

Suppression of TGF-beta activity with remobilization attenuates immobilization-induced joint contracture in rats

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Abstract

Background

Joint contracture is a common complication of joint injury. This study aimed to assess the effect of inhibiting the transforming growth factor- β (TGF- β) signaling during joint immobilization and remobilization on immobilization-induced joint contracture in rats.

Methods

The knees of rats were immobilized using Kirschner wires following trauma to the femoral condyles to generate joint contracture. After immobilization, levels of TGF- β and passive extension range of motion (ROM) were measured at different time points, joints were histologically analyzed by hematoxylin and eosin (H&E) and Masson trichrome staining, and the expression of inflammatory or fibrosis-related mediators, including interleukin-1 β (IL-1 β), phosphorylated Smad2/3 (p-Smad2/3), α -smooth muscle actin (α -SMA) and collagen types I (Col 1) and III (Col 3), were examined in joint capsules using immunohistochemistry and quantitative real-time polymerase chain reaction (qRT-PCR). Rats were also treated with LY2157299, a TGF- β receptor I kinase inhibitor, at different stages of immobilization and remobilization.

Results

TGF- β 1 levels in the serum and the number of p-Smad2/3⁺ cells in the joint capsule were significantly elevated after immobilization. ROM decreased during the 6 weeks of immobilization and partly recovered after remobilization. After treatment with LY2157299 during immobilization, the restricted ROM moderately increased, but this effect was stronger when combined with active motion. Mechanistically, the expression of IL-1 β , TGF- β , fibrosis-related factors, and the density of collagen significantly decreased after treatment with LY2157299.

Conclusions

Inhibiting TGF- β signaling paired with active motion effectively attenuated the formation of immobilization-induced joint contracture in rats.

Introduction

Joint contracture is a common clinical disease characterized by a restricted range of motion (ROM) in the joint [1–3]. It is currently regarded as the most devastating adverse outcome following joint damage, arthroplasty, sports injuries, and other musculoskeletal disorders. The most common etiological factor for joint contracture is joint immobilization, which is clinically used as an orthopedic treatment to ease joint

pain and reduce inflammation for patients with musculoskeletal damage [4]. The deterioration of joint contracture may drastically decrease the quality of life for patients as they eventually suffer from lifelong disability. Despite years of investigation by professionals involved in primary care and rehabilitation, the pathological factors leading to the aggravation and restriction of joint contracture remain to be elucidated [5, 6].

Based on the precise changes in joint tissues during immobilization, joint contracture is classified into two categories, myogenic contracture and arthrogenic contracture [7, 8]. As serious arthrogenic contracture is irreversible, the arthrogenic components, including the synovium, joint capsules, and ligaments, are the key study areas for attenuating joint contracture. It is widely accepted that inflammatory cytokines and profibrotic factors trigger the progression of fibrosis in the joint capsule and synovium [9–11]. Previous studies indicated that profibrotic growth factors are elevated during joint immobilization [12, 13]. Of these, transforming growth factor- β (TGF- β), as a dominating profibrotic factor of arthrofibrosis, induces the plasticity and proliferation of fibroblasts with upregulated collagen in muscle contractures and other fibrotic tissues [11–13]. Fibrous adhesion formation can be reduced by small proteoglycan, decorin, via the inhibition of TGF- β [14]. However, few studies have systematically investigated the effects of the inhibition of TGF- β signaling pathway on the rehabilitation of joint contracture after immobilization or remobilization.

In this study, we proposed that intervention of the TGF- β signal pathway by the administration of anti-fibrotic drugs, such as the TGF- β receptor I kinase inhibitor LY2157299, may alleviate the detrimental effects of immobilization-induced joint contracture. In the present experiments, we found that high levels of active TGF- β are involved in driving joint contracture progression. Inhibition of TGF- β activity, especially when combined with active movement, effectively attenuated the exacerbation of joint contracture in the rat model.

Materials And Methods

Experimental Animals

All animal experimental procedures in this protocol were approved by the Wuxi 9th People's Hospital Institutional Review Board (no. KT2019019). A total of 70 skeletally mature male Sprague-Dawley rats, aged 12 weeks, were purchased from Yangzhou University (Yangzhou, China). Rats were housed under a 12-hour light/dark cycle in a temperature ($22 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) controlled room. Standard rodent chow and water were provided *ad libitum*. The health status of each rat was monitored throughout the experiment by animal veterinary technicians. The rats were free of all viral, bacterial, and parasitic pathogens during the experimental schedule.

Joint Immobilization and Remobilization

The right knee joints of rats were immobilized according to the method described in previous studies [15, 16]. Briefly, after anesthesia by intraperitoneal injection of sodium pentobarbital, a lateral parapatellar

arthrotomy of the knee was performed. The patella was moved medially and the knee joint was flexed to expose the femoral condyles. Next, two 1.5-mm x 1.5-mm cortical bone defects were created at the non-cartilaginous portions of the medial and lateral femoral condyles to mimic an intra-articular fracture with hematoma formation. Then, the anterior and posterior cruciate ligaments were transected, and the joint was hyperextended to 45° to disrupt the posterior capsule. Finally, a 0.8 mm-diameter Kirschner wire (K-wire) was drilled from the tibia to the femur and curved at both ends to immobilize the knee joint at approximately 160° flexion (angle between tibia and femur is approximately 20°). The skin was then closed with 4.0 silk sutures. Following surgery, rats were allowed to move freely with all four limbs. Five rats were sacrificed at each of five time points after immobilization (1 week, 3 weeks, 6 weeks, 9 weeks and 12 weeks; n = 5 each; Fig. 1a). Six weeks after the initial surgery of immobilization, five additional rats underwent a second operation on their right knee to remove the K-wires and were allowed to move freely (remobilization) for 3 weeks (Fig. 1b). For the sham-operation, the right lateral parapatellar arthrotomy of knee was performed, but the tibia and femur were not fixed with K-wire. For all surgical groups, the contralateral (non-operated side) knee joints were used as controls [17].

An additional thirty rats were randomly divided into six groups (n = 5 each; Fig. 1b, c) to investigate the effect of LY2157299 (Selleck, Houston, USA) on joint contracture. The knees were immobilized or remobilized using the same methods as described above. After immobilization or remobilization, the rats were intraperitoneally injected with LY2157299 daily at a dose of 10 mg/kg body weight or an equivalent volume of vehicle (DMSO and PBS) according to the experimental protocol (Fig. 1c). Rats were all euthanized 9 weeks after surgery.

Measurement of ROM

To assess joint contracture, passive knee extension ROM was measured according to the methods described in the previous study [18]. Briefly, rats were euthanized by carbon dioxide (CO₂) inhalation. After the skin of the hindlimbs and K-wire were removed, rats were placed in a neutral spine position and the femur was manually fixed at a hip flexion of 90°. Then, a knee extension moment of 14.6 N/mm was applied by a pulley and weight to stretch the knee joint to reach its physiological restriction while avoiding damage to the soft tissue peripheral to the joint. The angle was measured by an arthrometer between femur and tibia and considered as passive extension ROM.

