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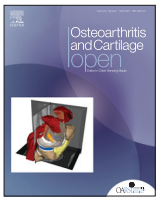

Mild treadmill exercise inhibits cartilage degeneration via macrophages in an osteoarthritis mouse model

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ABSTRACT

Objective: We previously reported how treadmill exercise can suppress cartilage degeneration. Here, we examined the changes in macrophage dynamics in knee osteoarthritis (OA) during treadmill exercise and the effect of macrophage depletion.
Design: OA mouse model, generated via anterior cruciate ligament transection (ACLT), was subjected to treadmill exercise of different intensities to investigate the effects on cartilage and synovium. In addition, clodronate liposomes, which deplete macrophages, were injected intra-articularly into the joint to examine the role of macrophages during treadmill exercise.
Results: Cartilage degeneration was delayed by mild exercise, and concomitantly, an increase in anti-inflammatory factors in the synovium was observed, with a decrease in the M1 and increase in M2 macrophage ratio. On the contrary, high-intensity exercise led to the progress of cartilage degeneration and was associated with an increase in the M1 and a decrease in the M2 macrophage ratio. The clodronate liposome-induced reduction of synovial macrophages delayed cartilage degeneration. This phenotype was reversed by simultaneous treadmill exercise.
Conclusions: Treadmill exercise, especially at high intensity, was detrimental to articular cartilage, whereas mild exercise reduced cartilage degeneration. Moreover, M2 macrophage response appeared necessary for the chondroprotective effect of treadmill exercise. This study indicates the importance of a more comprehensive analysis of the effects of treadmill exercise, not limited to the mechanical stress added directly to cartilage. Hence, our findings might help determine the type and intensity of prescribed exercise therapy for patients with knee OA.

1. Introduction

Knee osteoarthritis (OA) is a degenerative musculoskeletal disease characterized by the degeneration of articular cartilage. In Japan, 25.3 million people are diagnosed with OA, including asymptomatic OA, making the establishment of treatment methods a social imperative [1,2]. Although drug therapy, physical therapy, and physiotherapy are available as treatment strategies for OA, disease-modifying therapies to prevent the degeneration of articular cartilage are still far from being established.

Recently, several studies reported exercise as a form of physical therapy efficient in preventing the progression of OA. Moderate exercise has been shown to delay the progression of cartilage degeneration in animal models of OA [3–7]. However, the reason for the chondroprotective effect of treadmill exercise has not yet been fully elucidated. It has been recognized that mechanical loading from exercise is necessary for cartilage maintenance [7]. In the anterior cruciate ligament transection (ACLT) [8] and destabilization of the medial meniscus (DMM) [9] models, the articular cartilage is exposed to excessive mechanical loading, resulting in the development and progress of OA over time. Performance of treadmill exercise applies loading to the cartilage. Therefore, it is difficult to interpret the effects of treadmill exercise solely in terms of mechanical loading, and we hypothesized that other factors may be involved in the suppression of cartilage degeneration.

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Macrophages in the synovium have been reported to regulate the inflammatory state and to be greatly involved in the onset and progression of OA. In addition, macrophages can to be polarized into inflammatory (M1) or anti-inflammatory (M2) cells depending on their origin, function, and molecules produced during the programming process [10,11]. For example, M2 macrophages are characterized by the release of anti-inflammatory cytokines such as interleukin (IL)-4 and IL-10 and TGF-β [12]. Furthermore, studies using treadmill exercise in other fields have reported that treadmill exercise suppressed macrophage infiltration in adipose tissues and reduced the M1/M2 ratio [13]. Overall, treadmill exercise has been shown to change the phenotype of macrophages in injured tissues, and it is thought that similar changes might occur in macrophage phenotypes in the OA synovium.

This study aimed to define the biological effects of treadmill exercise in an ACLT mouse model with the specific objectives of examining (1) the biological response of cartilage and synovium in an exercise intensity-dependent manner and (2) the role of macrophages using clodronate liposomes.

2. Methods

2.1. Animals and surgical induction

This study was approved by the Animal Research Committee of Saitama Prefectural University (approval number: 2020-3), and animals were handled in accordance with the relevant legislation and institutional guidelines for humane animal treatment. This study included 64 adult (12-week-old) male ICR mice (Sankyo Labo Service Corporation, Tokyo, Japan) (Fig. 1). ACL transection procedures were performed on mice under the use of a combination of anesthetics including 0.375 mg/kg medetomidine (Nippon Zenyaku Kohgyo Co., Ltd. Fukushima, JPN); 2.0 mg/kg midazolam (Astellas Pharma Inc., Tokyo, JPN); and 2.5 mg/kg butorphanol (Meiji Seika Pharma Co., Ltd. Tokyo, JPN). To induce knee OA in the right hind limb, the medial capsule was exposed and the ACL was transected using scissors. OA mice were randomly assigned to either exercise (n = 40) or sedentary (n = 24) groups. Eight mice from each group were administered clodronate liposomes to the knee joint.

2.2. Treadmill exercise intervention

2.2.1. Mice allocated to the exercise groups were placed on a rodent treadmill MK-680

(Muromachi Kikai Co., Ltd. Tokyo, JPN). Six weeks after ACL transection, all mice were allowed to become familiarized with the treadmill environment for 3 d. As previously described [14], mice allocated to the exercise groups exercised on the treadmill at a constant speed of 10, 15, or 20 m/min (mild, moderate, and high, respectively) for 30 min/d, 3 d/week, for a total of 4 weeks. And the treadmill exercise in the clodronate group was performed at 10 m/min for 30 min/d, 3 d/week, for a total of 4 weeks.

2.2.2. Intra-articular injection of clodronate liposome

Clodronate liposomes (Hygieia Bioscience Co., Ltd. Osaka, JPN) were used to deplete synovial macrophages. Clodronate liposomes were administered directly into the knee joints of mice in the macrophages-depletion (MΦ-de) and exercise + MΦ-de (Ex + MΦ-de) groups (n = 8/group) using a Hamilton syringe (GL Sciences Co., Ltd. Tokyo, JPN) equipped with a 30G needle. Clodronate liposomes were administered under inhalation anesthesia with isoflurane (Mylan Seiyaku Ltd, Tokyo, JPN) by inserting a needle from inside the patellar tendon. After the injection, the needle was left in place for 1 min to prevent drug leakage. After removing the needle, the knee joint was flexed 10 times to allow the spread of clodronate liposomes in the joint. Clodronate liposomes were administered once per week at a dose of 6 μL (as reported by Blom [15]).

