its splenic subcapsular region is the monocyte reservoir (Swirski et al. 2009). Thus, to explore whether other systemic reactions occurred besides the circulating system, inflammatory monocyte percentages in spleens after orthodontic force stimuli were also analyzed. Spleen flow

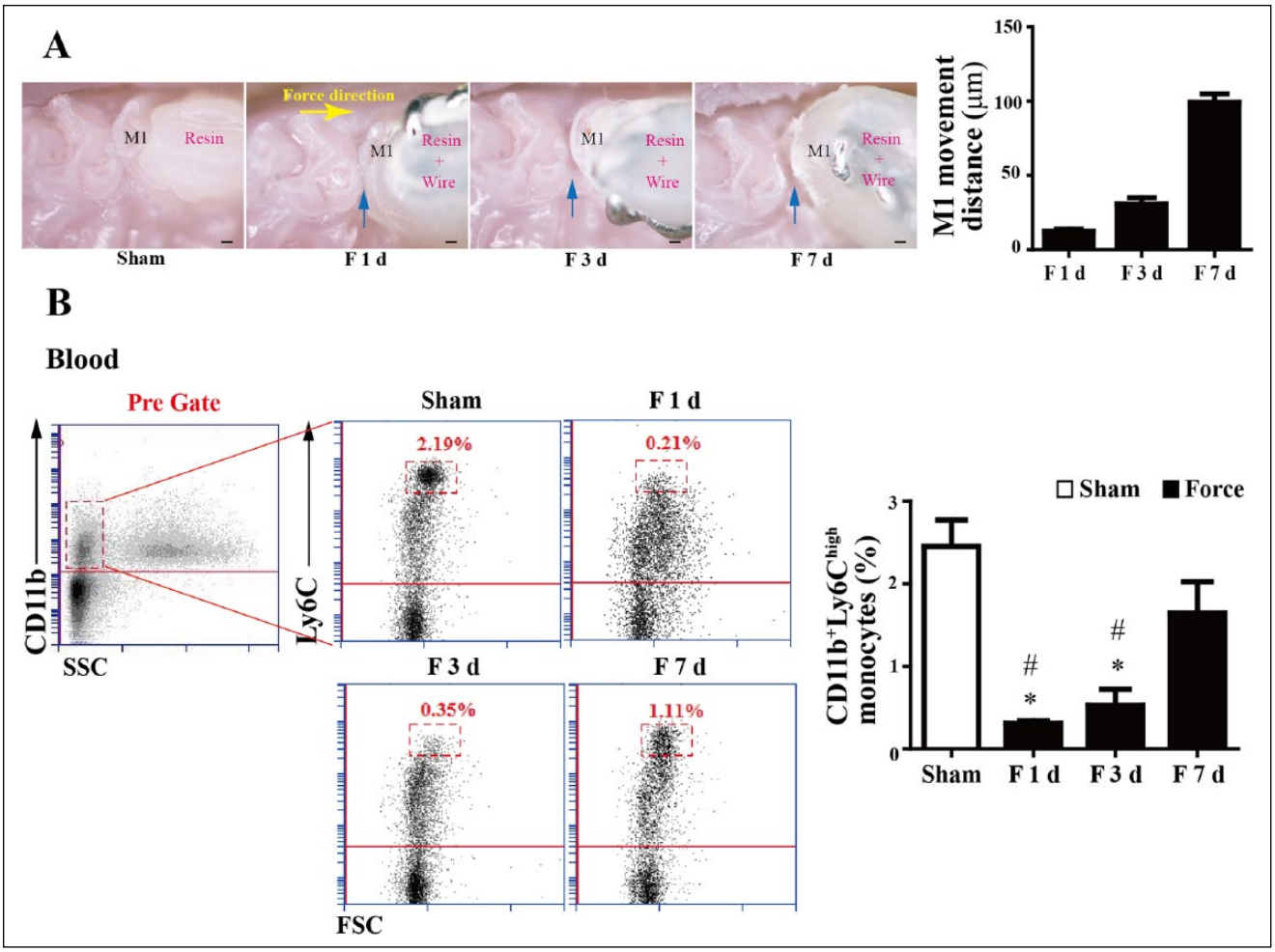


**Figure 1.** Peripheral blood inflammatory monocyte percentage in leukocytes initially decreased and then recovered along with increasing tooth movement distance under orthodontic force stimulus. (**A**) Tooth movement distance increased with time. Bar = 100 μm. F, force; M1, first molar. $n = 4$. (**B**) Flow cytometric analysis of peripheral blood inflammatory monocytes. Inflammatory monocytes were gated as $SSC^{low}CD11b^{+}Ly6C^{high}$. The left panel shows the characterizations of inflammatory monocyte percentage changes at different time points of orthodontic treatment. (Dotted boxes indicate gating. Numbers by boxes represent percentages.) The right panel shows the statistical result. The percentage dropped significantly on days 1 and 3 but recovered on day 7. $^{*}P < 0.05$ vs. sham; $^{\#}P < 0.05$ vs. force 7 d. $n = 11$ to 14. FSC, forward scatter; SSC, side scatter.

cytometry results showed that inflammatory monocyte percentage initially declined during the early stage of the response and then recovered later (Fig. 2A). Sham operations on days 1, 3, and 7 showed no statistical differences in the spleen inflammatory monocyte percentages (data