Anti-Arthritic Effect of TLR4 Inhibitor TAK-242 Through the Inhibition of Th1 and Th17 Cell Proliferation in a Curdlan-Induced Spondyloarthritis SKG Mouse Model

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Abstract

Background: Toll-like receptor 4 (TLR4) is involved in the pathogenesis of arthritic diseases. The TLR4 inhibitor TAK-242 has shown potential as a possible treatment for rheumatoid arthritis in an adjuvant-induced arthritis rat model. However, further investigation of the anti-arthritis effect of TAK-242 in other animal models of arthritis disease is required.

Objective: This study was conducted to examine the anti-arthritic effects and mechanisms of TAK-242 on curdlan-induced spondyloarthritis in SKG mice.

Methods: Curdlan-injected SKG ZAP-70^{W163C} mice were intraperitoneally administered with TAK-242 (3 mg/kg) every other day or anti-IL-23 antibody (100 μg) weekly for 9 weeks. The therapeutic effect of TAK-242 was evaluated in the SKG mice by measuring the inflammation level of foot, ileum, lung and tail by histology, the levels of proinflammatory cytokines in serum, bone mineral density (BMD) in femur and spine, and CD4+T cell subtypes Th1 and Th17 in spleen.

Results: TAK-242 treatment significantly reduced clinical score, paw width, and colon length in SKG mice. Consistent with the therapeutic effect, TAK-242 also reduced peripheral arthritis and IL-17 expression in the foot and ileum tissues. It also inhibited inflammation of the lungs, ileum, paws, and tail and reduced serum levels of pro-inflammatory cytokines such as IL-6 and TNF-α. TAK-242 recovered the reduced bone density in the femur but not in the spine. Treatment with TAK-242 also suppressed the populations of Th1 and Th17 cells in the spleen of SKG mice.

Conclusions: TAK-242 may show beneficial anti-arthritis effects by inhibiting the proliferation of pro-inflammatory Th1 and Th17 cells. Further research is needed for TAK-242 to be repositioned as a treatment for arthritis disease.

Introduction

Chronic inflammation in autoimmune diseases such as spinal arthritis (SpA) and rheumatoid arthritis (RA) causes damage to tissues and cells, resulting in the release of molecules such as damage associated molecular patterns (DAMPs) into nearby tissues. DAMPs further activate the inflammatory response through the signaling pathway regulated by toll-like receptors (TLRs) that are expressed in tissues or immune cells [1]. TLRs are expressed in most cells, which play an important role as the front line of innate immunity. Ten TLRs have been identified in humans, and the roles of TLR1–9 have been determined [2]. TLR4 triggers immune responses by recognizing invading organisms as pathogen-associated molecular patterns (PAMPs) [3]. TLR4 also recognizes various molecules, such as RNA or DNA released from damaged cells, to initiate immune responses as DAMPs. All the TLR-mediated inflammatory responses remove harmful stimuli or repair damaged tissues. However, under chronic inflammatory conditions, persistent innate damage signals could lead to harmful inflammation, resulting

in more destruction than the original injury [4]. Thus, inappropriate TLR activation has been suggested to be involved in the chronic and persistent inflammation of RA [5]. In our previous study, we found that the TLR4 inhibitor TAK-242 (resatorvid) significantly ameliorated inflammatory symptoms in joint tissues in an adjuvant-induced arthritis (AIA) rat model [6]. Although TAK-242 was initially developed for sepsis treatment [7], it has the potential to be applied for treatment of other inflammatory or non-inflammatory diseases such as cancer and hyperalgesia through drug repositioning [8–10].

Spondyloarthritis (SpA) refers to several clinical symptoms characterized as psoriatic arthritis, enteropathic arthritis, reactive arthritis, a subgroup of juvenile idiopathic arthritis, and ankylosing spondylitis (the prototypic and best studied subtype) [11]. The disease subtypes all show joint inflammation (peripheral and axial skeleton); skin, gut and eye manifestations; and the absence of diagnostic autoantibodies (seronegative) [12]. The pathogenesis of SpA also involves genetic polymorphisms and overexpression of TLRs. For example, TLR4 gene polymorphism may be related to the development of psoriatic arthritis [13]. TLR4 expression was increased in patients with ankylosing spondylitis [14]. The IL-23/IL-17 immune axis also plays an important role in the pathogenesis of SpA. Thus, IL-23 and IL-17 cytokines have been used as therapeutic targets by many researchers [15]. Several therapeutic antibodies against IL-23 and IL-17 such as ustekinumab, secukinumab, ixekizumab and brodalumal have been clinically used for the treatment of SpA patients [16–18] and for the ankylosing spondylitis and/or psoriatic arthritis subforms of SpA, with promising clinical efficacy.

