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# Mechanical load-induced H<sub>2</sub>S production by periodontal ligament stem cells activates M1 macrophages to promote bone remodeling and tooth movement via STAT1

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## Abstract

**Background:** Tooth movement is a unique bone remodeling process induced by mechanical stimulation. Macrophages are important in mediating inflammatory processes during mechanical load-induced tooth movement. However, how macrophages are regulated under mechanical stimulation remains unclear. Mesenchymal stem cells (MSCs) can modulate macrophage polarization during bone remodeling. Hydrogen sulfide (H<sub>2</sub>S) can be produced by MSCs and have been linked to bone homeostasis. Therefore, this study aimed to investigate whether H<sub>2</sub>S contributed to periodontal ligament stem cell (PDLSC)-regulated macrophage polarization and bone remodeling under mechanical stimulation.

**Methods:** An experimental mechanical load-induced tooth movement animal model was established. Changes in cystathionine-β-synthase (CBS), markers of M1/M2 macrophages, tooth movement distance, and the number of osteoclasts were examined. The conditioned medium of PDLSCs with or without mechanical loading was utilized to treat THP-1 derived macrophages for 24 h to further investigate the effect of PDLSCs on macrophage polarization. Different treatments with H<sub>2</sub>S donor, CBS inhibitor, or the inhibitor of STAT1 were used to investigate the related mechanism. Markers of M1/M2 polarization and STAT1 pathway expression were evaluated in macrophages.

**Results:** Mechanical load promoted tooth movement and increased the number of M1-like macrophages, M1-associated pro-inflammatory cytokines, and the expression of CBS on the compression side of the periodontal ligament. The injection of CBS inhibitor or H<sub>2</sub>S donor could further repress or increase the number of M1-like macrophages, tartrate-resistant acid phosphatase-positive osteoclasts and the distance of tooth movement. Mechanistically, load-induced PDLSCs enhanced H<sub>2</sub>S production, which increased the expression of M1-associated cytokines in macrophages. These effects could be blocked by the administration of CBS inhibitor. Moreover, load-induced H<sub>2</sub>S steered M1 macrophage polarization via the STAT1 signaling pathway.

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**Conclusions:** These data suggest a novel mechanism indicating that mechanical load-stimulated PDLSCs produce H<sub>2</sub>S to polarize macrophages toward the M1 phenotype via the STAT1 signaling pathway, which contributes to bone remodeling and tooth movement process. These results provide new insights into the role of PDLSCs in regulating macrophage polarization and mediating bone remodeling under mechanical stimulation, and indicate that appropriate H<sub>2</sub>S supplementation may accelerate tooth movement.

**Keywords:** Stem cells, Macrophage polarization, Hydrogen sulfide, Bone remodeling, Mechanical load, Cell signaling

## Background

Mechanical stimulation is important in promoting tissue development and maintaining tissue homeostasis [1–3]. Mechanical stimuli can regulate communications among different cell types and between the cells and tissue microenvironment [4, 5]. Among all the systems of our body, the skeletal system, including alveolar bone, specifically responds to changes in mechanical load [6, 7]. Mechanical load can enhance the expressions of inflammatory cytokines, chemokines, and  $\beta$ -2 adrenergic receptor in the surrounding tissues of alveolar bone and activate immune cells such as macrophages and T lymphocytes, which may influence alveolar bone remodeling [8–12]. However, the detailed mechanism of how mechanical stimuli modulate alveolar bone remodeling remains unclear.

Tooth movement is a unique bone remodeling process induced by mechanical stimulation. During this process, aseptic inflammation develops on the compression side of periodontal ligament, which leads to bone resorption on the compression side and bone formation on the tension side [8, 13]. Macrophages, the main immune cells and the precursors of osteoclasts, play a critical role in developing inflammation and mediating bone remodeling during tooth movement [14, 15]. Macrophages could be polarized into different phenotypes under different environmental elements and perform different functions [16]. “Inflammatory” classically activated M1 polarization is mainly induced by interferon (IFN)- $\gamma$  or lipopolysaccharides and mediates the inflammatory process by producing inflammatory elements such as tumor necrosis factor (TNF- $\alpha$ ) and nitric oxide (NO); furthermore, M2 polarization is mainly activated by interleukin (IL)-4 or IL-13 and plays an important role in tissue remodeling by producing IL-10 and arginase I [17–19]. Previously, we observed that M1 macrophage polarization contributes to bone remodeling and root resorption during tooth movement [10, 20]. However, how mechanical signals influence macrophage polarization and contribute to bone remodeling during tooth movement remains unclear.

Mesenchymal stem cells (MSCs) can modulate macrophage polarization by secreting several bioactive and immunomodulatory factors [21–24]. MSCs are exposed to

mechanical stimuli, and the function of MSCs could be modulated by mechanical stimuli [25]. Periodontal ligament stem/progenitor cells (PDLSCs), as the main MSCs in periodontal tissues, can respond to mechanical load and contribute to alveolar bone remodeling by differentiating into osteo-related cell components and promoting osteoclastogenesis [26, 27]. Previously, we have shown that periodontal ligament cells could promote inflammatory cytokines expressions in THP-1-derived macrophages under mechanical stimuli [10]. However, whether and how PDLSCs interact with macrophages under mechanical stimuli and contribute to tooth movement require further illustration.

Hydrogen sulfide (H<sub>2</sub>S), a signaling gas molecule, plays an important role in many physiologic and pathophysiologic processes, including maintaining mesenchymal stem cell function, limiting cardiovascular injury, regulating metabolic disorders, and mediating the inflammatory process [28–30]. H<sub>2</sub>S can be produced by MSCs and can regulate the MSC function to maintain bone homeostasis [28]. PDLSCs can produce H<sub>2</sub>S and express cystathionine- $\beta$ -synthase (CBS), a H<sub>2</sub>S-generating enzyme [31]. Moreover, PDLSCs express H<sub>2</sub>S under mechanical stimulation and regulate osteoclastic activities [32]. Furthermore, H<sub>2</sub>S can mediate macrophage polarization during wound healing process or cardiac tissue repair after infarction [29, 33]. Therefore, we hypothesized that mechanical load-induced H<sub>2</sub>S production by PDLSCs modulates macrophage polarization and contributes to alveolar bone remodeling and tooth movement. Given that the signal transducer and activator of transcription 1 (STAT1) signaling pathway is critical in the activation of inflammatory M1-like macrophage polarization [19], the role of STAT1 signaling pathway during bone remodeling and tooth movement was further explored.

In this study, we used the mechanical load-induced tooth movement animal model *in vivo* and continuous compressive loading *in vitro* to test the mechanism of how mechanical load-stimulated PDLSCs produce H<sub>2</sub>S to promote M1 macrophage polarization and therefore contribute to alveolar bone remodeling and tooth movement.

## Methods

### Animal models with mechanical stimulation

Six- to eight-week-old male C57BL/6 mice were used in the study. All the protocols were approved by the Peking University Ethical Committee (LA2013-92). The mice were divided into four groups. Each group comprised five to six mice. Mechanical force was applied to three groups of mice for 7 days as previously described [32], and the remaining group without loading served as the control. Briefly, a nickel–titanium coil spring with 0.2-mm wire size, 1 mm in diameter, and 1 mm in length (Smart Technology, China) was bonded between the upper first molar and incisors by flowable resin (3M, USA), which provided approximately 30 g force [34].