not shown). After force application, the inflammatory monocyte percentage significantly decreased to 0.31% ± 0.04% on day 1, compared with 0.66% ± 0.11% in the sham group ($P < 0.05$; Fig. 2B). On day 3, the percentage remained low at 0.27% ± 0.07%, which was also statistically lower as compared with the sham group ($P < 0.05$); on day 7, the percentage recovered to 0.67% ± 0.14% and showed no statistical difference when compared with the sham group (Fig. 2B). To further confirm the spleen monocyte changes, CD11b immunohistochemical staining was performed. In the sham group, CD11b-positive monocytes could be detected in the splenic subcapsular regions (Fig. 2B). During OTM, decreased numbers of CD11b-positive cells were detected on days 1 and 3 after force application as compared with the sham group, but this number recovered on day 7 (Fig. 2B).

This result, with the inflammatory monocyte blood changes, indicated that the mononuclear phagocytic system was affected by orthodontic treatment.

## *Systemic Inflammatory Monocytes Were Recruited to Periodontal Region and Differentiated into Osteoclasts during OTM*

Systemic inflammatory monocyte changes were observed after orthodontic force stimuli. To explore whether these responses participate in periodontal OTM reactions, we first stained OTM regional osteoclasts and counted them. TRAP staining showed that activated osteoclasts significantly increased in the compression side of the periodontal tissues on day 3 and continued