Histochemistry and Immunohistochemistry

After ROM measurements, the knee joints of rats were resected and kept at flexion of 90° and fixed in 10% buffered formalin at 4°C for 48 hours. The joints were then decalcified in 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4) for 4 weeks at room temperature. Specimens were dehydrated in graded alcohol and embedded in paraffin. A series of 5.0-µm sagittal-oriented sections were cut at the medial condylar region using a microtome. Sections were deparaffinized and processed for hematoxylin and eosin (H&E) and Masson trichrome staining.

Immunohistochemistry was performed on the remaining sections. Dewaxed paraffin sections were heated to 99°C for 20 minutes in Target Retrieval Solution (Beyotime, P0086) for antigen retrieval followed rehydration. After washing three times with PBS, the sections on slides were incubated with primary monoclonal antibodies against rat IL-1 β (abcam, ab9722), p-Smad2/3 (abcam, ab63399), α -SMA (abcam, ab124964), collagen I (abcam, ab34710), collagen III (abcam, ab7778) overnight at 4 °C in a humidified chamber. Following a washing step, all slides were incubated with secondary antibodies in blocking solution for 1 hour at room temperature. Finally, the sections were stained with 3,3'-diaminobenzidine for 1 minute and counterstained with hematoxylin. The slides were assessed under an optical microscope (DP75; Olympus Corporation, Tokyo, Japan), and the observer was blinded to which specimen was examined.

Histological Assessment of Joint Capsules

The synovial length and joint capsule thickness were measured with the H&E-stained sections as described in previous studies [2, 3].

Quantification of Joint Capsule Collagen Density

To elucidate the fibrotic changes in the posterior joint capsules, collagen was quantified using the method published by Kaneguchi et al [2]. To assess the deposition of collagen, Masson trichrome stained sections of the posterior joint capsule were photographed at 200 \times magnification with a light microscope (DP70; Olympus Corporation, Tokyo, Japan). The percentage of blue area in the total joint capsule area from each slice represented collagen density and was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples from rats at each time point after joint immobilization (1 week, 3 weeks, 6 weeks, 9 weeks and 12 weeks) and remobilization (3 weeks) were collected and centrifuged at 3000 rpm for 10 min at 4°C. Control samples were obtained from the sham group. All serum samples were collected and stored at -80°C until analysis. The concentrations of active TGF- β 1 and IL-1 β in the serum at all times points were measured using ELISA development kits (Mmbio, China) according to the manufacturer's instructions.

Gene expression analysis

We extracted total RNA from paraffin sections according to the methods of previous studies [2, 19]. In brief, Twenty-micrometer sagittal sections of the lateral side of the knee were cut using a microtome and the posterior joint capsule was isolated with forceps under a stereomicroscope. RNeasy Extract Kit (Solarbio, Beijing, China) was used to extract total RNA following the manufacturer's protocol. cDNA was prepared by reverse transcription using the HiScript II First-strand synthesis system (Vazyme biotech, Nanjing, China) according to the manufacturer's instructions and stored at -20 °C until used for quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR amplification was performed using

the SYBR Premix Ex Taq™ kit (Takara) in a 20 mL reaction containing 0.4 mL of each primer, 0.4 mL SYBR Green Dye and 2 mL of cDNA. The primer sequences are as follows: IL-1 β , TGTGATGTTCCATTAGAC, reverse primer AATACCACTTGTGGCTTA; TGF- β 1, forward primer CCCACTGATACGCCTGAG, reverse primer GACTGATCCATTGATTTCC; α -SMA, reverse primer CATCCGACCTTGCTAACG, reverse primer TCCAGAGTCCAGCACAATAC; COL1A1, forward primer ATGCCATCAAGGTCTACTGC, reverse primer AATCCATCGGTCATGCTCT; COL3A1, forward primer CCACCCTGAACTCAAGAGC, reverse primer TGAAGTCAAAGCCACCATT; β -actin, forward primer CACCCGCGAGTACAACCTTC, forward primer CCCATACCCACCATCACACC. qRT-PCR was carried out on a Roche Light Cycler 480II with the following program: 95°C for 30 sec followed by 40 cycles of 95 °C for 5 sec and 58°C for 34 sec. The relative gene expression level was calculated with 2-DDCt method using β -actin as an internal control.

Statistical Analysis

All data are presented as mean \pm standard deviation (SD). Statistical analyses were performed using SPSS software (version 25.0; IBM, Armonk, NY, USA) and all figures were created using GraphPad Prism 8.0 (GraphPad Software, Inc.). We performed comparisons using one-way analysis of variance (ANOVA) and Tukey's post-hoc test to determine significance between groups. For all tests, probability (p) values < 0.05 were considered statistically significant and marked as *.

Results

1. TGF- β activity is elevated after immobilization and remobilization

To demonstrate the pathogenesis of immobilization-induced joint contracture, the activity of interleukin 1 β (IL-1 β) and TGF- β 1 was examined throughout the entire process of joint contracture after immobilization and remobilization. As shown in Fig. 2a, one week after traumatic immobilization, the inflammatory response was triggered with a marked increase in IL-1 β expression. The level of TGF- β 1 was also significantly increased compared with the sham group at 1 week (Fig. 2b). The concentration of IL-1 β and TGF- β 1 gradually decreased 3 weeks later and remained stable until 6 weeks, but still maintained a higher level of expression than that in the sham group. Three weeks after the removal of K-wire, due to the constant stimulation of free movement to the traumatic femoral condyles, the activity of IL-1 β and TGF- β 1 both distinctly increased again in the rats who were remobilized, however, statistically significant differences were not found between immobilized and remobilized group (Fig. 2c,d).

H&E staining of the joint tissue showed altered joint architecture including swelling, inflammatory cell infiltration, thickening of the joint capsule, and contraction of the free joint space, with a prolonged fixation duration 3 weeks after post-traumatic immobilization (Fig. 3a). Masson trichrome staining is commonly used to evaluate fibrotic lesions as it characteristically reveals collagen fibers. In this experiment, the density of collagen fibers in the joint capsules were increased 3 weeks after immobilization and showed a statistically significant difference after 9 weeks (Fig. 3b, c). The length of

the synovium intima was remarkably reduced relative to sham-operated joints 1 week after surgery (Fig. 3d). The sham group had a thin posterior joint capsule, while the joint capsule was dramatically thickened in immobilized rats 3 weeks after surgery (Fig. 3e).

Immunohistochemistry assays and gene expression analysis demonstrated that, along with the accumulation of IL-1 β -positive inflammatory cells and expression of *IL-1 β* gene (Fig. 4a, b, and c), the number of cells containing phosphorylated Smad2/3-positive (p-Smad2/3⁺), a pivotal downstream signaling transducer of the TGF- β pathway, and the expression *TGF- β 1* gene were both significantly increased 3 weeks after immobilization (Fig. 4d, e, and f). Additionally, expression of α -SMA, a myofibroblast marker, and collagen 1 and 3 revealed that the number of myofibroblasts and collagenous fibers in the joint capsule was significantly increased relative to the sham group 3 weeks after immobilization (Fig. 4g-i, j-l, and m-o). Taken together, our results reveal that following the heightened immune activity and fibrillogenesis during post-traumatic joint immobilization, the activity of TGF- β increases and may play a key role in the progression of joint contracture.