The SKG mouse has a mutation in the gene encoding the SH2 domain of ZAP-70, a key signal transduction molecule in T cells. The mutation changes the thresholds of T cells to thymic selection, leading to the positive selection of otherwise negatively selected autoimmune T cells [19]. Curdlan is a water-insoluble linear β-1,3-glucan, a high-molecular-weight polymer of glucose. The injection of curdlan into the SKG mouse results in the development of SpA-like symptoms, including chronic inflammation of the peripheral joint, spine, skin, ileum and colon. These features resemble the characteristics of human SpA [20]. The mechanisms by which curdlan induces arthritis drives preferential differentiation and expansion of Th17 cells or dendritic cells (DCs) through TLR and dectin-1 [21, 22].

In this study, we examined whether TAK-242, a TLR4 inhibitor, has an anti-arthritic effect in the curdlan-induced spondyloarthritis SKG mouse model [23]. We showed that TAK-242 suppressed SpA in curdlan-injected SKG mice through inhibition of the proliferation of Th1 and Th17 cells. These findings suggest that TAK-242 has the potential to be developed as a treatment for SpA.

Materials And Methods

Induction of SpA in SKG mice and TAK-242 treatment

Female SKG mice were obtained from CLEA-Japan, Inc. The mice were maintained under specific pathogen-free conditions. SpA was induced in mice between 8 and 10 weeks of age by intraperitoneally (I.P) injecting 3 mg curdlan (Wako Chemicals, Osaka, Japan). Mice received either anti-IL-23 antibody or

TAK-242 injection. Mice were I.P injected with 100 μg of anti-mouse anti-IL-23(P19) monoclonal antibody (BioXcell, Seoul, Korea) 1 day before curdlan injection and weekly until mice were killed. In another group of mice, TAK-242 (3 mg/kg) was given I.P 1 day before curdlan injection and three times per week. All mice were handled according to protocols that were approved by the Committee on Animals of Kyung Hee University Hospital at Gangdong.

Clinical and histologic scoring

Clinical scores were monitored weekly and scored as follows: 0, no joint swelling or redness of finger, wrist and ankle joint; 0.1, swelling or redness of one finger joint; 0.5, mild swelling or redness of wrist and ankle; and 1.0, severe swelling or redness of the wrist and ankle. Mice were monitored by the same observer who was blinded with regard to treatment. Calipers were used to measure the wrist and ankle width. Scores for the affected joints of 4 legs were summed; the maximum score was 6. The hematoxylin and eosin (H&E)–stained sections of the ileum, tail, lung and peripheral joint were scored as described previously [24]: 0 = no inflammation, 1 = few infiltrating immune cells, 2 = 1–2 small patches inflammation, 3 = inflammation and reduced joint space throughout the ankle joint, and 4 = inflammation in > 70% of the tissue.

Histology

At the experimental endpoint of 9 weeks, the peripheral joint, lung, tail and ileum from control and treated mice were fixed in 10% formalin (Sigma-Aldrich, St. Louis, MO, USA) and embedded in paraffin. Tissue specimens of joint and ileum were sectioned at 7 μm and 4 μm thickness, respectively. The sections were dewaxed using xylene (Duksan General Science, Seoul, Korea), dehydrated using an alcohol gradient, and stained with H&E (Cancer Diagnostics Inc. Durham, NC, USA). For immunohistochemistry, sections were deparaffinized, hydrated and incubated in 20 μg/mL proteinase K (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37℃. Endogenous peroxidases were quenched in 3% hydrogen peroxide solution (Qiagen, Hilden, Germany) in methanol for 10 min. After washing with phosphate buffered saline (PBS), permeabilization with 0.4% Triton X-100 in PBS for 10 min and blocking with 1% bovine serum albumin (BSA) for 1 h at room temperature, the slides were incubated with primary antibody against IL-17A (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a 1:100 dilution for overnight at 4℃. Sections were then washed with PBS and incubated with horse anti-mouse/rabbit IgG antibody (H+L) (Universal, biotinylated, R.T.U [Vector Laboratories Inc., Burlingame, CA, USA]) for 30 min at room temperature. Next, 3,3'-diaminobenzidine peroxidase substrate solution was added, and dehydration, clearing and mounting was conducted by routine procedures. Samples were observed under a microscope and photographs were taken. Sections were analyzed, and inflammation was scored blindly by at least 3 independent researchers (CHM, HMD and LKM).