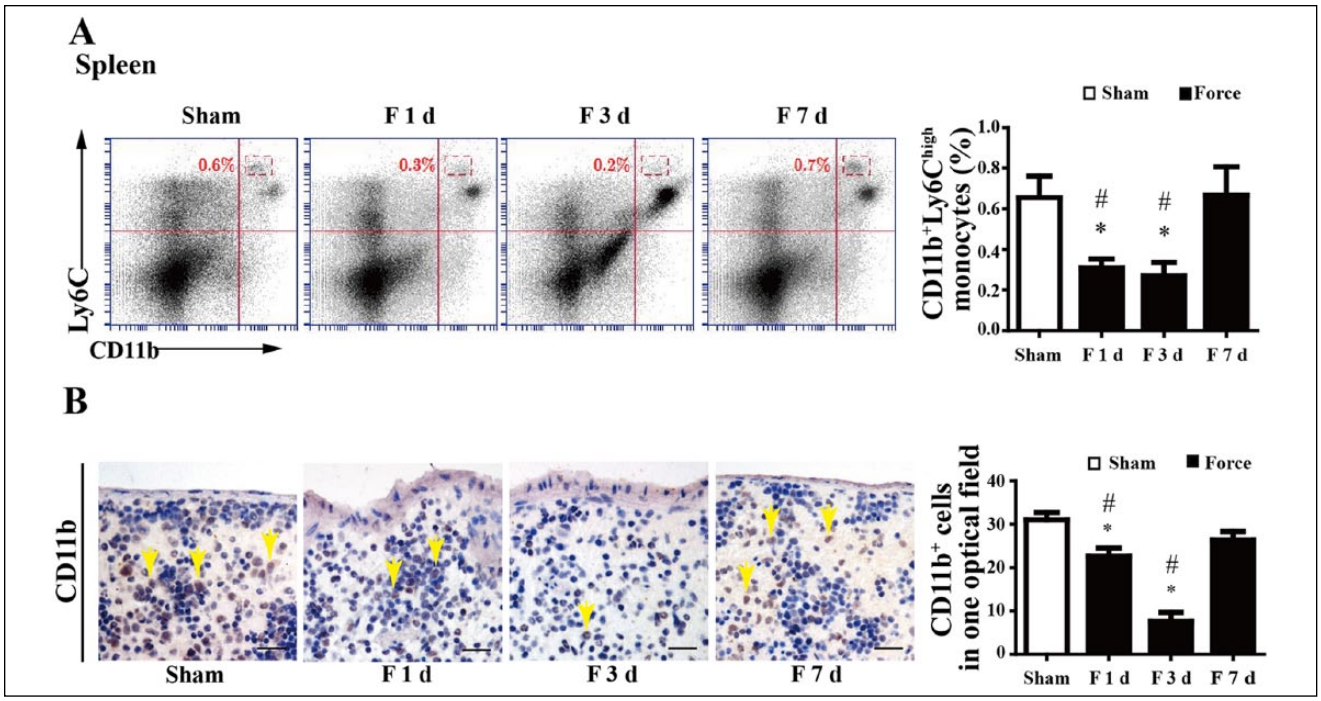


**Figure 2.** Spleen inflammatory monocyte percentage initially decreased and then recovered under orthodontic force stimulus. (**A**) Flow cytometric analysis of whole spleen inflammatory monocytes, which were gated as $CD11b^{+}Ly6C^{high}$. The left panel shows characterizations of inflammatory monocyte percentages at different time points of orthodontic treatment. (Dotted boxes indicate gating. Numbers by boxes represent percentages.) The right panel shows the statistical result. The percentage reduced significantly on days 1 and 3 but recovered on day 7. $^{*}P < 0.05$ vs. sham; $^{\#}P < 0.05$ vs. force 7 d. $n = 9$ to 11. F, force. (**B**) CD11b immunohistochemical staining of the splenic monocyte reservoir region. The left panel shows the characterizations of splenic monocytes at different time points of orthodontic treatment. Yellow arrows indicate $CD11b^{+}$ cells. Bar = 50 μm. The right panel shows semiquantitative assessment of splenic $CD11b^{+}$ monocytes. Spleen subcapsular $CD11b^{+}$ monocyte number decreased on day 1 and day 3 and then recovered on day 7. $^{*}P < 0.05$ vs. sham; $^{\#}P < 0.05$ vs. force 7 d. $n = 4$.

to increase on day 7 (Fig. 3A). To verify inflammatory monocyte recruitment, EGFP-labeled inflammatory monocytes were sorted from EGFP mice and transfused into wild-type orthodontic mice for tracking (Fig. 3B). In the force group, EGFP-positive cells were found in the compressed periodontal tissues on day 7, and these green fluorescent signals of EGFP-positive cells were also colocalized with TRAP-positive activated osteoclasts; in the sham group, no EGFP-positive cells were found in the periodontal tissues (Fig. 3B). These results suggested that regional recruitment of systemic inflammatory monocytes and their differentiation into osteoclasts contribute to OTM.