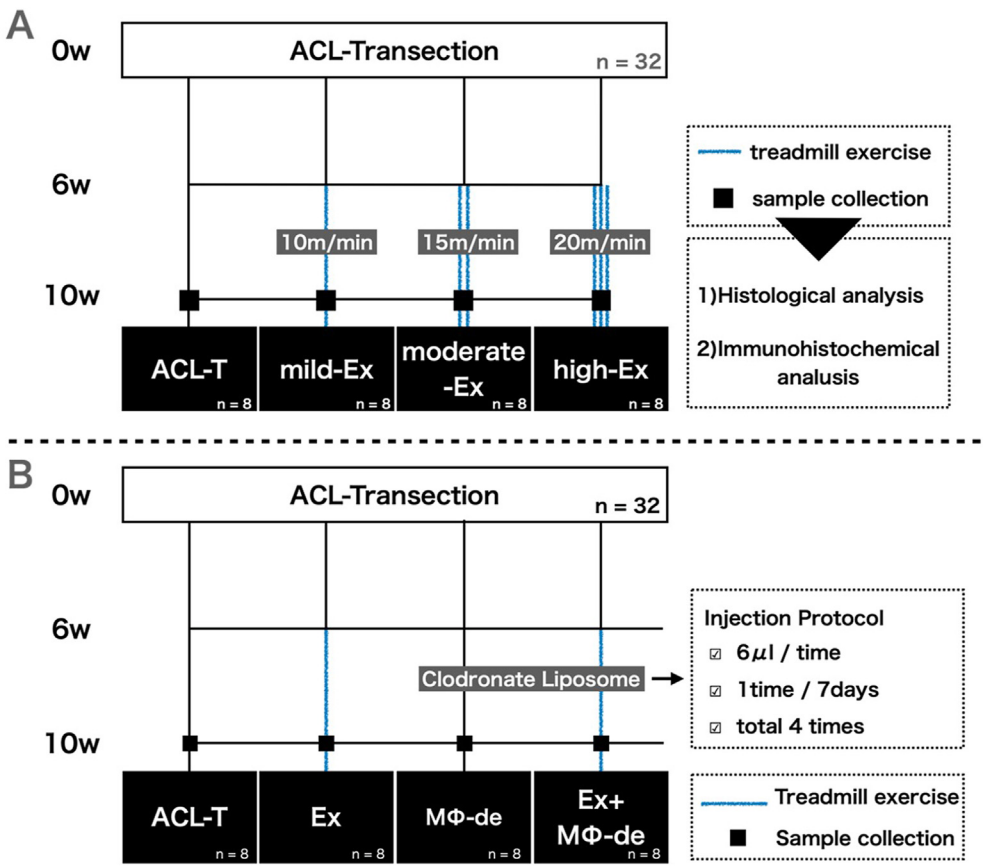

Fig. 1. Experimental design. (A) Three different intensities at different running speeds were set to verify the cartilage protective effect of treadmill exercise. Furthermore, to confirm the response of the synovium to treadmill exercise at moderate intensities, we examined the phenotypic changes in macrophages. (B) The role of macrophages on OA during treadmill exercise was tested by depleting macrophages in the joints. Histological analysis of the knee joint after treadmill exercise of different intensities. Each group, n = 8.

Mice in the ACLT and exercise (Ex) groups were treated with PBS (pH 7.4) in the same manner and used as control.

2.3. Histological analysis

Tissue sections of the knee joint were stained with Safranin-O Fast Green stain to observe the articular cartilage, according to the method described by Schmitz et al. [16] Hematoxylin and eosin (H&E) staining was performed to observe the synovium. Two sections of the articular cartilage were evaluated for each mouse, based on the scoring method recommended by the Osteoarthritis Research Society International (OARSI) system [17]. The synovium was evaluated in two sections of each individual against the synovium medial to the infrapatellar pad. The synovial inflammation score [19] is a rating scale that classifies synovitis on a 5-point scale (0–4) based on the number of synovial layers, degree of thickening, and degree of cellular infiltration. Two independent researchers performed the scoring of all sections in a blinded test, and the mean value was used as the score. Immunohistochemical staining was performed employing the avidin-biotinylated enzyme complex method and using the VECTASTAIN Elite ABC rabbit IgG kit (Vector Laboratories, CA, USA). Anti-gremlin-1 (ab231065; Abcam plc . MA , USA , 1:200), anti-MMP-13 (ab39012; Abcam plc . MA , USA , 1:250), anti-ADAMTS4 (bs-4191R; Bioss Antibodies Co., Ltd. MA, USA, 1:100), anti-CD68 (ab125212; Abcam plc. MA, USA, 1:100), anti-iNOS (2977S; Cell Signaling Technology, Inc., MA, USA, 1:200), anti-CD206 (18704-1-AP; Proteintech Group, IL, USA, 1:500), anti-TLR4 (ab13867; Abcam plc. MA, USA, 1:250), and anti-IL-4 (GTX66741; Gene Tex Inc., CA, USA, 1:100) were used as primary antibodies. Hematoxylin staining was also performed to visualize the nuclei. Data was calculated as the percentage of positive cells/total cells in a randomly selected area of the cartilage (10,000 (100 × 100) μm^2) and the synovium (2500 (50 × 50) μm^2).

2.4. Statistical analysis

Statistical analyses were performed using the SPSS software. All data were tested for normality using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) and Tukey's method were used as post-tests for the data of the OARSI score, synovitis score, gremlin-1 immunohistochemical staining, MMP-13, ADAMTS4, IL-4, and TLR4 positive cell. The Kruskal–Wallis test was used for calculating the CD68 positive cell count, iNOS, and CD206 positive cell, whereas the Steel-Dwass method was used for the post-test. Data with normality are shown as the mean [95% confidence interval], whereas data without normality are shown as the median [interquartile range]. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Mild exercise inhibited cartilage degeneration

In the assessment of articular cartilage using the OARSI score (Fig. 2), the mild-Ex group showed significantly lower values than those in the ACLT and high-Ex groups ([mild-Ex vs. ACLT]; $p = 0.022$, [mild-Ex vs. high-Ex]; $p < 0.001$). Moreover, the high-Ex group showed significantly higher values than the moderate-Ex group ([high-Ex vs. moderate-Ex]; $p = 0.006$).

Positivity for gremlin-1 was detected in the noncalcified layer in all groups. The higher the exercise intensity, the more gremlin-1 positive cells were observed. Therefore, the positive cell was significantly higher in the moderate-Ex group than in the ACLT group ([moderate-Ex vs ACLT]; $p = 0.003$). Furthermore, the high-Ex group showed significantly higher gremlin-1 positive cell than that in the ACLT and mild-Ex groups ([high-Ex vs ACLT, mild-Ex]; $p < 0.001$).