Vehicle (normal saline, NS), hydroxylamine (HA; 100 µg/mouse, Sigma), or GYY4137 dichloromethane complex (1 mg/mouse, Sigma) was intraperitoneally injected every other day since 1 day prior to the 7-day course of tooth movement in the three groups with mechanical loading [28]. After 7 days, the mice were sacrificed, and the maxillae were harvested. Tooth movement distance was measured from the occlusal view of the maxilla using a stereomicroscope (SWZ1000; Nikon) as previously described [9]. Briefly, the tooth movement distance was measured between the midpoint of the distal-marginal ridge of the first molar and midpoint of the mesial-marginal ridge of the second molar. The distance was measured thrice by a trained researcher who was blinded by the experimental design.

### PDLSC culture and mechanical loading

Isolation of human primary culture PDLSCs was performed as previously described [26]. The Peking University Ethical Committee has approved the protocols (PKUSSIRB-201311103), and informed consents were signed by the patients. Briefly, the periodontal ligament scraped from the root surface was digested in a mixture of 3 mg/ml type 1 collagenase (Worthington Biochem, Freehold, USA) and 4 mg/ml dispase II (Roche, Mannheim, Germany) for 1 h at 37 °C. The single-cell suspensions were then used for cell culture after passing the incubated mixture through a 70-µm strainer. The PDLSCs were identified as previously described [35] and used at the fourth passage.

When cell confluence was approximately 80%, 1 g/cm<sup>2</sup> continuous compressive force was applied to the PDLSCs for 24 h [36] by using glass layers and 50 mL plastic tube caps containing weighed metal balls following a modified previously described method [37] (Fig. 3a). The culture medium was collected from PDLSCs with or without loading for further experiments. H<sub>2</sub>S production in the culture medium from human PDLSCs was measured using a human H<sub>2</sub>S enzyme-linked immunosorbent assay (ELISA) kit (TSZ ELISA) according to the manufacturer's instructions.

### Culture and treatment of THP-1-derived macrophages

THP-1 human monocytic cells ( $1 \times 10^6$ ) (ATCC TIB-202) were treated with 50 ng/ml phorbol 12-myristate 13-acetate for 24 h to differentiate into macrophages.

To determine whether the production of H<sub>2</sub>S from load-stimulated PDLSCs can influence macrophage polarization, we cultured THP-1-derived macrophages with conditioned medium of force-treated PDLSCs with or without CBS inhibitor hydroxylamine (HA) (100 µM) [28]. Macrophages cultured with supernatant from PDLSCs without loading served as control (CS).

To investigate the mechanism of H<sub>2</sub>S on macrophage polarization, we treated THP-1-derived macrophages with sodium hydrosulfide (NaSH) (100 µM), HA (100 µM), and phospho-STAT1 inhibitor fludarabine (50 µM, S1491, Selleck) combined with NaSH for 24 h [28, 38]. Macrophages were pre-treated with fludarabine for 2 h. THP-1-derived macrophages were also cultured with the supernatant of force-treated PDLSCs with or without fludarabine (50 µM) for 24 h to further detect whether load-induced endogenous H<sub>2</sub>S production in PDLSCs influences macrophage polarization via STAT1 signaling pathway.

### Immunohistochemical staining

After sacrifice, the trimmed maxillae were fixed in 10% neutral buffered formalin for 24 h. After decalcified in ethylenediaminetetraacetic acid for 4 weeks, the tissues were then embedded by paraffin. Four-micrometer consecutive horizontal sections were obtained from the middle to apical third of the maxillary first molar, and sections from similar position of the roots were used for histological study. Immunohistochemistry was performed with a two-step detection kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) as previously described [39]. The positive staining cells were counted in five different slides from each sample ( $N = 5-6$ ). The final result came from the average of three tests.

The primary antibodies were anti-CBS (1:100, ab135626, Abcam), anti-TNF-α (1:100, ab1793, Abcam), anti-IFN-γ (1:50, sc1377, Santa Cruz), anti-IL-10 (1:200; sc-365858, Santa Cruz, Dallas, TX), and anti-CD206 (1:200, ab64693, Abcam).

### Immunofluorescence staining

Immunofluorescence staining was performed as previously described [39]. The sections were double stained with antibodies consisting anti-CD68 (1:600; MCA341GA, Serotec, UK), and anti-inducible nitric oxide synthase (iNOS) (1:100; ab-15323, Abcam), or anti-CD68 and anti-CD163 (1:100; sc-33560, Santa Cruz) to detect M1 or M2 macrophages. In addition, anti-phospho-STAT1 (Tyr701) (1:300; #9167, Cell Signaling) antibodies were used to detect the influence of H<sub>2</sub>S on the STAT1 signaling pathway.

The sections were then incubated with fluorescein isothiocyanate-conjugated or tetramethylrhodamine isothiocyanate-conjugated secondary antibody (1:200, Jackson Immuno Research Laboratories, West Grove, PA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Confocal microscopic images were acquired using a laser scanning microscope (LSM 510, Zeiss, Jena, Germany), and the images were processed using LSM 5 Release 4.2 software. The positively stained cells were counted in five different slides from each sample ( $N = 5-6$ ). The final result came from the average of three tests.

#### Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining was conducted according to a leukocyte acid phosphatase kit (387A-1KT; Sigma, USA). Only the TRAP-positive cells (> 2 nuclei) located within the compressive PDL and the surface of the adjacent alveolar bone were considered.

#### Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from cultured cells with TRizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. Reverse transcription and real-time PCR were performed as previously described [39]. The sequences of primers, which were designed by Primer Premier 5.0 software and commercially synthesized, were listed as follows.

Human GAPDH sense/antisense:

5'-ATGGGGAAGGTGAAGGTCG-3'/5'-GGGGTCATTGATGGCAACAATA-3'.

Human TNF- $\alpha$  sense/antisense:

5'-GAGGCCAAGCCCTGGTATG-3'/5'-CGGGCCGATTGATCTCAGC-3'.

Human IL-1 $\beta$  sense/antisense:

5'-ATGATGGCTTATTACAGTGGCAA-3'/5'-GTCGGAGATTCGTAGCTGGA-3'.

Human DECTIN sense/antisense:

5'-GGAAGCAACACATTGGAGAATGG-3'/5'-CTTTGGTAGGAGTCACACTGTC-3'.

Human arginase-1 sense/antisense:

5'-TGGACAGACTAGGAATTGGCA-3'/5'-CCAGTCCGTCAACATCAAACACT-3'.

The amplification specificity was confirmed by the melting curve. The efficiency of PCR was confirmed by sequencing the products.

#### Western blot analysis

Western blot tests were performed as previously described [10]. The primary antibodies included anti- $\beta$ -actin antibody (1:10000, a-5441, Sigma), anti-iNOS (1:500, abs130136, Absin), anti-TNF- $\alpha$  (1:500, ab1793, Abcam), anti-arginase-1 antibody (1:1000, sc-21050, Abcam), anti-STAT1 antibody (1:1000, #9172, Cell Signaling), and anti-

phospho-STAT1 antibody (1:1000, #9167, Cell Signaling). The blots were then developed by horseradish peroxidase-conjugated secondary antibodies. Finally, the blots were enhanced by chemiluminescence detection before photography. The relative density of the comparable results was measured by ImageJ 1.37v software (Wayne Rasband).