Micro-computed tomography (CT) imaging

The bodies of mice were stored in 4% formalin. The mice were washed twice with PBS and imaged at a resolution of 100 μm with a NanoSPECT/CT (Bioscan Inc., Santa Barbara, CA, USA). High resolution scans of the femurs of the mice were captured to evaluate the 3D structure. The BMDs of femur and spine were measured using the manufacturer's 3D analysis tools.

Cell preparation from spleen

Spleens were excised from killed mice and placed immediately in RPMI 1640 media. Using the plunger end of a syringe, the spleen was mashed through the cell strainer into a 50 ml tube. After centrifugation of the tube at 1,500 rpm, 4℃ for 5 min, the samples containing white blood cells were treated with Ammonium-Chloride-Potassium Lysing Buffer (BioLegend, San Diego, CA, USA) for the lysis of red blood cells. After centrifugation at 1500 rpm, 4℃ for 5 min, the lymphocytes were stained with antibodies for analysis of T cell subtypes.

Surface and Intracellular Staining and Flow Cytometry

IFN-γ-FITC, CD4-PE-Cy™5 (BD, Franklin Lakes, NJ, USA) and IL-17-PE/Cy7 antibodies (BioLegend, San Diego, CA, USA) were used for the analysis of T cell subtypes by fluorescence-activated cell sorter (FACS) analysis. For the analysis of Th1 and Th17 cells with intracellular cytokine staining, the cells were stimulated in culture medium containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1 ng/ml ionomycin (Sigma-Aldrich) and 1× golgistop (BD) for 4 h. The cells were treated with the intracellular fixation & permeabilization buffer set (BD) according to the manufacturer's protocol. The stimulated cells were treated with the transcription factor staining buffer set (eBioscience, San Diego, CA, USA). Cells were analyzed using a cytometric FC 500 flow cytometer (Beckman Coulter, Brea, CA, USA). Data were analyzed by Kaluza software.

Measurement of serum cytokines

Sera from mice were obtained from heart puncture. Komabiotech Co. (Seoul, Korea) analyzed the serum level of cytokines such as IL-17A, TNF-α, IL-6, IL-2, IL-10, IFN-γ and IL-1β using the Luminex® 200™ Total System (Luminex Corporation, Austin, TX, USA).

Statistical analysis

Experimental data are expressed as mean ± standard error of the mean (SEM). Differences between three groups were analyzed using the nonparametric Kruskal–Wallis test. If a statistical difference was detected ($P < 0.05$), post hoc pairwise group comparisons were performed using Dunn's test. Two-way analysis of the variance (ANOVA) was used to analyze TAK-242 effects on paw thickness and clinical score over time, following Turkey's post hoc test. Prism software v.5 (Graphpad Software, San Diego, CA,

USA) was used for statistical analysis and graphing. Differences were considered statistically significant at $P<0.05$.

Results

TAK-242 treatment prevented peripheral arthritis in SKG mice with SpA

To investigate whether the TLR4 inhibitor TAK-242 could ameliorate or prevent peripheral arthritic symptoms in the SpA mouse model, SKG mice were intraperitoneally injected with TAK-242 (3 mg/kg) one day before curdlan injection and then three times a week for 9 weeks. As shown in Fig. 1A and 1B, the clinical score and ankle width of hind leg was significantly reduced by TAK-242. However, the inhibitory effect of TAK-242 treatment was not stronger than that of anti-IL-23 Ab treatment. The inhibitory effect of TAK-242 and anti-IL-23 Ab was almost same after 8 weeks of treatment, suggesting that TAK-242 did not show a strong inhibitory effect at the initial stage of inflammation but may be as effective as anti-IL-23 Ab treatment at the late stage. The colon length was shortened in SKG mice with curdlan-induced SpA (Fig. 1C). The shortened colon length was recovered after the treatment of anti-IL-23 Ab or TAK-242 for 9 weeks.