### *MCP-1 Expression in Periodontal Tissue Increased After Orthodontic Force Application*

To investigate the mechanism of systemic inflammatory monocyte migration to periodontal region during OTM, MCP-1 expression was detected at different time points of OTM. In vivo immunohistochemical staining detected MCP-1 expression upregulation in the compression side of the periodontal ligament from day 1 to day 7, in which osteoclasts mainly appeared (Fig. 4A). When static compressive force was applied to primary cultured human PDLCs, the expression of MCP-1 mRNA was upregulated by force treatment as early as 1 h and peaked at 2 h (Fig. 4B). This result indicated that compressive force–induced upregulation of MCP-1 expression in periodontal tissues contributes to inflammatory monocyte recruitment at the initial stage of OTM.

## Discussion

OTM has been considered a classic alveolar osteoblastic and osteoclastic process for decades. Explanations of the underlying mechanism come from molecular, cellular, and tissue-level studies restricted to the periodontal region (Krishnan and Davidovitch 2006). The orthodontic response at the systemic level tended to be neglected for a long time. To date, few studies (MacLaine et al. 2010) attempted to detect the systemic signs in human. These studies tested serum C-reactive protein, tumor necrosis factor α, and interleukin 6 levels in human patients at 2-mo intervals after clinical appointments but reported negative results. Our study focused on early responses and demonstrated, for the first time, the responses of systemic inflammatory monocytes in the circulating system and spleen during OTM, suggesting the existence of systemic immune responses in orthodontic mice. This study extended conceptually our understanding of the tooth movement mechanism at the systemic level.