2. Suppression of TGF- β activity increases ROM in immobilization-induced joint contracture

After 1 week of immobilization, the extension ROM of the joints was $108 \pm 4^\circ$, which was significantly smaller than that in the sham group ($160 \pm 3^\circ$) (Fig. 5a). ROM decreased during immobilization in a time-dependent manner and reached $30 \pm 4^\circ$ on week 6, remaining constant afterward (Fig. 5a). Extension ROM was approximately 160° for the contralateral joint of rats (Fig. 5b). As expected, there was no significant change in the ROM of the contralateral joint across different periods (Fig. 5b), which is consistent with previous findings that the contralateral joint is a suitable control for ROM experiments [17, 20]. As described above, after 6 weeks of immobilization, the K-wire was removed and the knee joint was remobilized freely for 3 weeks. Following remobilization, the joint extension ROM was partially recovered compared to the immobilized group ($69 \pm 12^\circ$ vs $30 \pm 4^\circ$) (Fig. 5c), indicating that active motion could moderately increase the limited joint ROM.

We next examined whether inhibition of TGF- β activity mitigates the formation of joint contracture. A specific TGF- β receptor I inhibitor (LY2157299) was injected daily from the day of immobilization or remobilization (Fig. 1b, c). With the administration of LY2157299 during the 6 weeks of joint immobilization, the ROM was significantly higher than in vehicle-treated rats ($73 \pm 12^\circ$ vs $32 \pm 5^\circ$) (Fig. 5c). To study the inhibitory effects of LY2157299 during joint remobilization, we performed the experiment as explained in Fig. 1c. The results showed that injection of LY2157299 during joint remobilization markedly increased ROM compared to vehicle-injected rats ($112 \pm 15^\circ$ vs $70 \pm 13^\circ$) (Fig. 5d). However, there was no significant difference in joint ROM in rats receiving LY2157299 during both immobilization and remobilization phases compared to those treated during only remobilization (Fig. 5d). Altogether, our data indicate that inhibition of TGF- β activity during immobilization or remobilization stages recovers the restricted ROM in joint contracture. In addition, the ROM of the stiff joint is improved enormously after the administration of LY2157299 combined with active motion.

3. Suppression of TGF- β activity mitigates the progression of immobilization-induced joint contracture

To evaluate the effect of signaling pathways in the progression of joint contracture, immunohistochemistry staining and gene expression analysis were conducted with joint samples and the levels of IL-1 β and TGF- β in serum were tested by ELISA. Histologically, H&E staining showed that, compared with the vehicle-treated group, the joint capsule tissue in rats treated with LY2157299 was thinner and the free joint space became larger after remobilization (Fig. 6a, b). The Masson's trichome-stained sections showed that the collagen deposits tended to be alleviated in the remobilized joint capsules of the LY2157299 group relative to the vehicle group (Fig. 6c, d). However, there was no significant difference in the length of synovium intima, levels of serum IL-1 β and TGF- β 1 between the LY2157299-treated group and vehicle-treated group, albeit IL-1 β and TGF- β 1 were distinctly reduced (Fig. 6e, f, and g). Immunostaining showed that the number of IL-1 β ⁺ inflammatory cells and p-Smad2/3⁺ cells in the articular capsule tissue in rats treated with LY2157299 were significantly reduced relative to vehicle-treated rats after 6 weeks of immobilization and 3 weeks of remobilization, respectively (Fig. 7a, b, c, and d), demonstrating that LY2157299 also has anti-inflammatory action. Similar results were obtained in regards to α -SMA and collagen 1 and 3 (Fig. 7e-j). Furthermore, the gene expressions of *IL-1 β* , *TGF- β 1*, *α -SMA*, *COL-1A1*, and *COL-3A1* in joint capsules were all remarkably decreased in the LY2157299-treated groups (Fig. 7k-o). Collectively, these results demonstrate that inhibition of TGF- β signaling via the Smad2/3 pathway attenuates the progression of joint contracture, especially during the remobilization stage.

Discussion

Joint contracture is a common complication of joint immobilization. Once joint contracture is formed, functional incapacitation may occur, including restricted joint ROM, amyotrophy of muscles around the joint, and the development of an abnormal gait [21, 22]. It is pivotal to understand the pathomechanisms of immobilization-induced joint contracture to develop more effective therapeutic schedules. In this study, our data revealed the mechanism of joint contracture progression as excessive activation of TGF- β coupling with an inflammatory reaction to trauma. Systemic injection of an inhibitor targeting the TGF- β signal transduction pathway effectively attenuated the formation of joint contracture in multiple stages of joint immobilization and remobilization.

It has become evident in recent years that facilitating joint movement is effective in improving immobilization-induced joint contracture. Therefore, passive joint movements, such as stretching, are widely accepted in clinical practice following joint injury. However, it is still controversial whether passive movements are effective in the rehabilitation process of joint contracture [23, 24]. In contrast to passive joint movement, recent research showed that spontaneous active movement of joints during remobilization may have beneficial effects on immobilization-induced joint contracture [25, 26].

Based on the primary changes in muscular and articular components, joint contracture is divided into myogenic and arthrogenic subtypes [4]. In the early phase of joint immobilization, the myogenic limitation of joint contracture is dominant and can be reversed by active movement. However, after long-term immobilization, arthrogenic pathogenesis occurs and is often irreversible even with active movement. Thus, the formation of arthrogenic contracture is extremely serious. These conclusions are consistent with our observations: after the removal of the fixation device for 3 weeks with active movement, a normal ROM was restored, but only for the segment with myogenic contracture. Furthermore, the ROM was unexpectedly eased to a wider range when rats were administrated with an inhibitor of TGF- β activity paired with remobilization through active movement, compared to active movement alone, indicating that the TGF- β signaling pathway specifically targets the aggravation of arthrogenic joint contracture.

TGF- β s are involved in all multicellular organisms and maintain tissue homeostasis through growth regulation, cell migration, differentiation, epithelial-mesenchymal transition, extracellular matrix remodeling, and immune reactions. However, aberrant overproduction of TGF- β ligands has been identified in diseases such as cancer, fibrosis, and inflammation where hyperactive TGF- β drives disease progression by modulating cell growth, migration, or various phenotypes [27–29]. Our results showed that TGF- β was aberrantly expressed following joint immobilization, promoting an inflammatory reaction. Limited ROM of the joint, thickness of the synovium, and deposition of collagen were all dramatically improved by blocking the TGF- β signaling pathway. TGF- β has been widely documented to increase collagen synthesis and deposition by accumulating fibroblasts which secrete excessive amounts of extracellular matrix proteins. Thus, TGF- β has become a popular therapeutic target for fibrosis [30]. Overexpression of TGF- β 1 *in vivo* through adenovirus delivery in the knee joints induces arthrofibrosis with the upregulation of the collagen genes [31]. Moreover, similar to our findings, inhibition of the TGF- β 1 pathway alleviates fibrous adhesion and arthrofibrosis induced by intra-articular injury or arthritis [14, 32].