Furthermore, the mild-Ex group exhibited significantly lower matrix metalloproteinase (MMP)-13 positive cell compared with the other three groups ([mild-Ex vs. ACLT, moderate-Ex, high-Ex]; $p = 0.004$, $p < 0.001$, and $p < 0.001$, respectively). However, the moderate-Ex and high-Ex groups showed significantly higher values than those in the ACLT group ([moderate-Ex, high-Ex v. ACLT]; $p = 0.001$ and $p < 0.001$, respectively).

3.2. Mild exercise altered macrophage phenotype and suppressed synovitis

Histological images of the hematoxylin and eosin (H&E)-stained synovium and synovitis scoring are shown (Fig. 3). The synovium

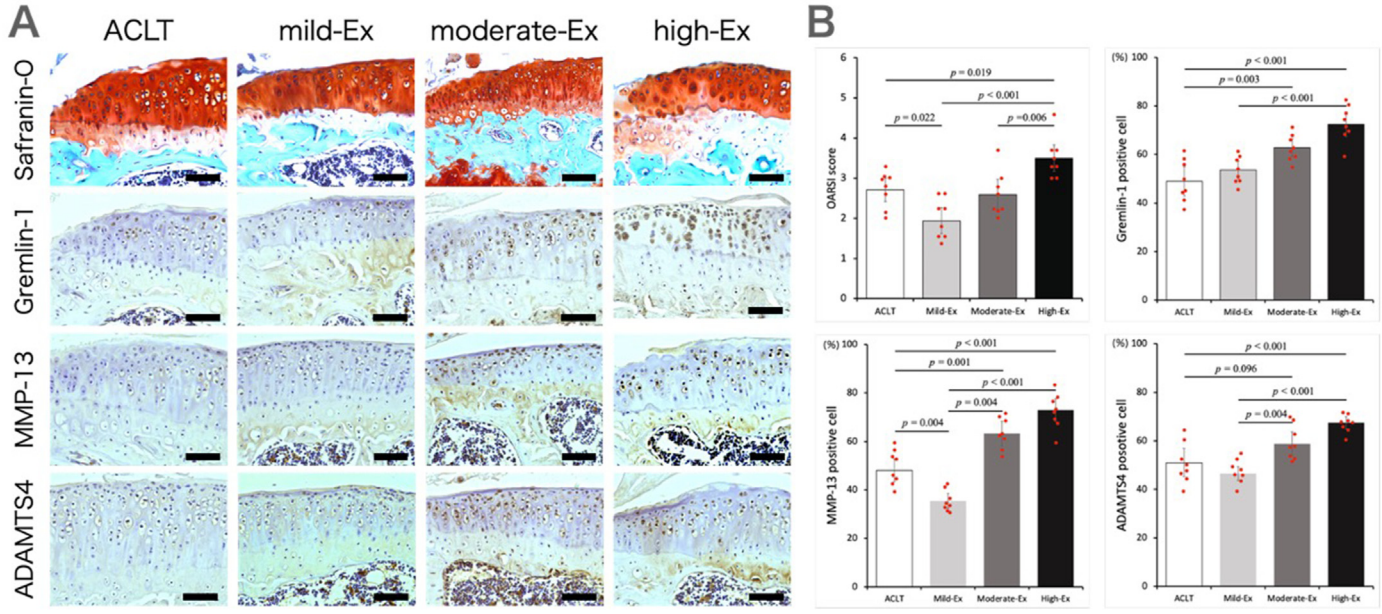

Fig. 2. Treadmill exercise affects cartilage degeneration. (A) Histological images of articular cartilage stained with Safranin-O Fast Green and immunohistochemical staining for Gremlin-1, MMP-13, and ADAMTs4 (Scale bars: 50 μm). (B) Scoring results by OARSI score to assess cartilage degeneration and positive cell rates for Gremlin-1, MMP-13, and ADAMTs4 (n = 8, in each group. One-Way ANOVA, post-hoc Tukey). Data are presented as means and 95% confidence interval. In mild-ex group, OARSI score, MMP-13, and ADAMTS4 positive cell rate were suppressed. Positive cell rate for gremlin-1 increased in an exercise intensity-dependent manner.