#### Statistical analysis

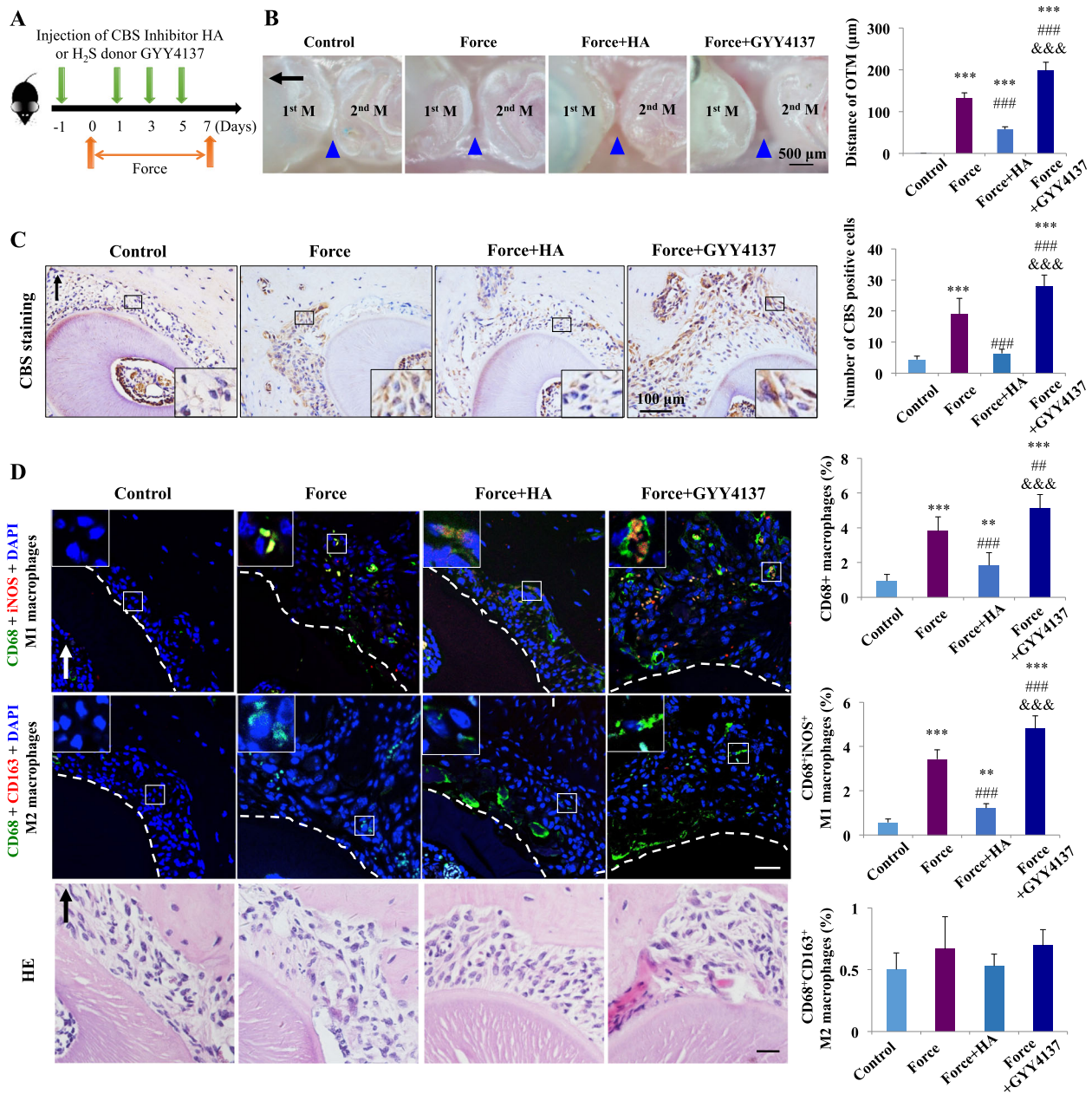
SPSS 20.0 was used to conduct statistical analysis. Data were presented as mean  $\pm$  standard deviation. The results were tested by one-way ANOVA and post hoc study was used to compare the differences between groups. Statistical significance was considered at  $P < 0.05$ .

## Results

### Mechanical load-induced M1-like macrophage polarization to promote alveolar bone remodeling and tooth movement depends on H<sub>2</sub>S production

To explore the influence of H<sub>2</sub>S on macrophage polarization under mechanical load, we blocked or enhanced the H<sub>2</sub>S level in mice by the systemic administration of CBS inhibitor HA or H<sub>2</sub>S donor GYY4137 during tooth movement (Fig. 1a). H<sub>2</sub>S concentration in the serum increased after GYY4137 administration and decreased after HA administration, which verified the validity of H<sub>2</sub>S blockage and enhancement (Supplementary Fig. 1). After force was applied for 7 days, the tooth movement distance reached  $132.3 \pm 12.5 \mu\text{m}$ , which decreased to  $57.8 \pm 6.2 \mu\text{m}$  after HA injection. GYY4137 injection could further enhance the tooth movement distance to  $197.7 \pm 19.8 \mu\text{m}$  ( $P < 0.001$ , Fig. 1b). Meanwhile, CBS expression on the compression side of periodontal tissue was elevated after loading ( $P < 0.001$ ), and it was suppressed after HA injection ( $P < 0.001$ ) and enhanced after GYY4137 injection ( $P < 0.001$ ) compared with the force group (Fig. 1c).

Concomitantly, immunofluorescence staining showed that CD68-positive macrophages accumulated on the compression side of periodontal ligament after force was applied for 7 days. HA injection significantly decreased the percentage of macrophages, and GYY4137 injection resulted in their increase compared with the force group ( $P < 0.001$ ). The percentage of CD68<sup>+</sup>iNOS<sup>+</sup> M1-like macrophages increased to  $3.39\% \pm 0.43\%$  after force was applied compared with  $0.6\% \pm 0.13\%$  in the control group. HA injection significantly decreased the percentage of M1-like macrophages to  $1.3\% \pm 0.3\%$  compared with the force group ( $P < 0.001$ ). The percentage of M1-like macrophages further increased to  $4.85\% \pm 0.52\%$  after GYY4137 injection ( $P < 0.001$ ; Fig. 1d). However, CD68<sup>+</sup>CD163<sup>+</sup> M2-like macrophages were hardly detected (Fig. 1d).



**Fig. 1** Mechanical load-induced M1 macrophage polarization during tooth movement depends on H<sub>2</sub>S production. **a** Schematic illustration. **b** Representative images and semiquantification analysis of the tooth movement distance in mice. Mechanical load increased the tooth movement distance, and this result was partially reversed by HA injection and enhanced by GYY4137 injection. *N* = 5–6. \*\*\**P* < 0.001 versus control; ###*P* < 0.001 versus force; &&&*P* < 0.001 versus force + HA. 1<sup>st</sup> M, the first molar; 2<sup>nd</sup> M, the second molar. Black arrow represents the direction of mechanical force. Blue triangles represent the tooth movement distance. Scale bar: 500 μm. **c** Representative immunohistochemical images and semiquantification analysis of cystathionine-β-synthase (CBS) expression on the compression side of distal roots. Large boxed areas show high-magnification views of the small boxed areas. *N* = 5–6. \*\*\**P* < 0.001 versus control; ###*P* < 0.001 versus force + HA. Scale bar: 100 μm. **d** Representative immunofluorescence images and the corresponding hematoxylin–eosin (HE) staining of the compression side of distal roots and semiquantification of positive cells. The changes in CD68- (green) and inducible nitric oxide synthase (iNOS)-positive (red) M1 macrophage polarization (merged yellow) and CD68- (green) and CD163-positive (red) M2 macrophage polarization (merged yellow) are presented. Dashed lines mark the root outline. Arrow represents the direction of mechanical force. Large boxed areas show high-magnification views of the small boxed areas. Scale bar: 50 μm. *N* = 5–6; the positive staining cells were counted on five different slides from each sample. The final result came from the average of three tests. \*\**P* < 0.01, \*\*\**P* < 0.001 versus control. ###*P* < 0.001 versus force. &&&*P* < 0.001 versus force + HA

The contribution of H<sub>2</sub>S to the expression levels of macrophage-associated markers on the compression side of periodontal tissues was also investigated. The expressions of M1-macrophage-associated pro-inflammatory cytokines, TNF- $\alpha$  and IFN- $\gamma$ , were upregulated after force was applied for 7 days ( $P < 0.001$ ). HA administration significantly decreased the expression levels of TNF- $\alpha$  and IFN- $\gamma$ , whereas GYY4137 administration resulted in their increase ( $P < 0.001$ , Fig. 2). Correspondingly, the upregulated expression of TRAP<sup>+</sup> osteoclasts in the force group was further suppressed by HA administration and enhanced by GYY4137 administration ( $P < 0.001$ , Supplementary Fig. 2), which was consistent with our previous studies [32].