To further examine the anti-inflammatory effect of TAK-242, the inflammation level of foot, ileum, lung and tail was investigated with H&E staining and IL-17 immunostaining. The level of tissue inflammation was assessed by the level of invasive immune cells and IL-17 expression (Fig. 2). Treatment of anti-IL-23 Ab decreased the inflammation level. TAK-242 treatment also reduced tissue inflammation as much as anti-IL-23 Ab treatment. Consistent with the level of inflammation observed by H&E staining, the level of IL-17 staining was significantly reduced by anti-IL-23 Ab or TAK-242 treatment (Fig. 3A). Furthermore, serum TNF-α level was also slightly decreased by treatment of anti-IL-23 Ab. In contrast, IL-6 level was significantly reduced by TAK-242 treatment (Fig. 3B). These results suggest that TAK-242 treatment inhibited the peripheral arthritis of SKG mice with SpA as effectively as anti-IL-23 Ab.

TAK-242 treatment recovered the BMD loss in femurs in SKG mice with SpA

Next, we investigated the effect of TAK-242 on the bone health of SKG mice with SpA by micro-CT. The BMD was significantly decreased in femurs of SKG mice with curdlan-induced SpA (Fig. 4). Both anti-IL-23 Ab and TAK-242 significantly restored inflammation-mediated BMD loss in the femur but not in the spine. In particular, BMD was not decreased in the spine of curdlan-injected SKG mice. This result also indirectly suggests that the bone formation of ankylosing spondylitis is progressing in the spine, so this bone formation can mask the reduction of BMD in the spine.

TAK-242 treatment inhibited the proliferation of inflammatory T cells in spleen of SKG mice with SpA

The mice with SpA were sacrificed after 9 weeks of TAK-242 treatment. In the spleen, Th1 and Th17 cells of the CD4 + T cell subtype were analyzed by FACS. The population of Th1 and Th17 cells was

significantly increased in SKG mice with SpA. Both TAK-242 and anti-IL-23 antibody treatment significantly inhibited the increased cell count of Th1 and Th17 (Fig. 5). This suggests that the inhibition of Th1 and Th17 cell proliferation by TAK-242 treatment may partially or significantly contribute to the inhibition of peripheral arthritis in SKG mice.

Discussion

TAK-242, a small molecule inhibitor of TLR4 signaling was developed for the treatment of sepsis. TAK-242 binds selectively to TLR4 and interferes with the interactions between TLR4 and its adaptor molecules [25]. However, TAK-242 failed in phase III clinical human trials for severe sepsis [7]. Another TLR4 antagonist, eritoran, also failed in clinical studies for severe sepsis [26]. We previously showed that the TLR4 inhibitor TAK-242 (resatorvid) inhibited arthritic disease in an AIA rat model [6]. In this study, we tested the potential anti-arthritis effect of TAK-242 in a curdlan-injected SKG mouse model with activated and proliferating autoimmune T cells. As shown in Fig. 1, curdlan injection induces severe inflammation in foot and ileum. TAK-242 treatment significantly inhibited the inflammation, reduced the increased ankle and increased the reduced ileum length.

SpA is chronic inflammatory disease, and SpA patients commonly have chronic gut inflammation. The links between bowel and joints could be explained by immunological mechanisms in which activated T cells migrate from gut into synovium. The migrated immune cells secrete excess pro-inflammatory cytokines such as IL-17 and IL-23 [27, 28]. Thus, we examined the expression of IL-17 in foot and ileum by IL-17 immunostaining. The expression level of IL-17 in foot was decreased by the treatment of TAK-242 as well as anti-IL-23 antibody. However, the expression in ileum was not significantly inhibited by anti-IL-23 antibody, contrary to our expectations. The IL-23/IL-17 axis was shown to play important role in the pathogenesis of SpA through genetic, experimental and clinical studies [29]. IL-23 stimulates various cells to produce IL-17, which contributes to inflammation by increasing the production of inflammatory cytokines such as IL-6 and TNF-α. However, there is an inconsistent result against the hypothesis. For example, the IL-17A inhibitor ixekizumab effectively inhibited axial SpA. In contrast, inhibition of IL-12/IL-23p40 and IL-23p19 both failed to inhibit axial SpA but inhibited inflammatory bowel disease (IBD) which was associated with failed IL-17 inhibition in previous studies [30]. As shown in Fig. 3B, we examined cytokines such as IL-17A, TNF-α, IL-6, IL-2, IL-10, IFN-γ and IL-1β in serum. TAK-242 treatment also decreased the increased serum level of TNF-α and IL-6 in curdlan-induced inflammation. Only TNF-α and IL-6 were detectable in serum. Other cytokines may have altered levels of expression but were not measured.