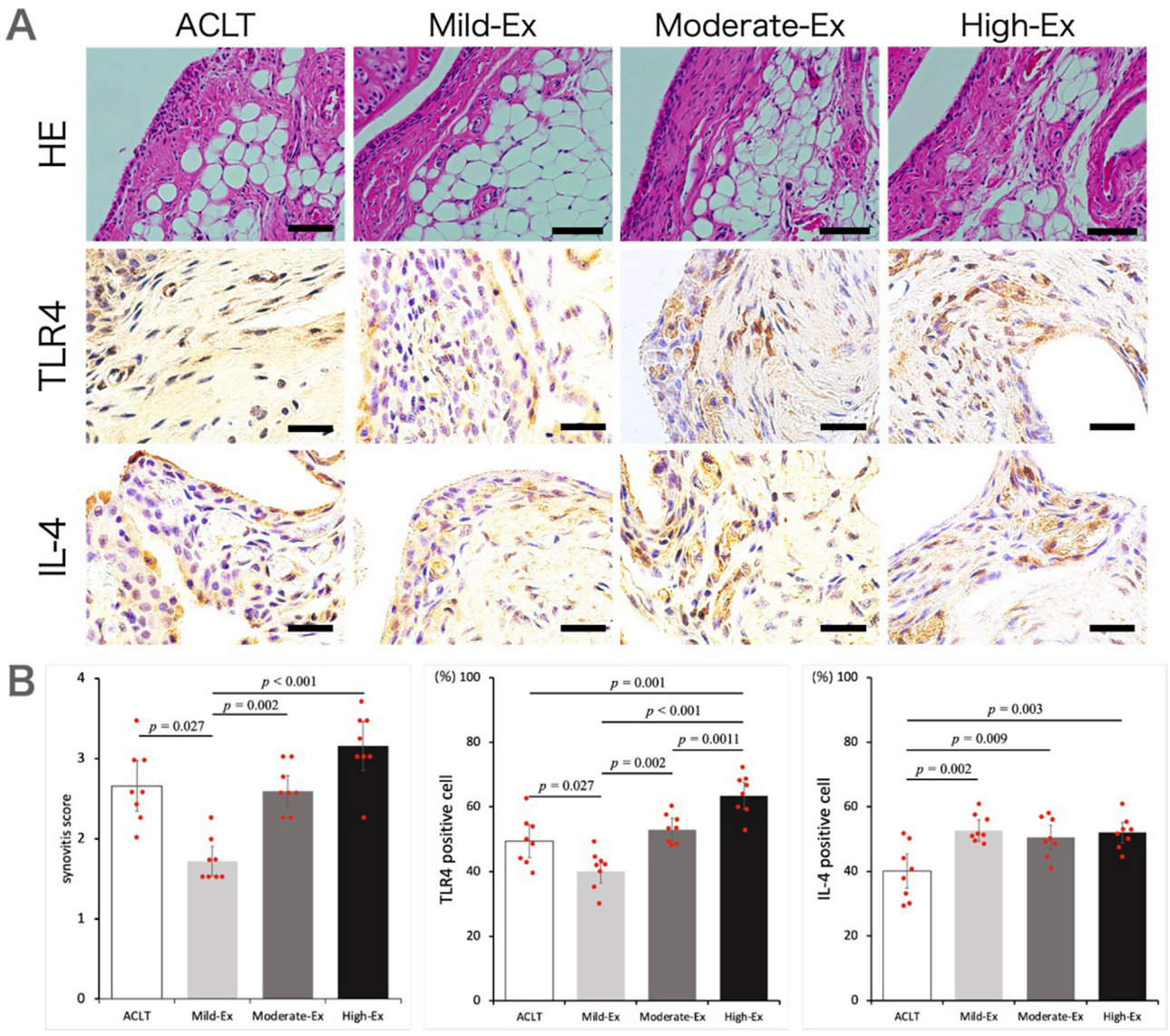

Fig. 3. Treadmill exercise affects synovitis and inflammatory conditions. (A) Histological images of synovium stained with H&E and immunohistochemical staining for TLR4 and IL-4 (Scale bars: 50 μm (HE staining), 10 μm (TLR4, IL-4)). (B) Results of scoring of synovitis and analysis of positive TLR4 and IL-4 cell rates (n = 8, in each group. One-Way ANOVA, post-hoc Tukey). Data are presented as means and 95% confidence interval. In mild-ex group, synovitis score, TLR4 positive cell rate were suppressed. Exercise groups were increased IL-4 positive cell rate.

showed thickening of the synovial tissue in all groups; however, both the thickening of the synovium and the increase in the number of cells were reduced in the mild-Ex group. We also observed that in the high-Ex group the boundary between the infrapatellar pad and synovial tissue was obscured resulting in synovial tissue infiltration into the infrapatellar pad.

Synovitis scores were significantly lower in the mild-Ex group relative to the other three groups ([mild-Ex vs. ACLT, moderate-Ex, high-Ex]; $p < 0.001$, $p = 0.001$, and $p = 0.003$, respectively). Moreover, the mild-Ex, moderate-Ex, and high-Ex groups exhibited significantly higher interleukin 4 (IL-4) positive cell than those in the ACLT group ([mild-Ex, moderate-Ex, high-Ex groups vs. ACLT]; $p = 0.002$, $p = 0.009$, and $p = 0.003$, respectively).

The results of the analysis on macrophages are shown (Fig. 4). CD68 was used to stain for macrophages. We observed that CD68-positive cells were not significantly different among the four groups (ACLT: 90 [79–95]; mild-Ex: 86 [80–109]; moderate-Ex: 91 [85–96]; high-Ex: 91 [81–116]; $p = 0.921$). Nevertheless, the inducible nitric oxide synthase (iNOS) staining specific for M1 macrophages was positive, mainly in the synovial surface layer, and the ratio of M1 to total macrophages was significantly lower in the mild-Ex group compared with that in the other three groups ([mild-Ex vs. ACLT, moderate-Ex, high-Ex]; $p = 0.024$, $p = 0.042$, and $p < 0.001$, respectively). However, the ratio of M2 macrophages calculated from CD206-positive cells was significantly higher in the mild-Ex group relative to that in the other three groups ([mild-Ex vs. ACLT, moderate-Ex, high-Ex]; $p = 0.024$, $p = 0.042$, and $p < 0.001$, respectively).

3.3. Intra-articular injection of clodronate liposomes attenuated the chondroprotective effect of mild exercise

Histological images of the Safranin-O Fast Green-stained articular cartilage and OARSI scores are shown (Fig. 5). We found that the OARSI score was significantly lower in the Ex and MΦ-de groups than in the ACLT group ([Ex group, MΦ-de group vs. ACLT]; $p < 0.001$ and $p = 0.002$, respectively). In addition, the Ex + MΦ-de group showed significantly higher OARSI scores compared with those in the other three groups ([Ex + MΦ-de vs. ACLT, Ex, MΦ-de]; $p = 0.007$, $p < 0.001$, and $p < 0.001$, respectively). Gremlin-1 positive cells were observed in all groups, mainly in the non-calcified layer, with the Ex group presenting a