However, the expression of M2-macrophage-associated markers IL-10 and CD206 showed no change in the four groups (Fig. 2). Specifically, the administration of HA or GYY4137 without loading cause no change in the number of CD68-positive macrophages or TRAP-positive osteoclasts (Supplementary Fig. 3). These data indicate that both endogenous H<sub>2</sub>S level and exogenous H<sub>2</sub>S administration regulated M1-like macrophage polarization and the subsequent alveolar bone remodeling and tooth movement process under mechanical stimuli.

#### Mechanical load-stimulated PDLSCs produce H<sub>2</sub>S to promote M1 macrophage polarization in vitro

Mechanical load can upregulate H<sub>2</sub>S expression in PDLSCs [32]. H<sub>2</sub>S concentration in the supernatant of PDLSCs was significantly upregulated after compressive loading and suppressed by the additional treatment of HA with compressive loading (Fig. 3b). Whether H<sub>2</sub>S expression in force-treated PDLSCs could promote THP-1-derived macrophages to polarize toward M1 phenotype was further explored in this study. To this end, THP-1-derived macrophages were cultured with the supernatants from force-treated primary PDLSCs with or without CBS inhibitor HA (FS + HA or FS). Macrophages cultured with supernatant from PDLSCs without loading served as the control (CS) (Fig. 3a).

The mRNA expression levels of M1 markers TNF- $\alpha$  and IL-1 $\beta$  of THP-1-derived macrophages were dramatically upregulated in the FS group ( $P < 0.001$  versus CS) and downregulated in the FS + HA group ( $P < 0.001$  versus FS), although the expression of TNF- $\alpha$  was still upregulated compared with the CS group ( $P < 0.001$ ). Conversely, the mRNA expression of M2 marker arginase-1 showed no change, and the expression of DECTIN was upregulated in the FS + HA group ( $P < 0.001$ ) (Fig. 3c).

Western blot analysis showed that the protein expression of M1-associated markers iNOS and TNF- $\alpha$  increased in the FS group and decreased in the FS + HA

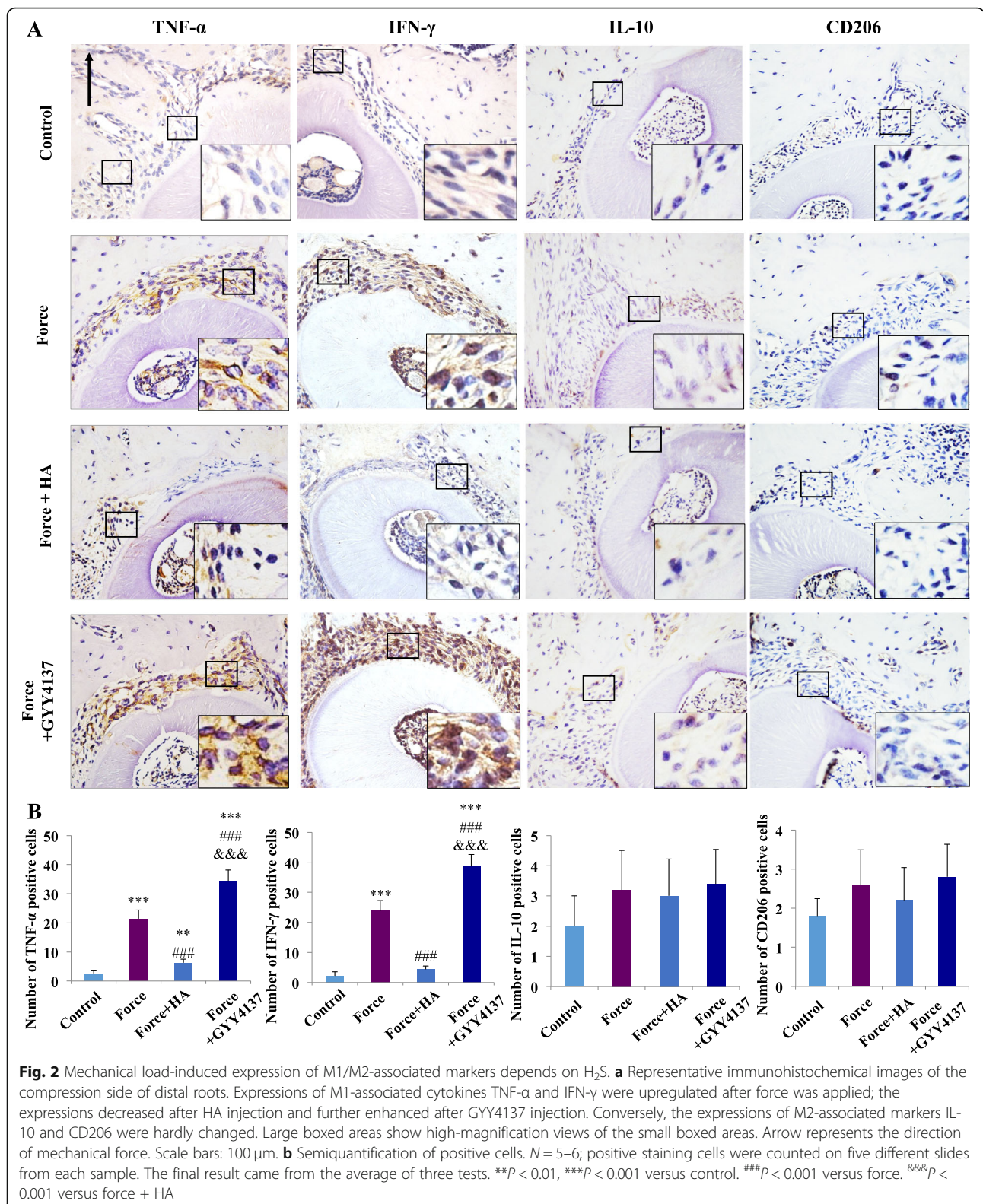
group. However, no changes were observed in the expression of arginase-1 (Fig. 3d). Specifically, we applied HA with PDLSC-conditioned medium without loading to treat THP-1 macrophages, and no significant changes in the expressions of TNF- $\alpha$  and arginase-1 were detected (Supplementary Fig. 4A). These findings suggest that upregulated H<sub>2</sub>S production in mechanical load-stimulated PDLSCs can promote THP-1-derived macrophages to polarize into M1 phenotype.

#### Mechanical load-induced H<sub>2</sub>S contributes to M1 macrophage polarization via the STAT1 signaling pathway

We further examined the mechanism of how exogenous H<sub>2</sub>S and endogenous H<sub>2</sub>S productions in mechanical load-stimulated PDLSCs promote M1 macrophage polarization. The STAT1 signaling pathway plays a critical role in M1 macrophage polarization [19]. To detect whether exogenous H<sub>2</sub>S promotes THP-1-derived macrophages to polarize toward M1 phenotype via the STAT1 signaling pathway, we treated THP-1-derived macrophages with H<sub>2</sub>S donor NaSH and CBS inhibitor HA and used NaSH + phospho-STAT1 inhibitor fludarabine to test the function of STAT1 pathway (Fig. 4a). The level of H<sub>2</sub>S in the culture medium of THP-1-derived macrophages was enhanced by NaSH treatment and decreased after HA treatment (Supplementary Fig. 5).