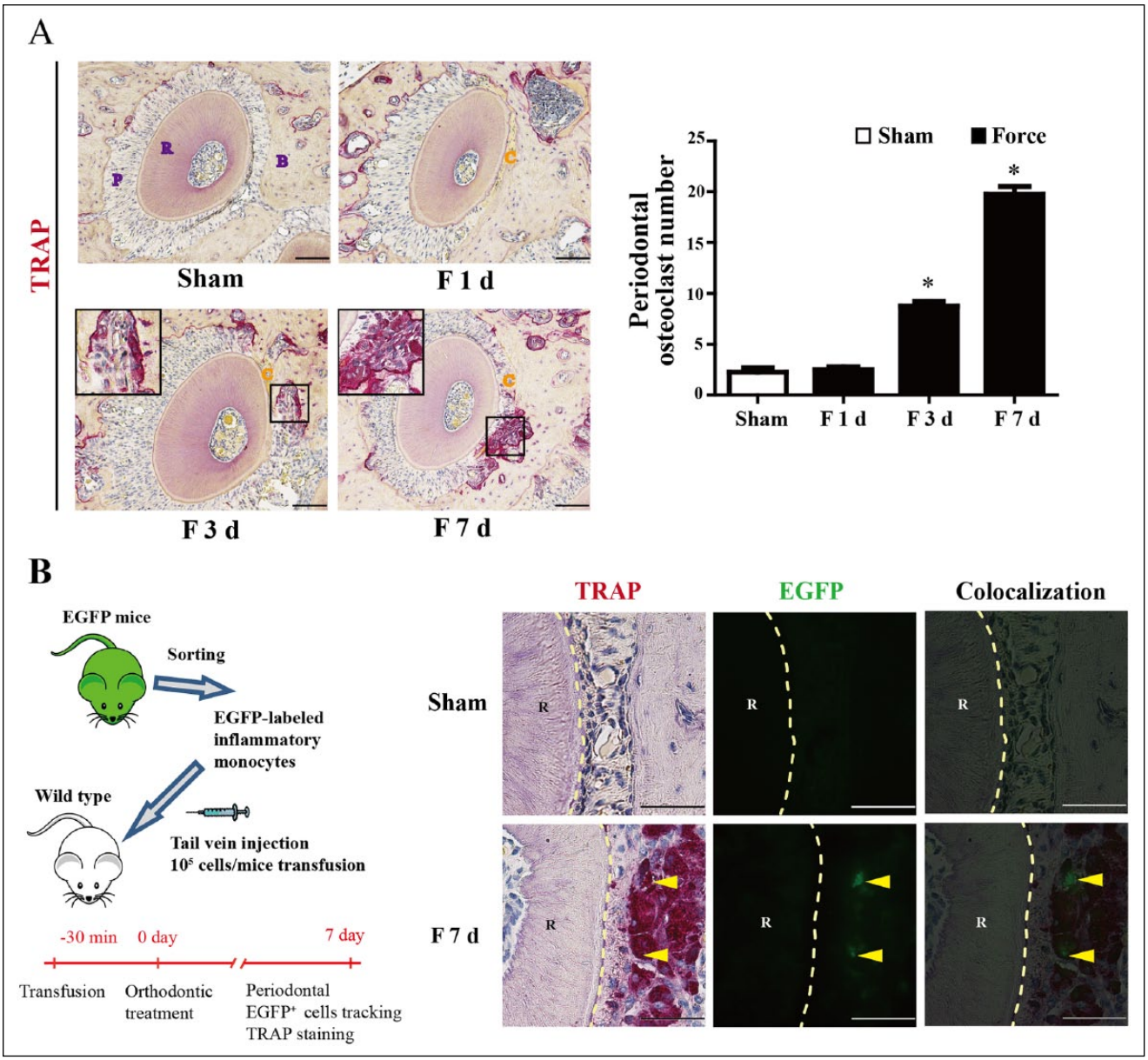


**Figure 3.** Systemic inflammatory monocytes were recruited to the periodontal region and differentiated into osteoclasts under orthodontic force stimulus. (**A**) Periodontal tartrate-resistant acid phosphatase (TRAP) staining. Left panel shows characterizations of TRAP-positive osteoclasts at different time points of orthodontic treatment. On day 3 and day 7, TRAP-positive cells remarkably increased and were presented in the compression periodontal side, near the alveolar bone surface. B, alveolar bone; C, compressed periodontal ligament; F, force; P, periodontal ligament; R, root. Bar = 100 μm. Right panel is the semiquantitative assessment of osteoclasts. $^{*}P < 0.05$ vs. sham. $n = 4$. (**B**) Tracking of transfused systemic enhanced green fluorescent protein (EGFP)–labeled inflammatory monocytes. The left panel is the scheme of sorting and transfusion of EGFP-positive inflammatory monocytes. The right panel shows the periodontal histology. Fluorescence (green) transfused EGFP-positive inflammatory monocytes were recruited to the compression side of the periodontal tissues and were also TRAP-positive (red) osteoclasts (yellow arrowhead). Bar = 50 μm.

In our study of mononuclear phagocyte system responses during OTM, the percentages of inflammatory monocytes were observed with early decline and later recovery in the blood and spleen. The decline occurred on as early as day 1 of treatment, indicating that the systemic recruitment of monocytes to region occurred at the initial stage of OTM. After induction of inflammation, inflammatory monocyte recruitment can be as rapid as 2 h (Henderson et al. 2003). Our in vitro static compression study also showed the capability of PDLCs to recruit monocytes in the first few hours after force stimulus by detecting early expression of MCP-1. We observed that systemic inflammatory monocyte percentages remained reduced on day 3 and then later recovered on day 7. This result is in accordance with a study on the time needed for inflammatory monocytes to recover their normal number in circulation after depletion, in which 4 d were needed for the bone marrow to produce new immune cells to fill the gap (Sunderkotter et al. 2004).