LY2157299 (also called galunisertib), a small molecule inhibitor of the serine/threonine kinase of the TGF- β receptor I, was shown to inhibit the phosphorylation of Smad2/3, pivotal transducers of TGF- β downstream signaling [33]. In recent years, there has been a growing interest in targeting the TGF- β pathway using LY2157299 for its anti-tumor effect in various preclinical and clinical studies. LY2157299 is a potent inhibitor of both canonical and non-canonical TGF- β pathways in a variety of hepatocellular carcinoma cells *in vitro*, and reverse E-cadherin secretion and the epithelial-mesenchymal transition, which are linked to tumor invasion and metastasis [34–36]. In a model of taurocholate-induced acute pancreatitis in rats, LY2157299 attenuated the severity of the disease by inhibiting TGF- β signaling and the secretion of pro-inflammatory cytokines [37]. It has also shown promising results in clinical trials due to its acceptable safety profile. It produced a prolonged overall survival outcome in phase 1/2 trials for patients with hepatocellular carcinoma, myelodysplastic syndrome, and other neoplastic diseases [38–42].

Many studies have found that the NF- κ B pathway, which plays a crucial role in the pro-inflammatory response, is positively regulated by TGF- β [43, 44]. Therefore, targeting the TGF- β signal could result in NF- κ B inactivation, and might be an effective treatment for reducing inflammation. In the present study,

we verified the anti-fibrosis and anti-inflammatory effects of LY2157299 and demonstrated that, when combined with remobilization, it has synergistic effects on suppressing the progression of immobilization-induced contracture in rats; it mediates this action through interfering with downstream signaling via inhibition of Smad phosphorylation. Employment of LY2157299 to block TGF- β signaling appears to modulate fibrosis and has a meaningful preclinical benefit for joint contracture in rats. The measurement of TGF- β levels may be valuable in the clinic for identifying individuals at risk for post-traumatic joint contracture, as the severity of joint contracture was positively correlated with TGF- β levels in serum.

This study has some limitations. In the experiments assessing arthrogenic joint contracture during remobilization, TGF- β levels and effect of LY2157299 were only measured at one time point (3 weeks). The dynamic changes of TGF- β and other inflammatory and fibrosis-related factors that give rise to contracture during active movement is unclear. The optimal dose and any potential side effects on normal physiological conditions in response to LY2157299 treatment remain need to be elucidated. Altogether, our results provide a strong rationale for the involvement of TGF- β in joint contracture and highlight preclinical data of LY2157299 for its treatment. Therefore, the inhibition of TGF- β signaling paired with active remobilization could be a potential therapeutic strategy to mitigate the progression of joint contracture in human patients.

Abbreviations

TGF- β : Transforming growth factor- β ; IL-1 β : Interleukin-1 β ; α -SMA: α -smooth muscle actin; Col 1: Collagen types I; Col 3: Collagen types III; ROM: Range of motion; ELISA: Enzyme-linked immunosorbent assay

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

This study was approved by the Wuxi 9th People's Hospital Institutional Review Board (no. KT2019019).

Consent for publication

All the authors agreed to publish this study in this journal.

Competing interests

The authors declare that there are no conflicts of interest.

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Author Contributions

Dong Mao and Fengfeng Li designed the experiments. Dong Mao performed most of the experiments, analyzed the results, and prepared the manuscript. Jingyi Mi and Xiaoyun Pan helped to collect the samples and did the statistical analyses. Fengfeng Li and Yongjun Rui supervised the experiments. All authors approved the final version to be published.

Acknowledgments

None

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Figures

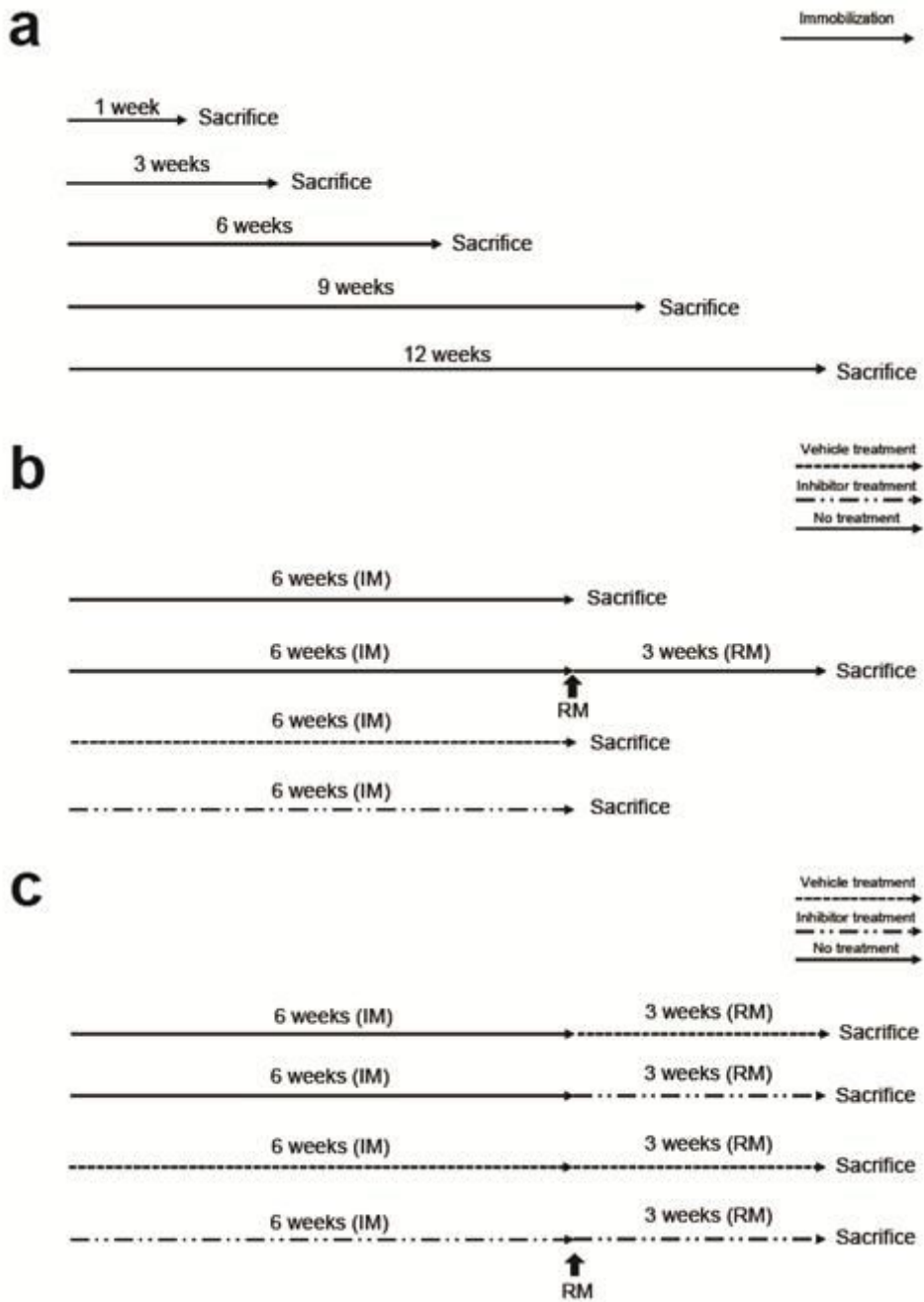


Figure 1

Schematic of experimental protocols for (a) measuring TGF- β 1 levels in rat serum following immobilization at five time points, (b) investigating the effects of active movement and LY2157299 on joint contracture during remobilization and immobilization, respectively, and (c) investigating the effects of active motion combined with LY2157299 treatment on joint contracture. IM, immobilization; RM, remobilization; inhibitor, LY2157299.