As shown in Fig. 4, BMD was significantly decreased in femurs of SKG mice with curdlan-induced SpA. Inflammation may induce osteoporosis, as it does to many other chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, ankylosing spondylitis, systemic lupus erythematosus, multiple sclerosis, inflammatory bowel diseases, pemphigus vulgaris and others [31]. Bone loss is tightly related to immune system activation or inflamm-aging in the form of acute, chronic active or chronic blazing inflammation. Increased pro-inflammatory cytokines could stimulate osteoclastogenesis. Briefly, TNF-α increases the

expression of RANKL in osteoblasts, which induces osteoclast differentiation and stimulates bone resorbing activity [32]. TAK-242 treatment decreased systematic inflammation, which could contribute to inhibition of bone loss.

TLR activation is involved in the pathogenesis of arthritic diseases [1]. TL4 signaling in T cells seems to be important to induce autoimmune inflammation [33]. In particular, when TLR4 was knocked out in only CD4 + T cells, the disease symptoms were almost completely blocked. The suppression of disease symptoms was primarily through Th17 reduction, with fewer Th1 responses, suggesting that Th17 and Th1 cells are more important to induce autoimmune diseases than other cells. Likewise, activation of TLRs is involved in T cell development and differentiation. In particular, T cell receptor (TCR) activation in distinct T cell subsets with different TLRs could lead to different results. For example, TLR4 activation in regulatory T cells (Tregs) enhances the suppressive function of the cells, while TLR6 activation inhibits the suppressive function of Tregs [23]. In addition, TLR-2 and TLR-4 agonists induced significant cell proliferation and increased the production of IL-6, IL-17 and IL-21 from CD4 + T cells in neuromyelitis optica spectrum disorder patients, a severe humoral autoimmune disease of the central nervous system [24]. Thus, TLR4 signaling in T cells also plays an important role in promoting autoimmune inflammation through direct regulation of T cell activation and survival [25]. Therefore, TAK-242 treatment could inhibit the activated TLR4 signaling pathway of T cells in curdlan-induced inflammation. As shown in Fig. 5, TAK-242 treatment inhibited the increased level of Th1 and Th17 cells. The inhibition degree of TAK-242 was different from that of anti-IL-23 antibody treatment. Furthermore, TLR4 signaling is closely related to activation of STAT3 as a key event of TLR4 activation [34], which regulates the differentiation of CD4 + T cells into Th17 cells [35, 36]. Therefore, targeting the STAT3 signaling pathway could be a promising therapeutic strategy for immune diseases as well as numerous cancers [37, 38]. Novel STAT3 inhibitors are being developed [38, 39]. These inhibitors could be tested as a treatment for arthritic disease in the future.

Conclusion

We previously demonstrated that TAK-242 has an anti-arthritic effect in AIA rats through the inhibition of NF-κB and AP-1 activation. In this study, we further established the anti-arthritic effect of TAK-242 in curdlan-injected SKG mice through the inhibition of proliferating Th1 and Th17 cells. To extend TLR4 inhibitors to clinical trials for patients with arthritic diseases, the mechanisms of the therapeutic effects should be clarified in various arthritis animal models. However, some patients may be resistant to existing drugs and thus new drugs need to be developed for patients with lower responsiveness to existing drugs. The TLR4 inhibitor TAK-242 could have potential to be developed as a therapeutic agent against arthritic diseases.