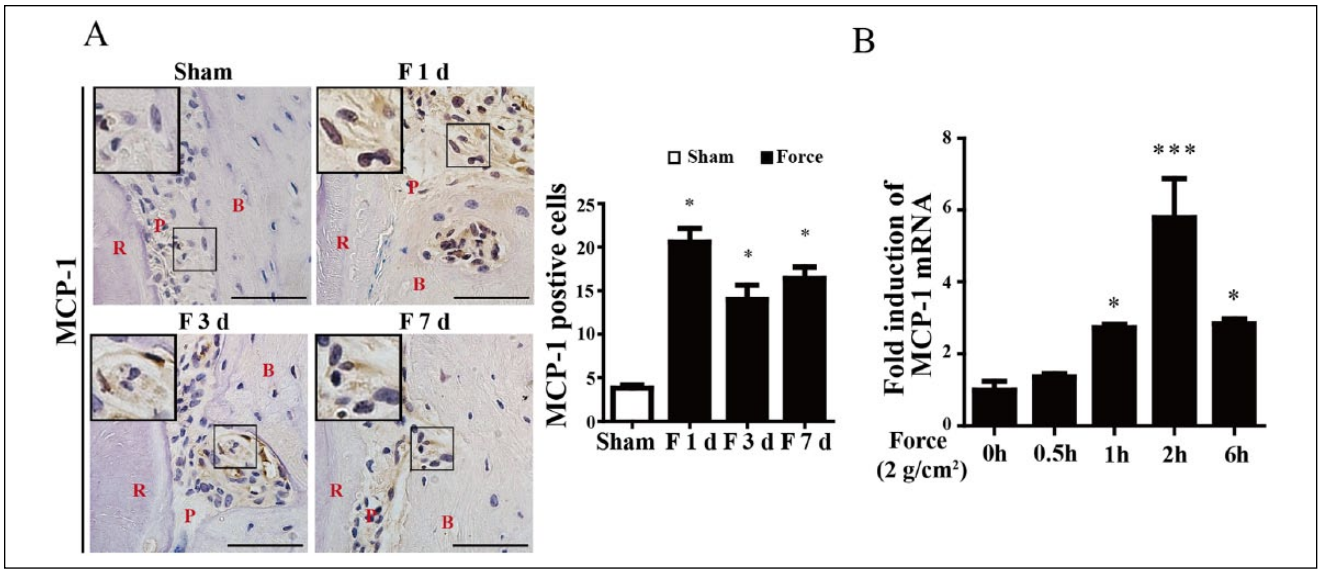


**Figure 4.** Monocyte chemotactic protein 1 (MCP-1) expression in periodontal tissue increased under orthodontic force stimulus. (**A**) Immunohistochemistry of MCP-1 in periodontal tissues. In mice periodontal tissues, MCP-1 expression was upregulated in the compression side under force stimulus. Most of the positively stained cells were fibroblast-like cells. F, force. Bar = 50 μm. *n* = 4. (**B**) MCP-1 mRNA expression in human PDLCs detected by real-time polymerase chain reaction. After compressive force application (2 g/cm$^2$), MCP-1 mRNA levels were upregulated with prolonged force treatment time and peaked at 2 h. n = 3. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. 0 h.

As discussed above, the systemic decline of inflammatory monocytes could be considered a result of system-to-region redistribution of immune cells. The experimental result of transfusion of EGFP-labeled inflammatory monocytes further suggested that these systemic cells redistributed to the periodontal region. In addition, the transfusion result revealed that the inflammatory monocytes directly participate in OTM by undergoing osteoclasts differentiation. Meanwhile, the increasing number of osteoclasts and continuous upregulation of MCP-1 expression indicated that the redistribution process was persistent. This is in accordance with the fact that teeth move over time. In brief, these results showed the important role of the participation of the systemic immune system in OTM.

Regional force stimuli induced systemic immune responses that directly participated in OTM. Our in vivo experiment showed persistent upregulation of MCP-1 expression in periodontal tissues during OTM. MCP-1 is an important monocyte chemokine produced either constitutively or by various cell types after induction by oxidative stress, cytokines, or growth factors (Deshmane et al. 2009). The in vitro experiment showed that upregulation of MCP-1 expression could be induced after direct force stimuli in PDLCs. Thus, MCP-1 may play an important role in the underlying mechanism of converting force stimulus signals to immune signals in OTM. Other studies proposed that orthodontic treatment could result in the production of more than one type of periodontal chemokine—namely, CCL2, CCL3, and CCL5—in rats (Alhashimi et al. 1999), mice (Andrade et al. 2009; Taddei, Andrade, et al. 2012; Taddei et al. 2013), and humans (Garlet et al. 2008; Madureira et al. 2012). Blocking the receptors of transgenic gene knockout mice significantly affected the number of osteoclasts and the distance of OTM (Andrade et al. 2009; Taddei, Andrade, et al. 2012; Taddei et al. 2013). Hence, MCP-1 is important for osteoclast recruitment in orthodontic treatment but may not be the only chemokine involved. Furthermore, the influence of force on immune regulation has been reported in other studies—both in production of immune cytokines of tumor necrosis factor α and interferon γ from PDLCs and in its direct modification of integrins at the nanoscale (Kook et al. 2011; Chen et al. 2012; He et al. 2015). Therefore, force stimulus in OTM induces immune reactions that are not limited to upregulation of MCP-1 expression in periodontal tissues. The systemic responses in OTM may not be restricted to the mononuclear phagocyte system and need further exploration.

It should be noted that our study was based on mice orthodontic models and may be different from orthodontic clinical works. However, this study yielded valuable insights for orthodontists when evaluating the treatment necessity of patients. In particular, for patients who already suffer from other systemic diseases, the risks of the monthly orthodontic force stimulus on their activated or suppressed immune system must be considered carefully.

In conclusion, orthodontic force induces systemic immune responses related to inflammatory monocytes, and systemic inflammatory monocytes can be recruited to periodontal tissues by orthodontic force stimulus.

## Author Contributions

M. Zeng, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; X. Kou, R. Yang, D. Liu, X. Wang, contributed to design, data analysis, and interpretation, critically revised the manuscript; Y. Song, contributed to design and data analysis, critically revised the manuscript; J. Zhang, contributed to data acquisition and analysis, critically revised the manuscript; Y. Yan, F. Liu, contributed to data acquisition, critically revised the manuscript; D. He,

contributed to data analysis and interpretation, critically revised the manuscript; Y. Gan, contributed to data interpretation, critically revised the manuscript; Y. Zhou, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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