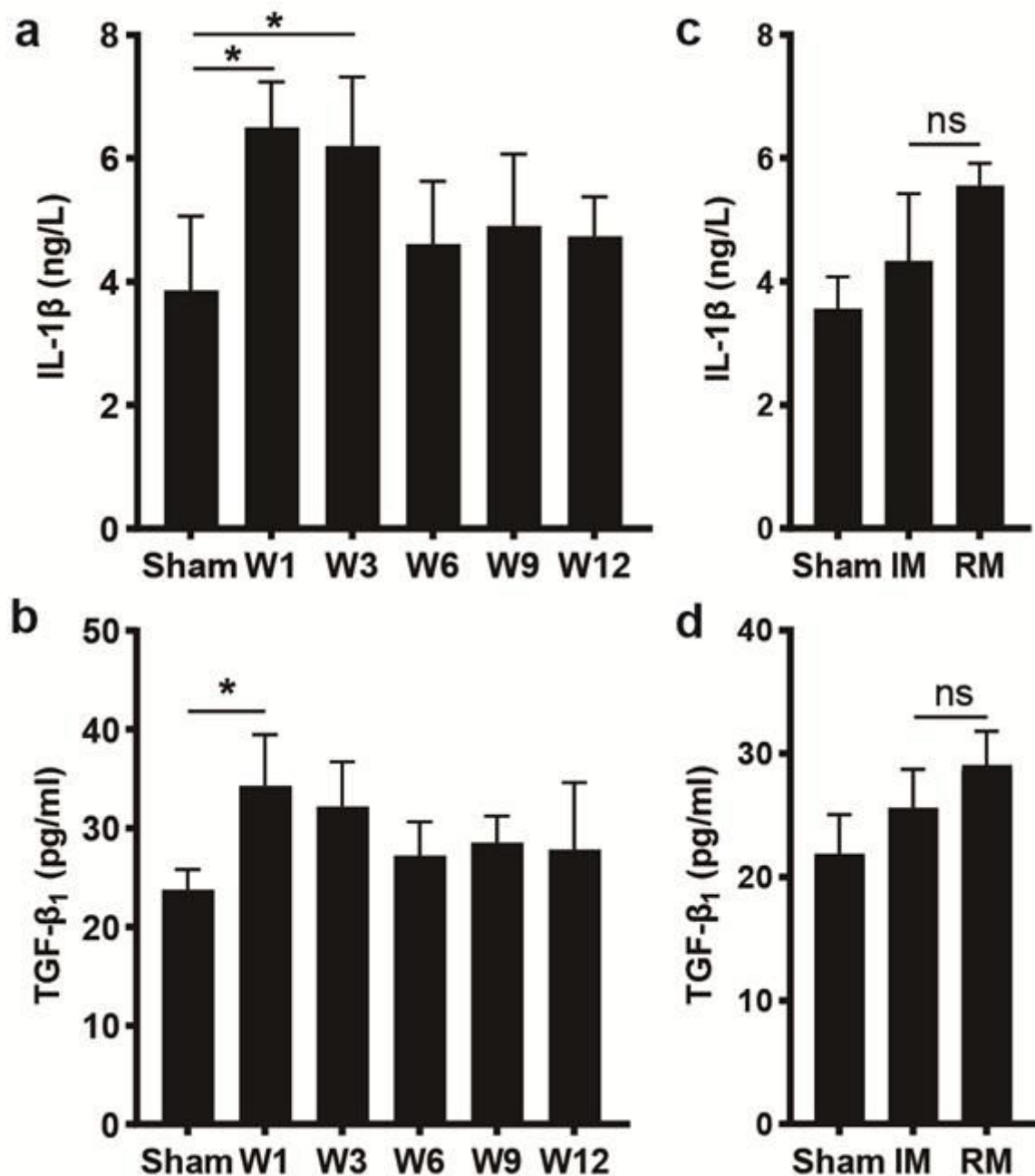


Figure 2

Quantitative analysis of IL-1 β and TGF- β 1 in rat serum using ELISA. Levels of IL-1 β (a) and TGF- β 1 (b) were significantly elevated after one week of immobilization and then gradually decreased. Levels of IL-1 β (c) and TGF- β 1 (d) were mildly increased again after three weeks of remobilization compared with six weeks of immobilization. IM, immobilization; RM, remobilization; ns, not significant. n = 5 per group. All data are presented as mean \pm SD. * P < 0.05