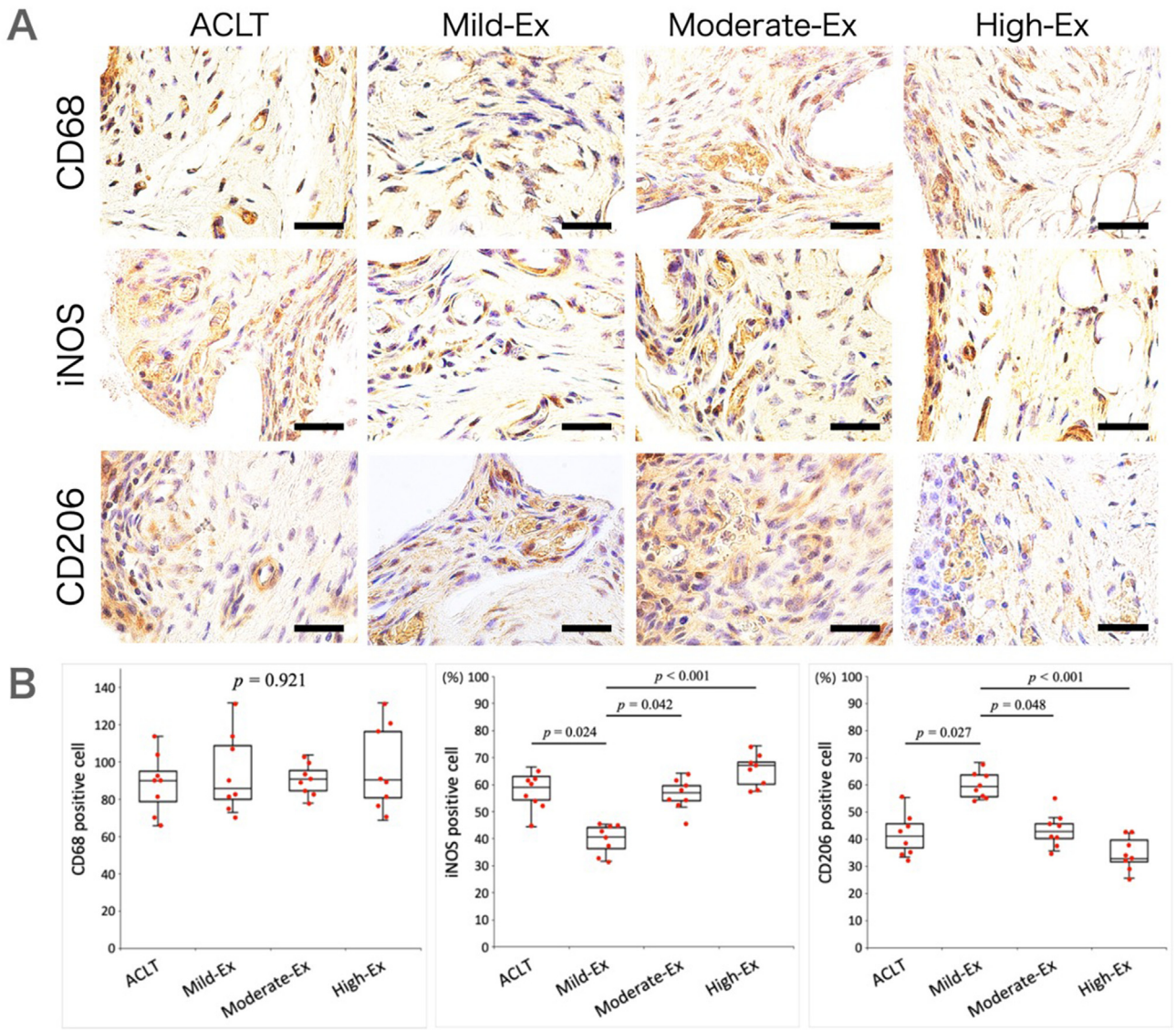

Fig. 4. Treadmill exercise affects macrophage polarization. (A) Images of immunohistochemical staining for CD68, iNOS, and CD206 and analysis of positive cell rates (Scale bars: 10 μm). (B) CD68 staining sections was evaluated for the number of positive cells. iNOS and CD206staining sections were calculated as the percentage of positive cells to the number of positive cells for CD68 (n = 8, in each group. Kruskal-Wallis test, post-hoc Steel Dwass test). Data are presented as median and interquartile range. Mild-Ex group showed suppressed positivity for iNOS and increased positivity for CD206.

significantly higher positive cell than the ACLT group ($p = 0.047$). The Ex + MΦ-de group showed a significantly higher positive cell than the MΦ-de group ([Ex + MΦ-de vs MΦ-de]; $p = 0.036$).

We also observed strong MMP-13 positive staining in the Ex + MΦ-de group. In particular, the Ex and MΦ-de groups exhibited significantly lower positive cell than that of the ACLT group ([Ex group, MΦ-de group vs. ACLT]; $p = 0.002$ and $p = 0.050$, respectively). However, the Ex + MΦ-de group showed significantly higher values compared with those in the other three groups ([Ex + MΦ-de vs. ACLT, Ex, MΦ-de]; $p = 0.035$, $p < 0.001$, and $p < 0.001$, respectively).

3.4. Intra-articular injection of clodronate liposomes attenuated the inhibitory effect of mild exercise on synovitis

Histology analysis of the H&E-stained synovium upon clodronate liposomes treatment was performed, and synovitis scoring was assigned (Fig. 6). We observed synovial thickening in all groups. However, we found that. in the Ex + MΦ-de group, the boundary between the synovium and the fat body became indistinct, indicating the infiltration of the synovium in the fat body. Moreover, a decrease in the number of cells in the synovial surface layer was detected in both the MΦ-de and Ex + MΦ-de groups. In terms of synovitis score, the Ex group showed a significantly lower synovitis score compared with that in the other three groups ([Ex vs. ACLT, MΦ-de, Ex + MΦ-de]; $p = 0.025$, $p = 0.009$, and $p < 0.001$, respectively). However, the Ex + MΦ-de group showed significantly higher synovitis scores relative to that in the ACLT and MΦ-de groups ([Ex + MΦ-de, ACLT group vs. MΦ-de]; $p = 0.001$ and $p = 0.003$, respectively). We observed a limited number of TLR4 positive cells in the synovial surface layer of the Ex group. We also observed a higher number of IL-4 positive cells in the exercise group with their rate in the Ex and Ex + MΦ-de groups being significantly higher than that in the ACLT and MΦ-de groups ([Ex, Ex + MΦ-de vs. ACLT, MΦ-de]; $p = 0.001$ and $p = 0.003$, as well as $p = 0.001$ and $p = 0.005$, respectively).

The results of the analysis on macrophages are shown (Fig. 7). An increased number of CD68 positive cells in the synovial surface layer in the ACLT and Ex groups was detected; however, few positive cells were observed in the MΦ-de and Ex + MΦ-de groups that received macrophage-depleting agents. In particular, the number of CD68 positive cells was significantly lower in the MΦ-de and Ex + MΦ-de groups compared with the ACLT and Ex groups ([MΦ-de, Ex + MΦ-de vs. ACLT,