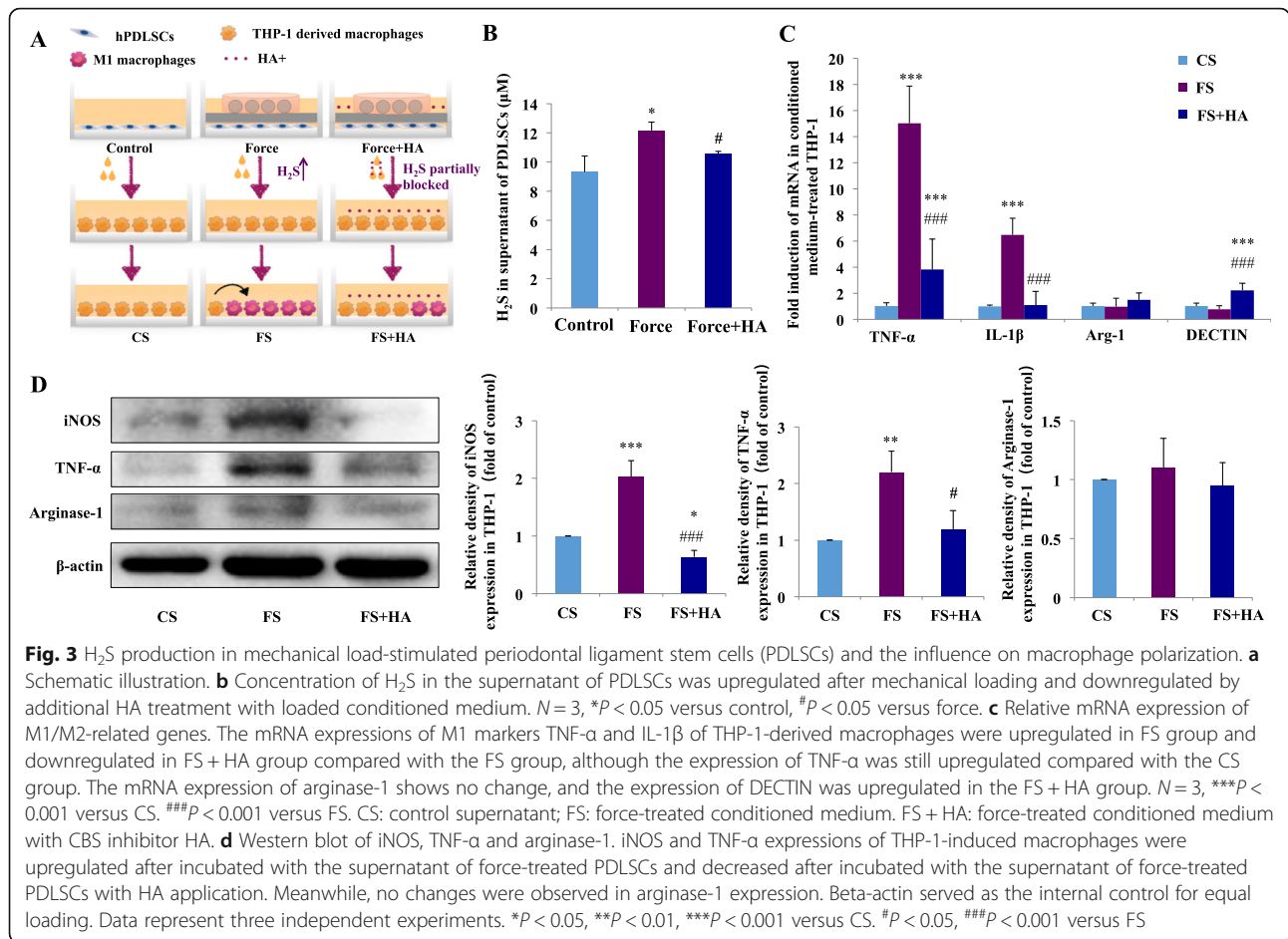
The mRNA expression levels of the M1 markers TNF- $\alpha$  and IL-1 $\beta$  in THP-1-derived macrophages were upregulated after NaSH treatment ( $P < 0.001$  and  $P < 0.01$ , respectively), which were reversed after treatment with HA and NaSH + phospho-STAT1 inhibitor ( $P < 0.001$  versus NaSH). Conversely, the mRNA expression levels of M2 markers arginase-1 and DECTIN showed no change (Fig. 4b).

Immunocytochemical analyses showed that the proportion of CD68<sup>+</sup>iNOS<sup>+</sup> M1 macrophages increased significantly after NaSH treatment ( $P < 0.001$ ) and decreased significantly after HA or NaSH + phospho-STAT1 inhibitor treatment ( $P < 0.001$  versus NaSH). Meanwhile, the proportion of CD68<sup>+</sup>CD163<sup>+</sup> M2 macrophages showed no changes (Fig. 4c). Western blot analysis showed that the expressions of iNOS and TNF- $\alpha$  were significantly upregulated after NaSH treatment ( $P < 0.001$  and  $P < 0.05$ , respectively) and downregulated after HA treatment ( $P < 0.05$ ). The upregulation of iNOS and TNF- $\alpha$  was partially reversed by the additional administration of phospho-STAT1 inhibitor with NaSH ( $P < 0.001$  and  $P < 0.05$  versus NaSH, respectively). Meanwhile, the proportion of phospho-STAT1/STAT1 was also upregulated after NaSH treatment ( $P < 0.01$ ) and was partially reversed by HA treatment or by additional administration of phospho-STAT1 inhibitor ( $P < 0.01$  versus NaSH). However, no changes were observed



in arginase-1 expression (Fig. 4d). These results indicate that macrophages were polarized toward M1 phenotype after treatment with NaSH.

To confirm whether load-induced endogenous H<sub>2</sub>S production in PDLSCs promotes THP-1-derived macrophages to polarize toward M1 phenotype via the STAT1



signaling pathway, we cultured THP-1-derived macrophages with the supernatants from force-treated PDLSCs with or without phospho-STAT1 inhibitor fludarabine (FS + In or FS). Macrophages cultured with supernatant from PDLSCs without loading served as the control (CS) (Fig. 5a).