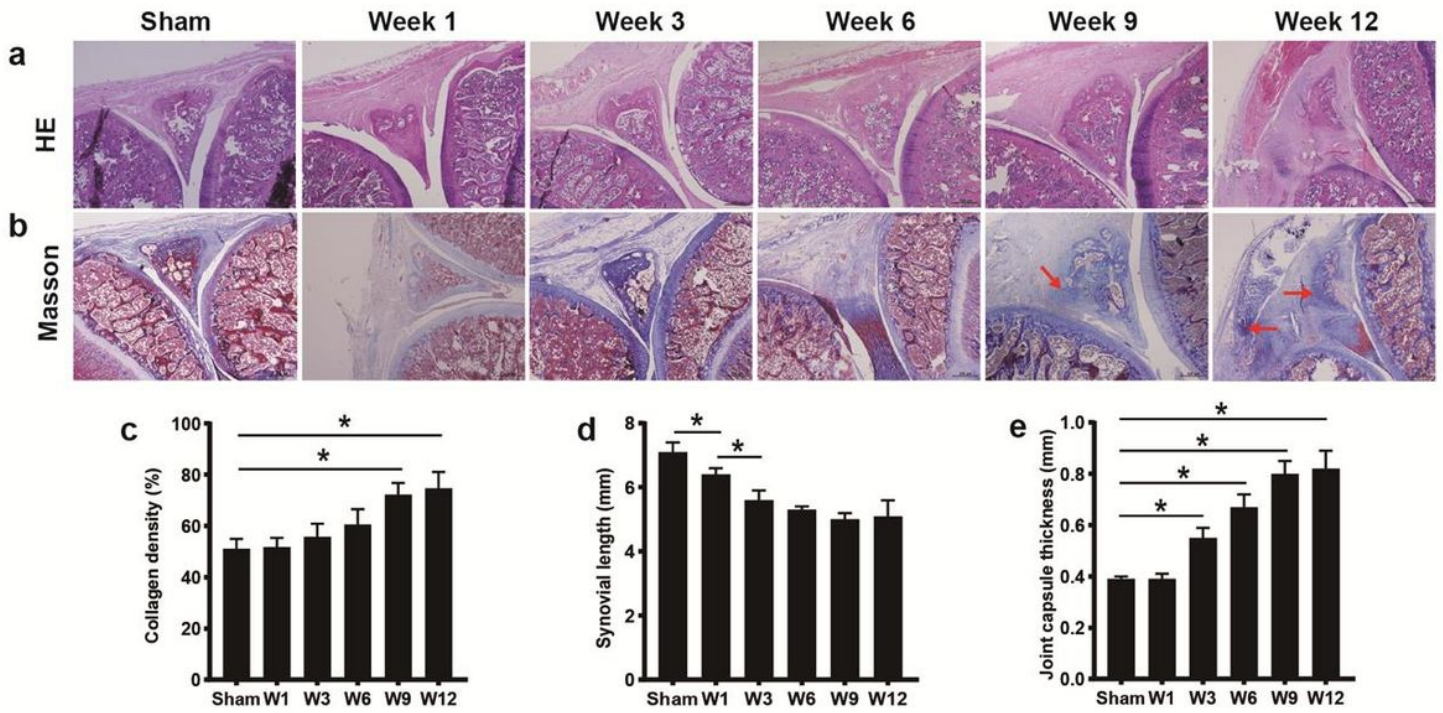


Figure 3

H&E (a) and Masson's trichome (b) staining of knees were performed at different stages to observe the progression of joint contracture. Scale bars: 250 μ m. Red arrowheads point to collagen deposition. (c) Collagen density was gradually increased during immobilization. (d) The total synovial length was markedly reduced after 3 weeks of immobilization. (e) Joint capsule thickness was significantly increased after 3 weeks of immobilization. $n = 5$ per group. All data are presented as mean \pm SD. * $P < 0.05$.

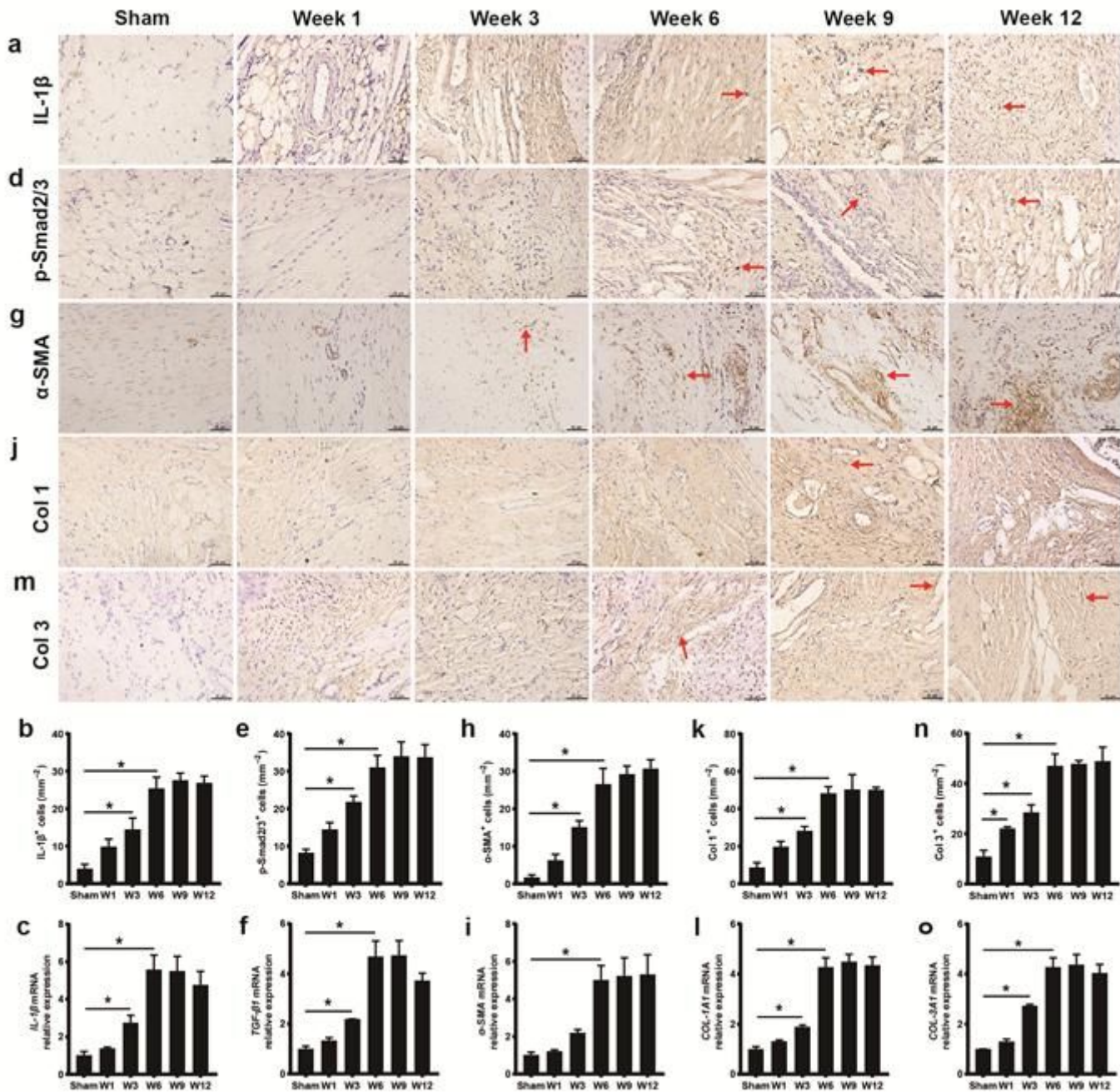


Figure 4

Elevated TGF- β levels are associated with increased fibrillogenesis in the formation of joint contracture. Immunohistochemical staining and quantification of (a, b) IL-1 β ⁺ and (d, e) p-Smad2/3⁺ cells after sham operation or joint immobilization. (g, j, m) α -SMA⁺, Col 1⁺, and Col 3⁺ cells in the joint capsules of sham or immobilized rats and (h, k, n) their quantification. Scale bars: 25 μ m. Red arrowheads point to positive cells. (c, f, i, l, o) Relative expression of IL-1 β , TGF- β 1, α -SMA, COL-1A1, and COL-3A1 in posterior joint capsules at different stages of immobilization. n = 5 per group. All data are presented as mean \pm SD. * P < 0.05.