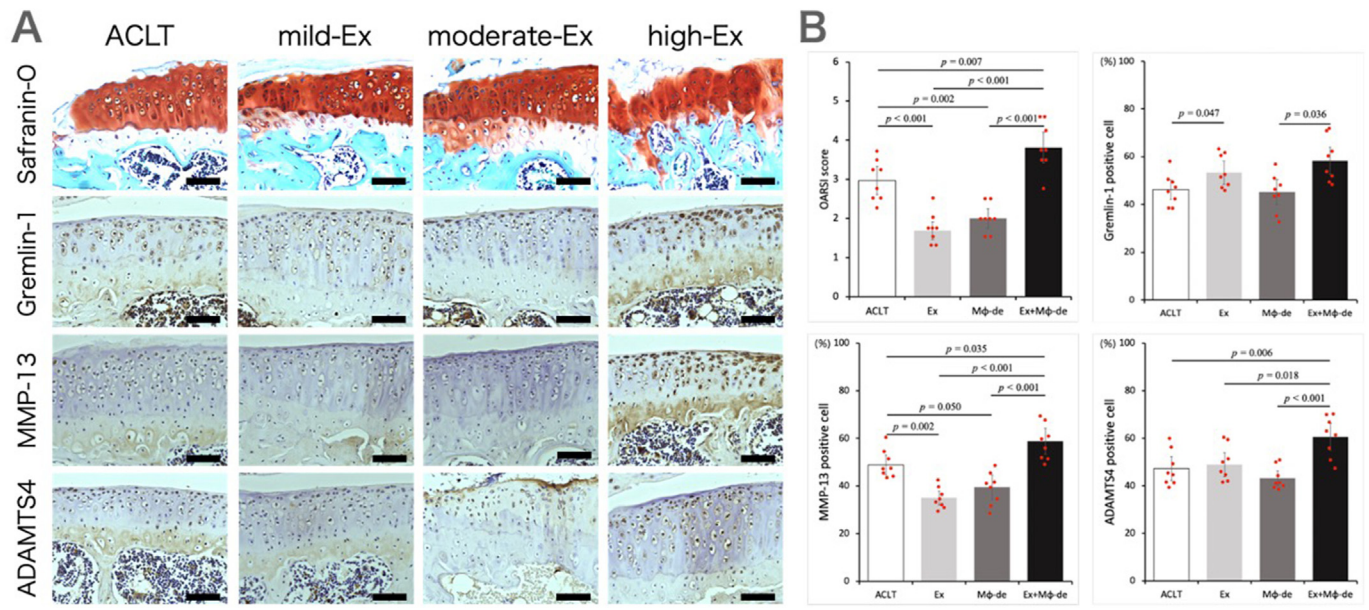

Fig. 5. Treadmill exercise and clodronate liposomes affect cartilage degeneration. (A) Histological images of articular cartilage stained with Safranin-O Fast Green and immunohistochemical staining for Gremlin-1, MMP-13, and ADAMTs4 (Scale bars: 50 μm). (B) Scoring results by OARSI score to assess cartilage degeneration and positive cell rates for Gremlin-1, MMP-13, and ADAMTs4 (n = 8, in each group. One-Way ANOVA, post-hoc Tukey). Data are presented as means and 95% confidence interval. Ex and MΦ-de group showed lower OARSI scores and lower MMP-13 positive cell rates. Gremlin-1 positive cell rate increased in the exercise group.

Ex]; p = 0.006 and p = 0.006, as well as p = 0.003 and p = 0.002, respectively). Furthermore, the ratio of the iNOS-positive M1 macrophages was significantly lower in the Ex group relative to that in the other three groups ([Ex vs. ACLT, MΦ-de, Ex + MΦ-de]; p = 0.010, p = 0.002, and p = 0.003, respectively). On the contrary, the ratio of CD206 positive M2 macrophages was significantly higher in the Ex group compared with that in the other three groups ([Ex vs. ACLT, MΦ-de, Ex + MΦ-de]; p = 0.010, p = 0.002, and p = 0.003, respectively).

4. Discussion

In the present study, we examined a mouse model of knee OA upon treadmill exercise of different intensities to determine the biological responses of articular cartilage and synovial tissue to exercise. Furthermore, we conducted experiments using clodronate liposomes to verify the role of synovial macrophages in response to treadmill exercise.

In the OA model used in this study, degeneration of articular cartilage was delayed in the mild-Ex group, which had the lowest of the investigated intensities. Previous studies examining the effects of exercise on rat OA models reported that moderate-intensity treadmill exercise suppressed cartilage degeneration [7,19]. In addition, intense exercise has been reported to contribute to cartilage degeneration [20–23]. In our previous study, we also found that treadmill exercise at an intensity of 18 m/min increased the expression of articular chondrogenic catabolite factors, such as MMP-13 and IL-1β, and markedly advanced cartilage degeneration in OA mice compared with non-exercise mice. In this study, we found that ACLs were more susceptible to cartilage degeneration than ACLs in the non-exercise group [14]. However, considering the different size of mice and rats, it is possible that the exercise intensity of 18 m/min was not adequate to compare the two studies. Therefore, here, to address the effect of different exercise intensities, we set up a group of mice subjected to lower exercise intensity. We accordingly observed that cartilage degeneration was suppressed in the mild-Ex group, with the lowest intensity and running distance among the exercise groups. In addition, the levels of MMP-13 and ADAMTS4, which are matrix-degrading enzymes in the articular cartilage, were also reduced in the mild-Ex group.

However, the reason for the suppression of OA following mild exercise is still not fully understood. In the present study, the percentage of expression of gremlin-1, which is commonly upregulated in OA, was linearly increased in the ACLT, mild-Ex, moderate-Ex, and high-Ex groups, suggesting that mechanical loading on the articular cartilage became stronger in an exercise intensity-dependent manner; however, the severity of cartilage degeneration had a nonlinear relationship. This finding suggested that the exercise is associated with additional factors other than the commonly examined mechanical loading.

In addition to the articular cartilage, we also examined the synovium. Synovitis is characterized by intimal hyperplasia, fibrosis, and neovascularization [24]. Arthroscopy, MRI, and ultrasonography have been used to reveal the presence of synovitis in OA joints, and have been suggested as predictors of the progression of structural changes in the joint [25–29]. Synovitis scoring showed that synovitis was suppressed in the mild-Ex group and was associated with an increase in the levels of IL-4 and a decrease in the expression of the inflammatory factor TLR4 in the synovium.

In accordance with our current findings, Castrogiovanni et al. reported that moderate-intensity treadmill exercise suppressed synovitis, increased the levels of IL-4, and reduced IL-1β in a OA rat model [30]. In the present study, we found that exercise affected macrophages M1/M2 polarization. M1 macrophages are induced by inflammatory factors and promote inflammation [31]. In contrast, M2 macrophages are induced by anti-inflammatory factors, such as IL-4 and IL-13, and play a role in preventing osteophyte maturation and cartilage wear [32–34]. In addition, studies in other fields have reported that aerobic exercise reduced the M1/M2 ratio and improved inflammation. Aerobic treadmill exercise in obese mice on a high-fat diet led to a decrease in the mRNA levels of inflammatory cytokines, such as TNF-α and IL-6, a decrease in the number of M1 macrophages, and an increase in the number of M2 macrophages in adipose tissues compared with non-exercise obese mice [13]. Moreover, Bobinski et al. reported that examining a mouse model of peripheral nerve injury subjected to treadmill exercise, the M1/M2 ratio was improved after treadmill exercise upon IL-4 depletion, indicating that IL-4 plays a role in this process [35]. These results suggested that treadmill exercise might alter the phenotype of macrophages in the