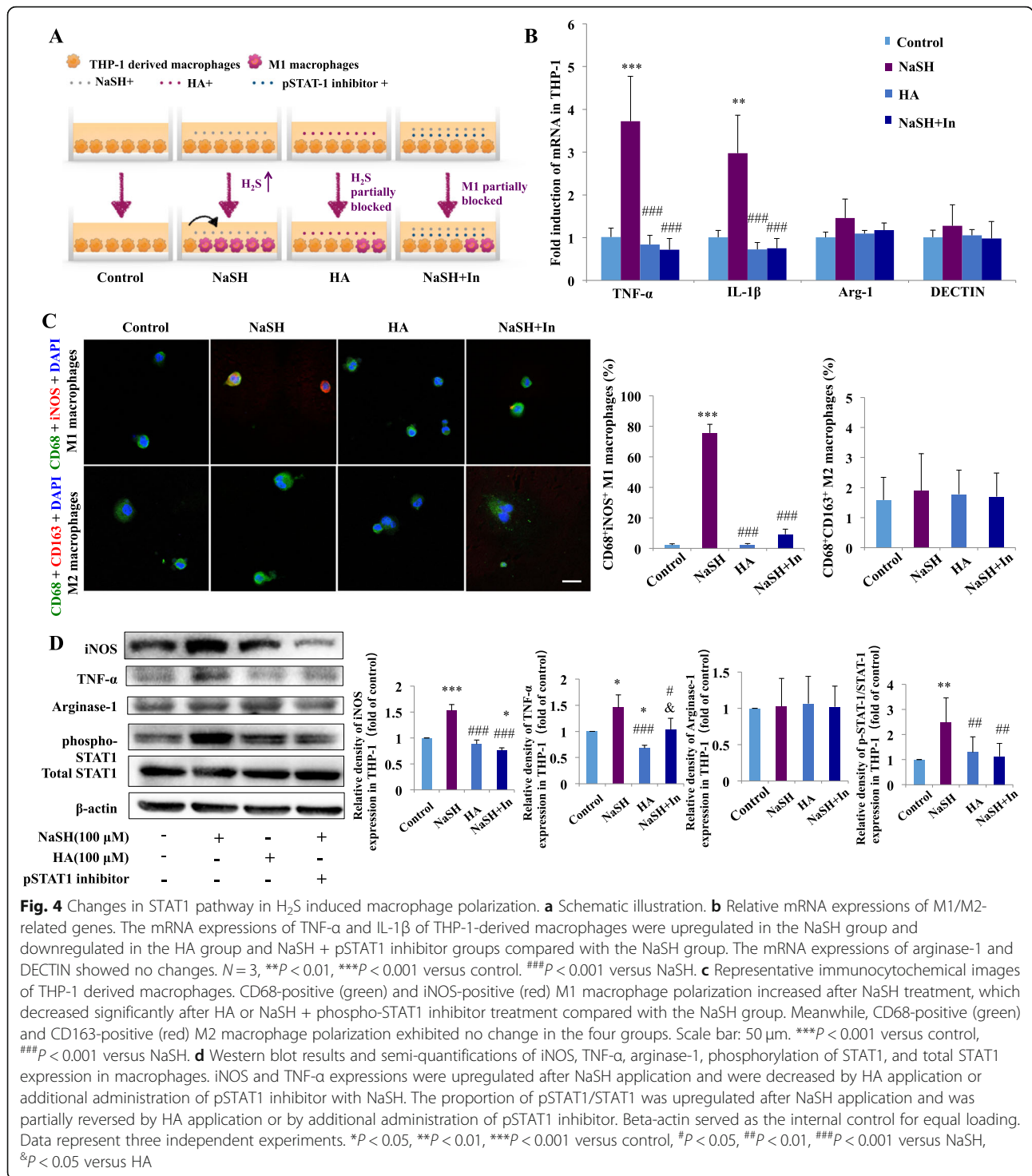
Real-time PCR showed that the mRNA expressions of TNF-α and IL-1β in THP-1-derived macrophages were upregulated after incubation with the force-treated conditioned medium of PDLSCs (*P* < 0.001 versus CS) and downregulated after incubation with the force-treated conditioned medium with fludarabine (*P* < 0.001 and *P* < 0.01 versus FS). Conversely, the mRNA expressions of arginase-1 and DECTIN remained unchanged (Fig. 5b).

The western blot results showed that the protein expression levels of iNOS and TNF-α and the proportion of phospho-STAT1/STAT1 were significantly upregulated after incubation with the force-treated conditioned medium of PDLSCs (*P* < 0.01). These enhancing effects were partially reversed by the incubation with the force-treated conditioned medium with fludarabine (*P* < 0.01 versus LM). Meanwhile, no changes were

observed in arginase-1 expression (Fig. 5c). Specifically, we applied fludarabine with PDLSC-conditioned medium without loading to treat THP-1 macrophages, and no significant changes were detected in the expressions of TNF-α and arginase-1 (Supplementary Fig. 4B). These data indicate that exogenous H<sub>2</sub>S and endogenous H<sub>2</sub>S production in force-treated PDLSCs could promote M1 macrophage polarization by activating the STAT1 signaling pathway.

To further confirm the involvement of STAT1 pathway in the macrophages in vivo, we performed immunofluorescence staining to show the co-expression of STAT1 with CD68 on the compression side of the periodontal tissues. CD68<sup>+</sup>phospho-STAT1<sup>+</sup> macrophages accumulated on the compression side of periodontal tissues during tooth movement, with positive cells of 3.03% ± 0.48% in the force group compared with 0.56% ± 0.16% in the control group (*P* < 0.001). The percentage of CD68<sup>+</sup>phospho-STAT1<sup>+</sup> macrophages significantly decreased to 1.52% ± 0.36% after HA application compared with the force group (*P* < 0.001) but remained higher than that in the control group (*P* < 0.01). Moreover, the application of GYY4137 dramatically increased



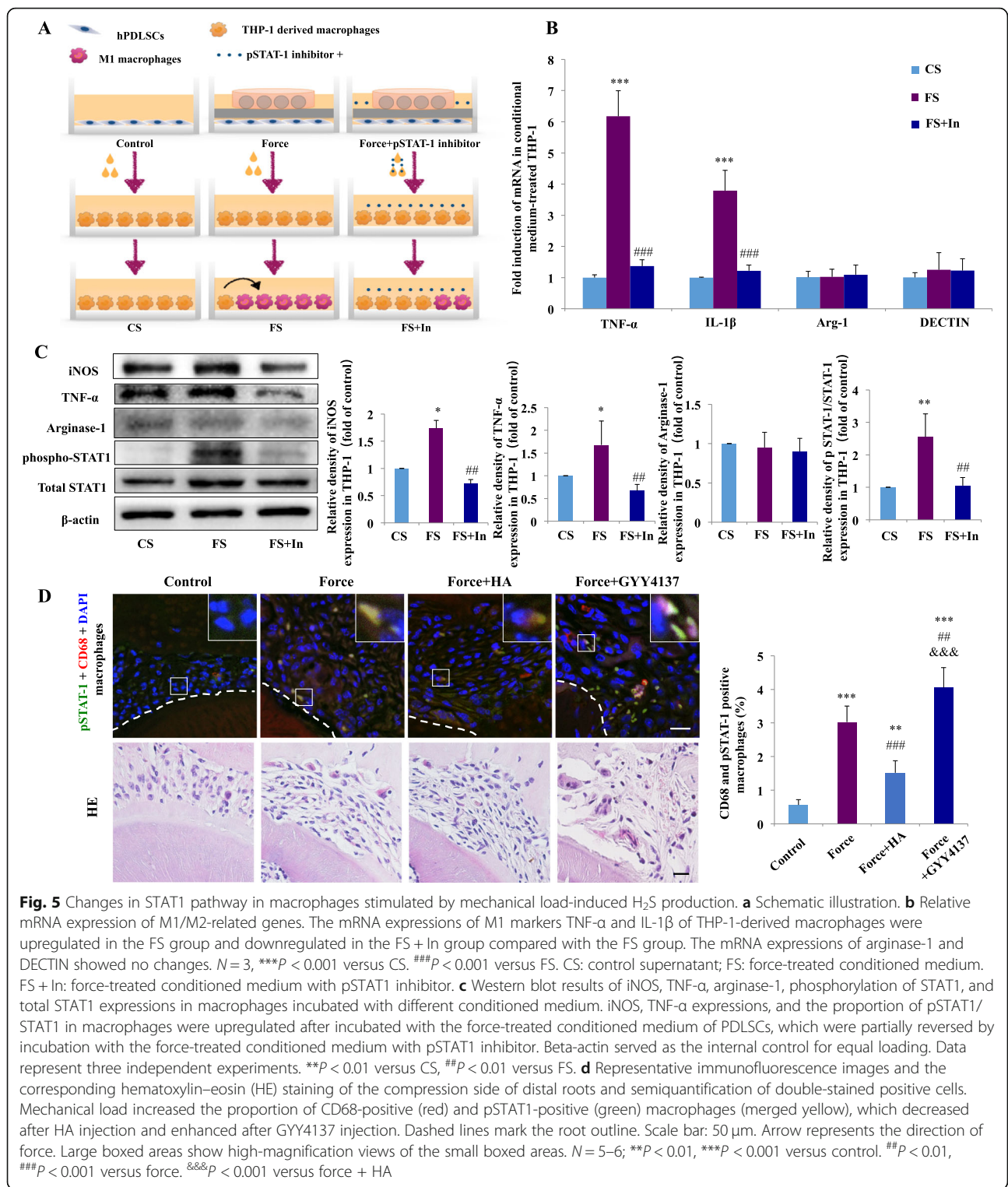


the percentage of CD68<sup>+</sup> phospho-STAT1<sup>+</sup> macrophages to 4.05%  $\pm$  0.59% ( $P < 0.001$ , Fig. 5d).