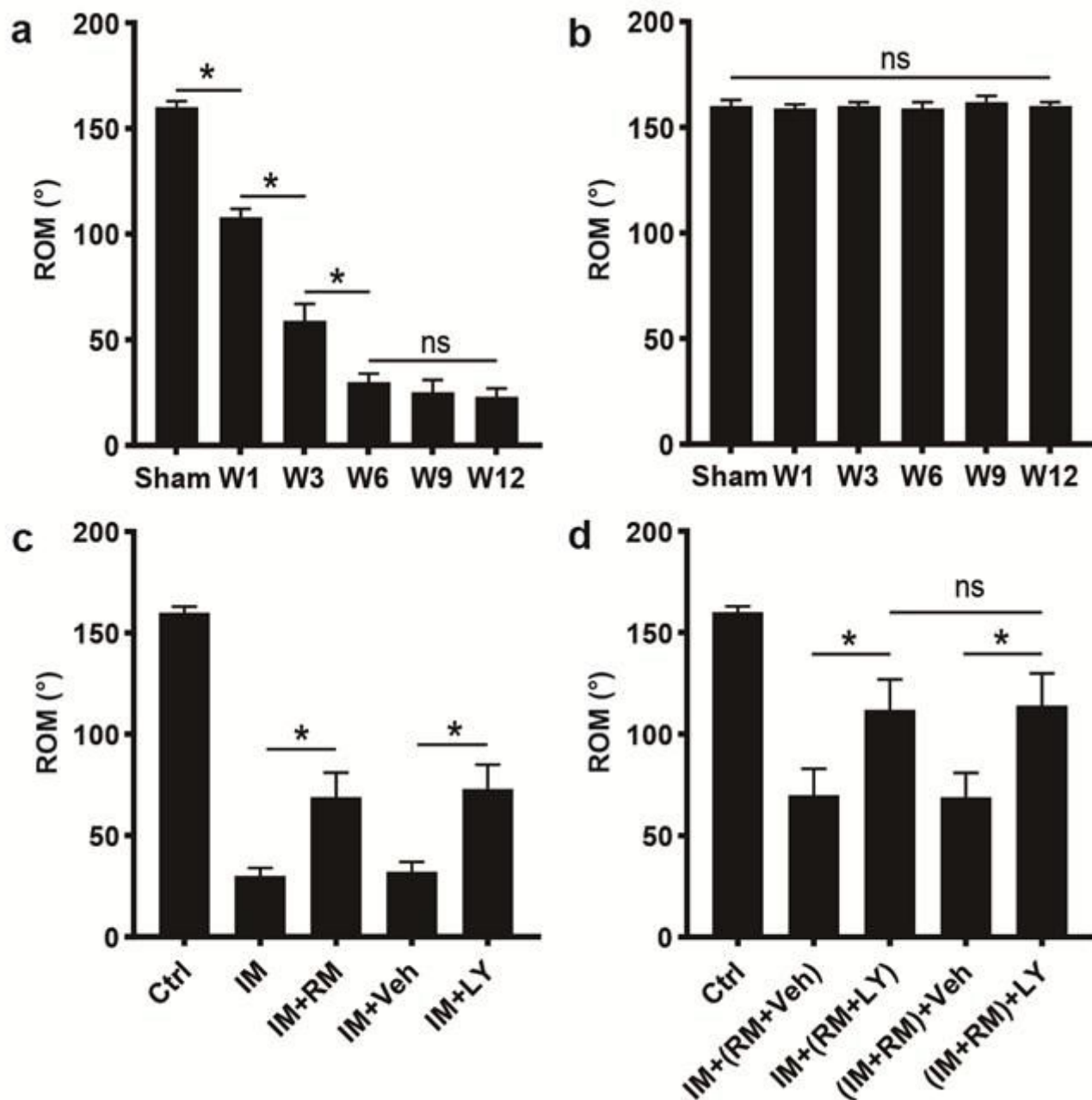


Figure 5

Systemic injection of TGF- β receptor I inhibitor (LY2157299) recovers restricted ROM in immobilization-induced joint contracture. (a) The ROM of the immobilized knee joint was gradually decreased with time of immobilization. (b) No difference in ROM was seen over time in contralateral knees from immobilized rats and two knees from sham-operated rats. (c) ROM was partly increased after 3 weeks of remobilization or 6 weeks of immobilization with LY2157299 treatment. (d) ROM was recovered when rats were treated with LY2157299 combined with remobilization. Ctrl, contralateral knees from immobilized rats; IM, immobilization; RM, remobilization; LY, LY2157299 treatment; Veh, vehicle treatment; IM + (RM + LY), rats were treated with LY2157299 only during remobilization (3 weeks) after 6 weeks of immobilization. (IM + RM) + LY, rats were treated with LY2157299 throughout the period of

immobilization (6 weeks) and remobilization (3 weeks). ns, not significant. n = 5 per group. All data are presented as mean \pm SD. * P < 0.05.