Abbreviations

AIA: Adjuvant-induced arthritis; TLR: Toll-like receptor; BMD; Bone mineral density; SpA: Spondyloarthritis; IBD: Inflammatory bowel disease

Declarations

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Figures

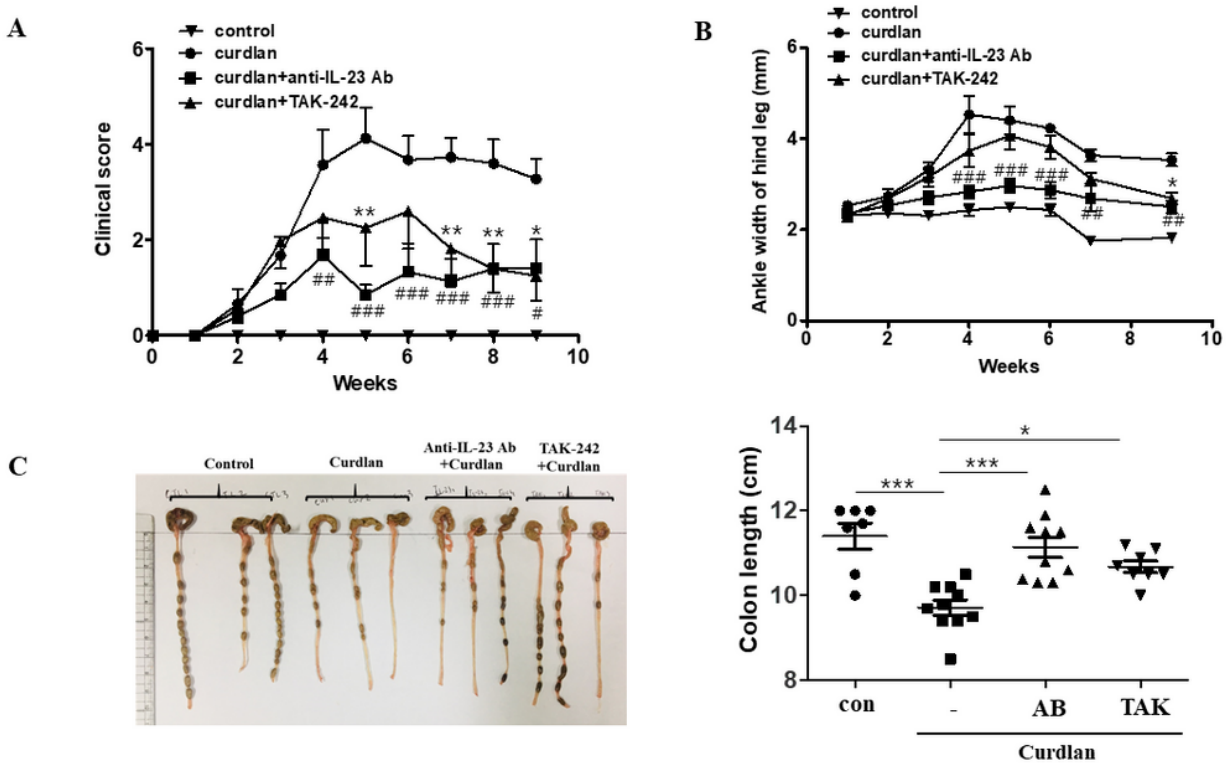

Figure 1

TAK242 inhibits peripheral arthritis in SKG mice with spondyloarthritis. (A) Clinical score. (B) Ankle width of hind leg. (C) Colon lengths were measured after treatment with TAK-242 or anti-IL-23 antibody. Mice were i.p administered TAK242 (3 mg/kg per mouse) 3 times and anti-IL-23 antibody (100 μg) once a week after 9 weeks of curdlan (3 mg) injection. Two repeated experiments showed similar results. Values are expressed as mean ± standard error of the mean (S.E.M). Two-way analysis of the variance (ANOVA) was used to analyze TAK-242 effects on paw thickness and clinical score over time, followed by Turkey's post hoc test. Differences were considered statistically significant at $P < 0.05$. *P or #P < 0.05; **P or ##P <

0.01; ***P or ###P < 0.001 versus curdlan-injected group. Colon length differences between the four groups were analyzed using the nonparametric Kruskal–Wallis test. If a statistical difference was detected (P < 0.05), post hoc pairwise group comparisons were performed using Dunn's test. Differences between the four groups were considered statistically significant at P < 0.05. *P or #P < 0.05; **P or ##P < 0.01; ***P or ###P < 0.001 versus curdlan-injected group; ns, not significant. Con, control; AB, anti-IL-23 antibody treatment; TAK, TAK242 treatment.