Taken together, our results indicate that mechanical load-stimulated PDLSCs could enhance H<sub>2</sub>S production to promote M1 macrophage polarization during tooth movement by activating the STAT1 signaling pathway (Fig. 6).

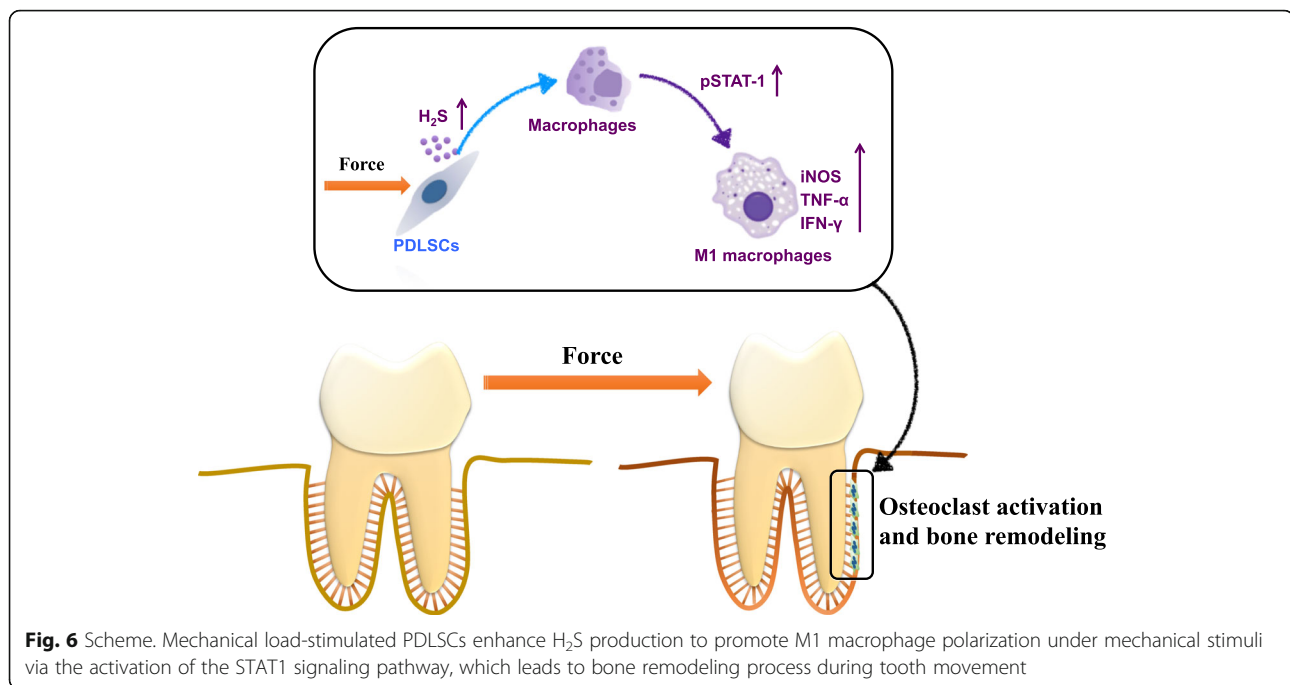
### Discussion

The conversion of mechanical stimulation into biochemical reactions is essential for intercellular communication, and this process may control bone remodeling in mammals [6, 7]. Tooth movement is a unique aseptic inflammation-associated bone remodeling process induced by mechanical stimulation [8, 13]. During this



process, PDLSCs and macrophages are the main cellular components within the microenvironment of periodontal tissues. PDLSCs are the unique MSCs that can respond to mechanical stimuli and express multiple cytokines, chemokines, and other inflammatory elements

to influence bone remodeling [27, 32, 40]. The detailed mechanism of how PDLSCs contribute to bone remodeling process under mechanical stimuli has not been illustrated. Macrophages, especially M1 phenotype, play critical roles in mechanical load-induced bone



**Fig. 6** Scheme. Mechanical load-stimulated PDLSCs enhance H<sub>2</sub>S production to promote M1 macrophage polarization under mechanical stimuli via the activation of the STAT1 signaling pathway, which leads to bone remodeling process during tooth movement

remodeling and tooth movement [10]. However, whether and how PDLSCs interact with macrophages under mechanical stimulation remain largely unknown. In this study, we showed a novel mechanism in which PDLSCs produce H<sub>2</sub>S to modulate M1 macrophage polarization to promote bone remodeling and tooth movement under mechanical stimuli. First, mechanical load promoted M1 macrophage polarization on the compression side of periodontal ligament during tooth movement, accompanied by increased CBS expression. The enhancement or blockage of H<sub>2</sub>S led to the increased or repressed of the expression of M1 macrophages and distance of tooth movement. Second, mechanical stimulation enhanced H<sub>2</sub>S production in PDLSCs, which increased the expression of M1-associated cytokines in macrophages. These effects could be blocked by the administration of CBS inhibitor HA. Finally, we confirmed that mechanical load-induced H<sub>2</sub>S promoted M1 macrophage polarization via the STAT1 signaling pathway. These data suggest that mechanical load-stimulated PDLSCs produced H<sub>2</sub>S to polarize macrophages toward M1 phenotype via the STAT1 signaling pathway, thus contributing to alveolar bone remodeling and tooth movement.

Although M1 macrophage polarization is considered important in mechanical load-induced bone remodeling during tooth movement [10], how M1 macrophages polarize under mechanical stimuli remains poorly understood. The phenotypes of macrophages change depending on different environmental elements [16, 17]. Inflammatory-associated elements, such as IFN-γ or lipopolysaccharides, cause the activation of M1

polarization, whereas IL-4 or IL-13 can induce the activation of M2 polarization [17–19]. Here, we observed that the changes in H<sub>2</sub>S level under mechanical stimuli and exogenous H<sub>2</sub>S application or inhibition could modulate the number of M1-like macrophages in periodontal tissues and influence bone remodeling and tooth movement. Further in vitro experiments showed that H<sub>2</sub>S production was upregulated in loaded PDLSCs, which promoted macrophages to polarize into M1 phenotypes. Our study uncovered a novel finding indicating that mechanical stimuli could modulate M1 macrophage polarization by inducing H<sub>2</sub>S production in PDLSCs, therefore contributing to alveolar bone remodeling and tooth movement.

Raising evidence has shown that H<sub>2</sub>S serves as a gas-transmitter that regulates various signaling pathways [41]. H<sub>2</sub>S can also mediate macrophage polarization during wound healing or cardiac tissue repair after infarction, probably through mitochondrial biogenesis, fatty acid oxidation, and the nuclear factor-κB signaling pathway [29, 33]. However, the molecular mechanism of how H<sub>2</sub>S regulates the differentiation of M1 macrophages under mechanical stimulation remains unclear. In the present study, both load-induced endogenous H<sub>2</sub>S production in PDLSCs and the exogenous administration of H<sub>2</sub>S could promote M1 macrophage polarization via the STAT1 signaling pathway, which is a critical signaling pathway in M1 macrophage polarization [19]. Our results uncovered the novel mechanism of H<sub>2</sub>S regulation on M1 macrophage polarization via the STAT1 signaling pathway under mechanical stimuli, which differs from

the previously reported biochemical regulatory mechanism.