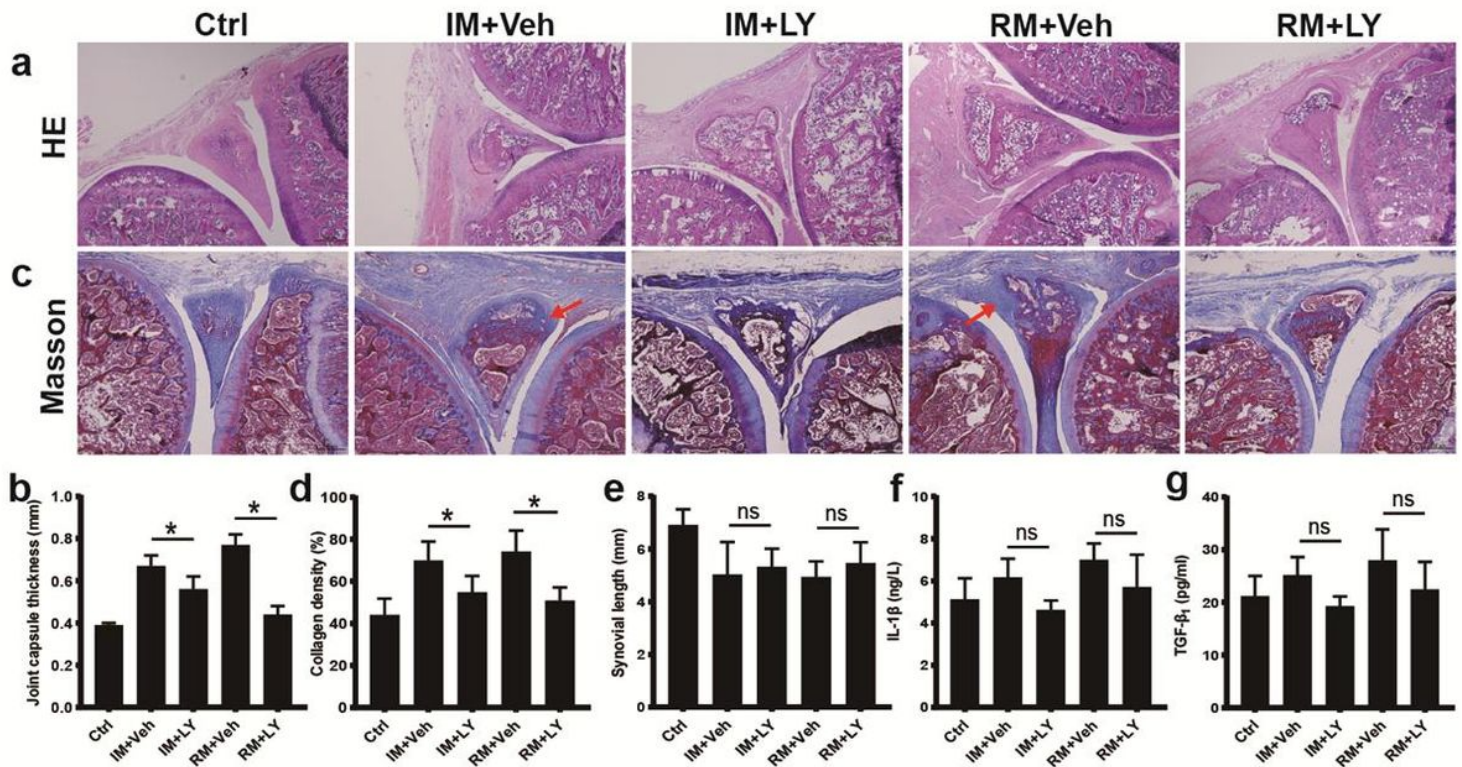


Figure 6

Systemic injection of TGF- β receptor I inhibitor (LY2157299) attenuates the incassation of the joint capsule and deposition of collagen. H&E (a) and Masson's trichome (c) staining of knees were performed to observe the alleviation of joint contracture in the rats treated with LY2157299. Scale bars: 250 μ m. Red arrowheads point to collagen deposition. (b, d, e) Joint capsule thickness, collagen density, and synovial length were measured after treatment with vehicle or LY2157299. Quantitative analysis of IL-1 β (f) and TGF- β 1 (g) in rat serum using ELISA. IM, immobilization; RM, remobilization; LY, LY2157299 treatment; Veh, vehicle treatment; Ctrl, contralateral knees from immobilized rats; IM + LY, rats were treated with LY2157299 during the 6 weeks of immobilization. RM + LY, rats were treated with LY2157299 during the 3 weeks of remobilization. ns, not significant. n = 5 per group. All data are presented as mean \pm SD. * P < 0.05.

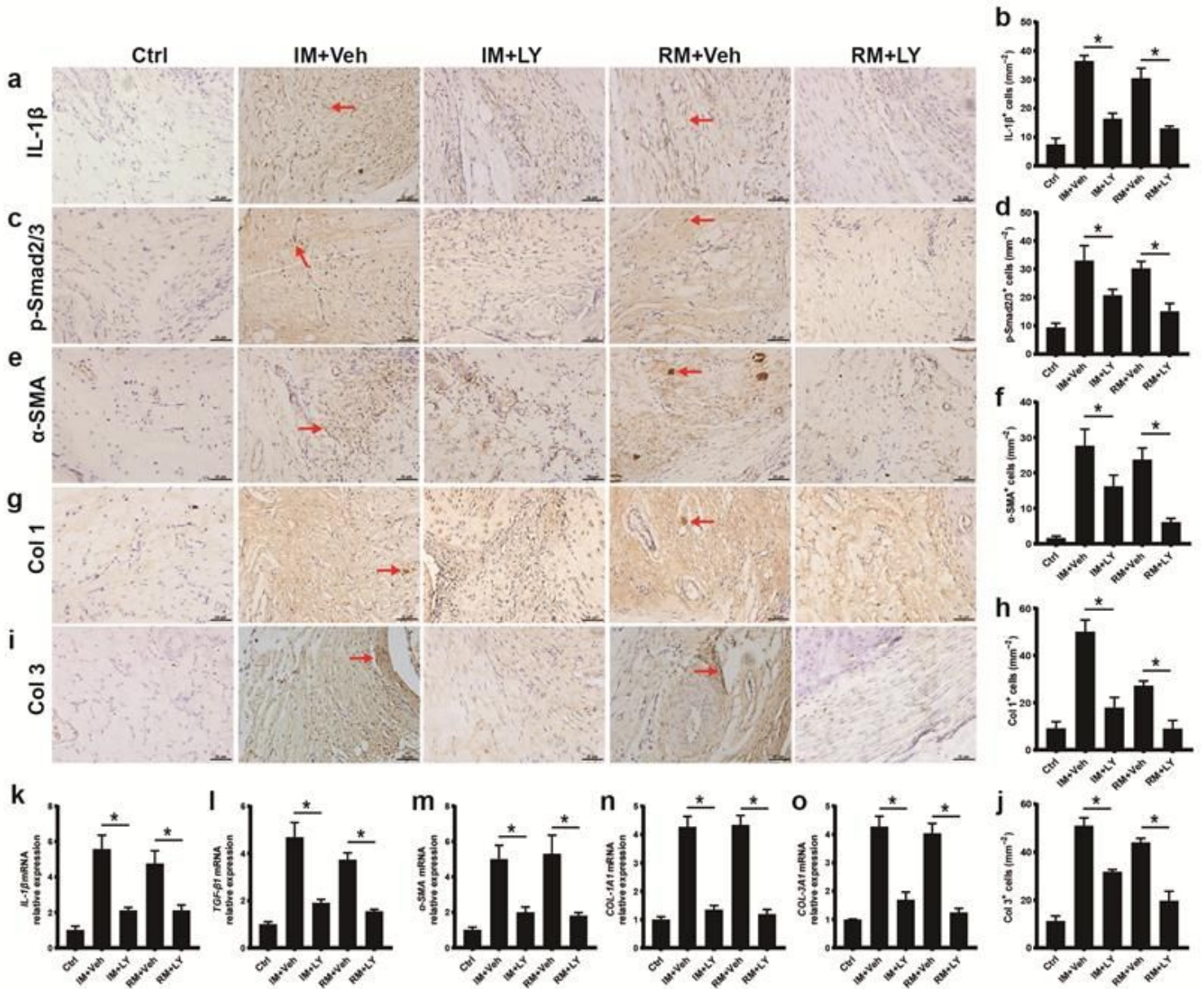


Figure 7

Systemic injection of TGF- β receptor I inhibitor (LY2157299) down-regulates the expression of inflammatory and fibrosis-related factors. (a, c, e, g, i) Immunostaining and (b, d, f, h, j) quantification of IL-1 β ⁺, p-Smad2/3⁺, α -SMA⁺, Col 1⁺, and Col 3⁺ cells in the joint capsule of knees. Scale bars: 25 μ m. Red arrowheads point to positive cells. (k, l, m, n, o) Relative expression of IL-1 β , TGF- β 1, α -SMA, COL-1A1, and COL-3A1 in joint capsules in LY2157299 or vehicle-treated group. IM, immobilization; RM, remobilization; LY, LY2157299 treatment; Veh, vehicle treatment; Ctrl, contralateral knees from immobilized rats; IM + LY, rats were treated with LY2157299 during the 6 weeks of immobilization. RM + LY, rats were treated with LY2157299 during the 3 weeks of remobilization. n = 5 per group. All data are presented as mean \pm SD. * P < 0.05.