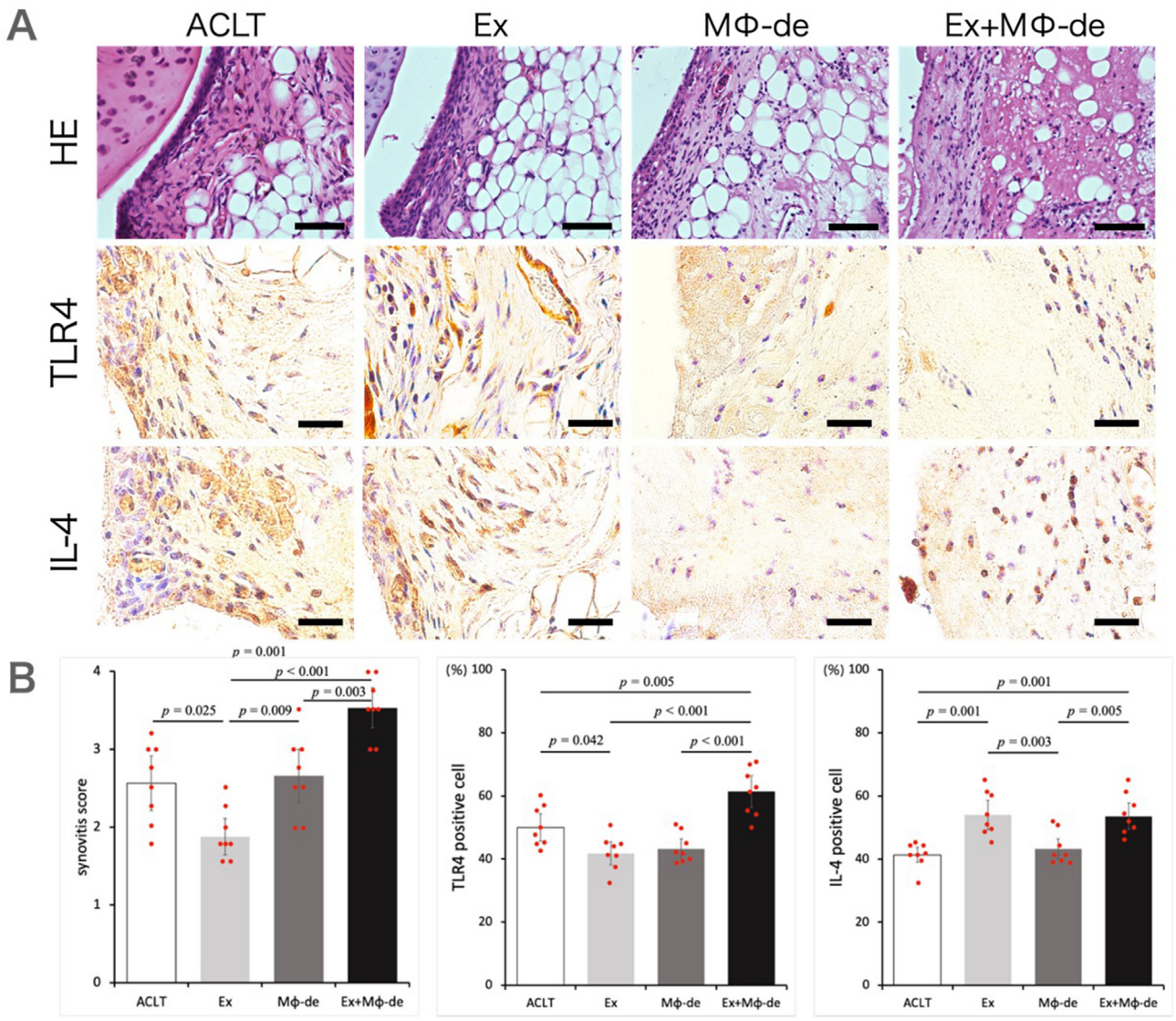

Fig. 6. Treadmill exercise and clodronate liposomes affect synovitis and inflammatory conditions. (A) Histological images of synovium stained with H&E and immunohistochemical staining for TLR4 and IL-4 (Scale bars: 50 μm (HE staining), 10 μm (TLR4, IL-4)). (B) Results of scoring of synovitis and analysis of positive TLR4 and IL-4 cell rates. Ex group showed lower synovitis score and TLR4 positive cell rate and increased IL-4 positive cell rate (n = 8, in each group. One-Way ANOVA, post-hoc Tukey). Data are presented as means and 95% confidence interval. Ex + MΦ-de group showed increased IL-4 positive cell rate but markedly worse synovitis score.

OA synovium, regulate the inflammatory state of the joint, and suppress subsequent cartilage degeneration. Macrophages are classified as either resident macrophages or bone marrow-derived macrophages. In general, bone marrow-derived macrophages are induced by inflammatory stimuli and differentiate into an M1 or M2-like phenotype after settling in tissues. Therefore, the macrophages mobilized in this study were most likely bone marrow-derived macrophages. Although M1 to M2 macrophages are generally converted from M1 to M2 macrophages, no evidence has been found for the conversion of M1 macrophages to M2 macrophages in tissues due to exercise. A previous study reported that exercise increases anti-inflammatory factors and M2 macrophages [13]. Furthermore, similar macrophage changes did not occur in IL-4 knockout mice, suggesting that IL-4 mediates the phenotypic change [35]. Based on these findings, it is likely that exercise also increased IL-4 in the present study, resulting in M2-like changes in bone marrow-derived macrophages.

As macrophages have been suggested to be involved in the chondroprotective effect of treadmill exercise, we attempted to deplete macrophages using clodronate liposomes in the knee joint. Notably, although cartilage degeneration was delayed in the Ex group and MΦ-de group following administration of clodronate liposomes and treadmill exercise alone, cartilage degeneration in the Ex + MΦ-de group worsened when these were combined. These results suggest that macrophages are necessary for the treadmill exercise-related effect of delaying cartilage degeneration in knee OA and may to contribute to a new potential treatment for knee OA. Glucocorticoids are known to increase the expression of CD163 (M2 macrophage marker) in synovial macrophages of patients with OA [36], and Utomo et al. [37] reported that dexamethasone added to synovium specimens of patients with OA suppressed M1 macrophages, whereas increased the number of M2 macrophages. The relationship between OA, macrophages, and exercise, as well as the relationship between OA and macrophages and between exercise and macrophages is becoming clearer; however, there have been no reports that verify the relationship between exercise and macrophages in the field of OA. In contrast, this study showed that the effects of treadmill exercise and clodronate liposomes administration canceled each other out, indicating that dynamic changes in macrophages are involved in exercise stimulation. Exercise in a macrophage-depleted state potentially