The activation of STAT1 is related to various signaling pathways, including the IFN- $\alpha/\beta$  and IFN- $\gamma$  pathways [5, 12, 13]. Meanwhile, H<sub>2</sub>S serves as a gasotransmitter to regulate a variety of signaling pathways. H<sub>2</sub>S can control cellular Ca<sup>2+</sup> levels and affect multiple transient receptor potential calcium channels [42]. Whether H<sub>2</sub>S activates STAT1 through regulating the extracellular messenger molecule binding to specific cell surface receptors or by regulating ionic channels needs to be further explored.

The interactions between MSCs and immune cells are important in bone and tissue regeneration and the healing of multiple pathological processes [43–45]. MSCs can modulate macrophage polarization by reducing inflammation and mediating tissue repair through the secretion of extracellular vesicles, exosomes, or cytokines such as transforming growth factor- $\beta$  [21–24]. PDLSCs are unique MSCs that constantly receive mechanical stimulation. These cells are sensitive to mechanical stimulation both in vitro and in vivo and can express high levels of inflammatory cytokines, chemokines, and gas molecular H<sub>2</sub>S under mechanical stimuli [27, 32, 40]. However, how PDLSCs interact with immune cells and contribute to bone remodeling under mechanical stimuli still needs further illustration. Our study revealed that H<sub>2</sub>S concentration in the supernatant of loaded PDLSCs was upregulated by approximately 1.3-fold, which increased the expressions of M1-associated cytokines in macrophages. The increased H<sub>2</sub>S production and M1 cytokine expression could be partially reversed by the additional application of HA. These results confirm that PDLSCs can promote M1 macrophage polarization by producing the gaseous signaling molecule H<sub>2</sub>S under mechanical stimulation, thus shedding light on the novel mechanism of how MSCs regulate macrophage polarization under mechanical stimulation. Nevertheless, further experiments should be conducted to explore whether other cytokines produced by PDLSCs would influence macrophage polarization under mechanical stimuli.

Regarding the relationship between macrophage polarization and alveolar bone resorption, macrophages and osteoclasts are derived from myeloid precursor cells of hematopoietic origin [46]. Previous research has shown that M1 macrophage-related cytokines such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$  could induce osteoclastogenesis. These cytokines could directly increase osteoclast precursor numbers and differentiation and indirectly promote osteoblasts and other stromal cells to increase RANKL production [47, 48]. On the contrary, M2 macrophage-related cytokines, such as IL-4 and IL-10, can inhibit osteoclastogenesis [47]. On the compressive side of periodontal ligament during tooth movement, M1 macrophages accumulated after mechanical stimulation, which contributed to the expression of

inflammatory cytokines. Furthermore, PDLSCs can increase the expressions of TNF- $\alpha$ , IL-1 $\beta$ , and the RANKL/osteoprotegerin ratio under mechanical loading [9, 49]. Therefore, the accumulation of M1 macrophage polarization on the compression side of periodontal tissues during mechanical load-induced tooth movement may lead to bone resorption. Nevertheless, the underlying molecular mechanisms of the interaction between macrophages and osteoclasts still require elucidation. Macrophages and osteoclasts are assumed to be competing differentiation outcomes from myeloid progenitors. Several nuclear receptors/transcription factors could promote osteoclast differentiation but suppress macrophage activation [50]. Further studies should be conducted to illustrate the relationship between macrophages and osteoclasts.

## Conclusions

In conclusion, mechanical load-induced H<sub>2</sub>S production by PDLSCs polarizes macrophages toward M1 phenotype via the STAT1 signaling pathway, which contributes to bone remodeling and tooth movement. These results aid in elucidating the mechanism of how PDLSCs influence macrophage polarization and contribute to bone remodeling under mechanical stimuli. The findings also provide new insights indicating that appropriate H<sub>2</sub>S supplementation may accelerate tooth movement.

## Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s13287-020-01607-9>.

**Additional file 1:** Figure S1. H<sub>2</sub>S concentration in the serum of mice. H<sub>2</sub>S concentration in the serum increased after GYY4137 administration and decreased after HA administration. \**P* < 0.05 versus control. #*P* < 0.05 versus GYY4137.

**Additional file 2:** Figure S2. Representative tartrate-resistant acid phosphatase (TRAP) staining images of the compression side of distal roots. (A) The number of TRAP-positive osteoclasts was upregulated after force was applied, which decreased after HA administration and further enhanced after GYY4137 administration. Large boxed areas show high-magnification views of the small boxed areas. Arrow represents the direction of force. Scale bars: 100  $\mu$ m. (B) Semiquantification of positive cells. *N* = 5–6; the positive staining cells were counted in five different slides from each sample. The final result came from the average of three tests. \*\*\**P* < 0.001 versus control. ###*P* < 0.001 versus force. &&&*P* < 0.001 versus force + HA.

**Additional file 3:** Figure S3. Representative immunohistochemical and tartrate-resistant acid phosphatase (TRAP) staining images of different treatments in mice without force application. (A) No significant changes of the number of TRAP positive osteoclasts and the expressions of CD68 were detected in the control group and groups with HA or GYY4137 application. Data showed the compression side of distal roots. Large boxed areas show high-magnification views of the small boxed areas. Arrow represents the direction of force. Scale bars: 100  $\mu$ m. (B) Semiquantification of positive cells. *N* = 5; the positive staining cells were counted in five different slides from each sample. The final result came from the average of three tests.

**Additional file 4:** Figure S4. Western blot results of THP-1-derived macrophages. (A) No significant changes were observed in TNF- $\alpha$  and arginase-1 expressions in THP-1-induced macrophages after incubated

with the supernatant of PDLSCs with or without HA application. (B) No significant changes were observed in TNF- $\alpha$  and arginase-1 expressions in THP-1-derived macrophages after incubated with the supernatant of PDLSCs with or without pSTAT1 inhibitor application. Beta-actin served as the internal control for equal loading. Data represent three independent experiments.

**Additional file 5:** Figure S5. H<sub>2</sub>S level in the supernatant of THP-1-derived macrophages. The level of H<sub>2</sub>S in the culture medium of THP-1-derived macrophages was enhanced or decreased after treated with NaSH or HA. \* $P < 0.05$  versus control. \*\* $P < 0.01$  versus NaSH.

### Abbreviations

CBS: Cystathionine- $\beta$ -synthase; HA: Hydroxylamine; H<sub>2</sub>S: Hydrogen sulfide; IFN- $\gamma$ : Interferon- $\gamma$ ; MSCs: Mesenchymal stem cells; NaSH: Sodium hydrosulfide; NO: Nitric oxide; PDLSCs: Periodontal ligament stem cells; STAT-1: Signal transducer and activators of transcription 1; TNF- $\alpha$ : Tumor necrosis factor

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### Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the manuscript and the supplementary materials.

### Authors' contributions

DH contributed to the conception and design, collection and assembly of data, and data analysis and interpretation, drafted the work, and revised the manuscript critically. FL, SC, NJ, and HY contributed to the collection and assembly of data, data analysis, and interpretation and revised the manuscript critically. YZ, LY, and XK contributed to the conception and design and revised the manuscript critically. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All the protocols were approved by the Peking University Ethical Committee (LA2013-92).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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