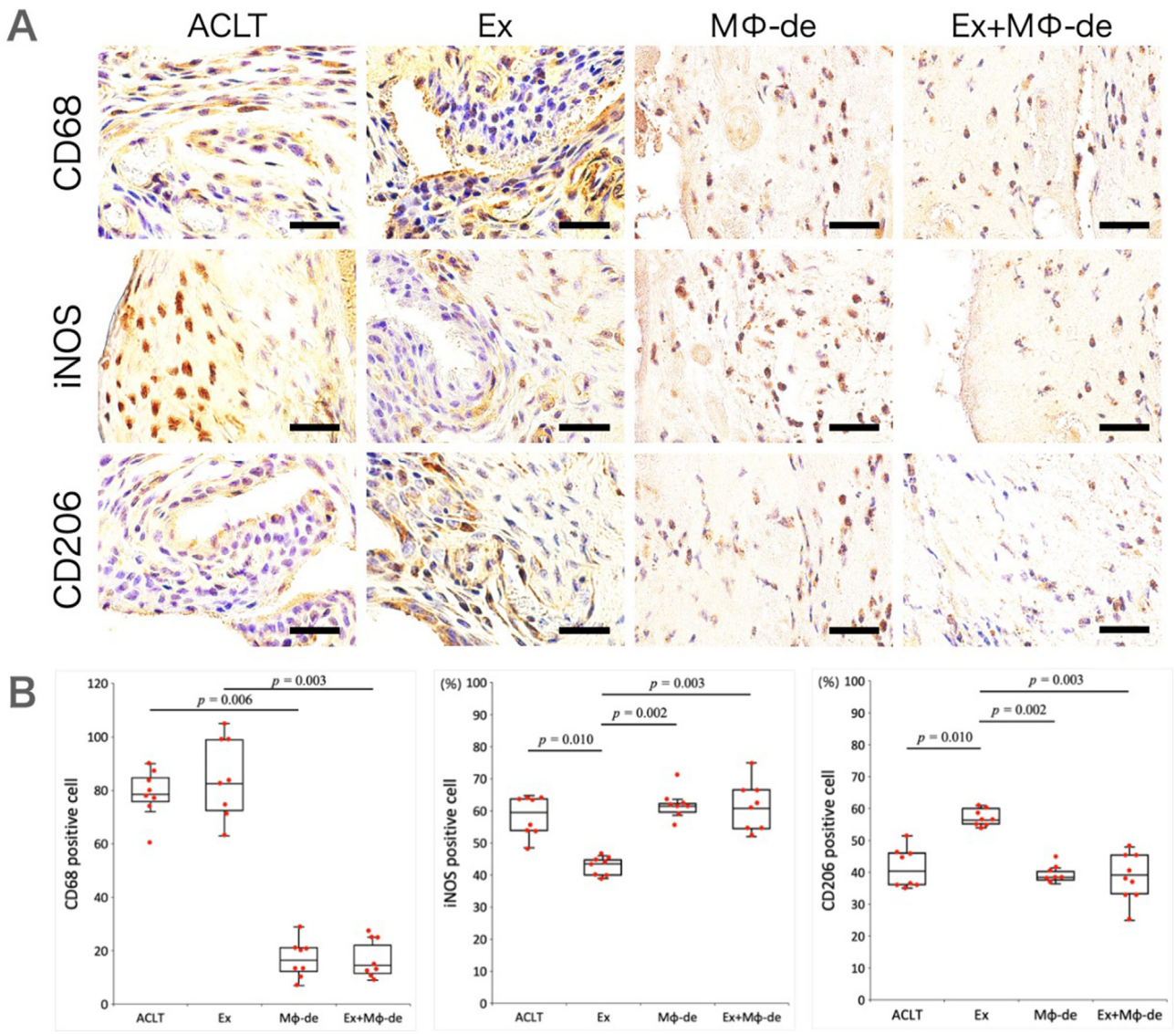

Fig. 7. Treadmill exercise and clodronate liposomes affect macrophage polarization. (A) Images of immunohistochemical staining for CD68, iNOS, and CD206 (Scale bars: 10 μm). (B) CD68 staining sections was evaluated for the number of positive cells. iNOS and CD206staining sections were calculated as the percentage of positive cells to the number of positive cells for CD68 (n = 8, in each group. Kruskal-Wallis test, post-hoc Steel Dwass test). Data are presented as median and interquartile range. The number of CD68-positive cells decreased in the clodronate liposome treated group. Ex group showed suppressed positivity for iNOS and increased positivity for CD206.

causes cartilage degeneration, as it lacks the ability to convert the inflammatory environment of the knee joint into an anti-inflammatory one. The increase in the levels of IL-4 in the Ex group might have altered the phenotype of macrophages and therefore the inflammatory state in the knee joint. However, in the Ex + MΦ-de group, the lack of any anti-inflammatory action by M2 macrophages promoted the retaining of the inflammatory state in the joint. Although treadmill exercise increased anti-inflammatory factors, cartilage degeneration could not be delayed when macrophages were depleted.

When comparing the MΦ-de group treated only with clodronate liposomes with the Ex + MΦ-de group, we observed an increase in the expression of anti-inflammatory factors in the synovium in the Ex + MΦ-de group an increase in both the OARSI score and the expression of chondrogenic factors. Macrophages were depleted in both groups following clodronate administration. Moreover, the percentage of gremlin-1 positive cells was higher in the Ex + MΦ-de group than in the MΦ-de group, suggesting that gremlin-1 was expressed in chondrocytes under excessive mechanical loading, leading to cartilage degeneration [38]. In the Ex + MΦ-de group, treadmill exercise added additional mechanical loading to the articular cartilage, which increased the levels of chondrogenic factors in chondrocytes and accelerated cartilage degeneration. Overall, treadmill exercise had a negative effect on the articular cartilage by weakening the function of macrophages in the knee joint.

This study bears few limitations. In this study, clodronate liposomes were used to deplete macrophages. PBS was also used for the control group. The use of liposomes for control is recommended, it is necessary to validate the use of such liposomes in the future. However, since some previous studies have used PBS or saline in the control group, it is not expected to have a significant effect on the results of the experiment.

With regard to macrophages, treadmill exercise caused a change in the ratio of macrophages, but it is difficult to determine that the change is attributable to exercise. If we can determine from which tissues the changed macrophages accumulated and differentiated, and what influenced them, we will be able to pursue the effects of exercise.

Specifically, it is not clear whether the altered macrophages in this study were resident macrophages or bone marrow-derived macrophages. Since it is generally known that bone marrow-derived macrophages

accumulate in inflammatory conditions and regulate inflammation, the macrophages in this study may be due to the response of bone marrow-derived macrophages, but it would be desirable to determine this by staining for specific factors. Further analysis will be required to determine whether the change to M2 macrophages is a switch from M1 to M2 or differentiation to M2 by IL-4 or other factors.

In this study, we observed two factors affecting cartilage degeneration, loading added to the articular cartilage and the macrophage response. However, because treadmill exercise was used, it was not possible to capture separately the effects caused by mechanical loading and those caused by whole-body exercise. Further investigation combining treadmill exercise, underwater exercise, and unloading techniques may address this limitation.

In conclusion, mild treadmill exercise prevented the progression of cartilage defects and synovitis in an OA mouse model. In addition, when treadmill exercise was performed following clodronate liposome-induced depletion of macrophages, cartilage defects progressed, suggesting that macrophages are involved in the chondroprotective effects of treadmill exercise.

Author contributions

YO, KM, TKo, and NK designed the study. YO, KO, YM, KA, and KT collected the data. YO, KO, YM, TKa, and AK performed the histological analysis. YO, KM, TKo, and NK contributed to the manuscript composition. All authors have read and approved the final manuscript.

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