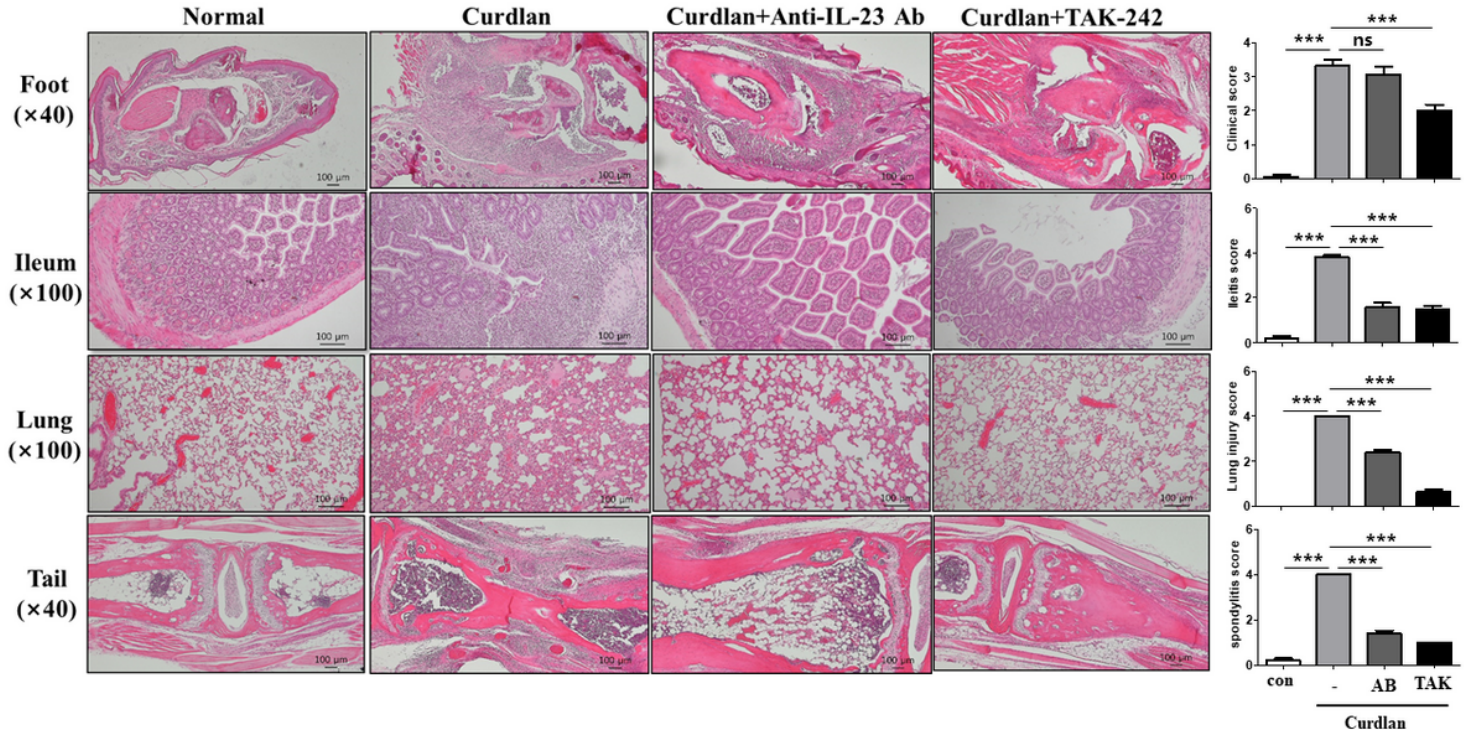

Figure 2

Anti-inflammatory effect of TAK-242 was analyzed by histological analysis in tissues of SKG mice with spondyloarthritis. Inflammation degree of foot, ileum, lung and tail was analyzed by H&E staining. Differences between the four groups were analyzed using the nonparametric Kruskal–Wallis test. If a statistical difference was detected (P < 0.05), post hoc pairwise group comparisons were performed using Dunn's test. Differences were considered statistically significant at P < 0.05. *P or #P < 0.05; **P or ##P < 0.01; ***P or ###P < 0.001; ns, not significant. Con, control; AB, anti-IL-23 antibody treatment; TAK, TAK242 treatment.

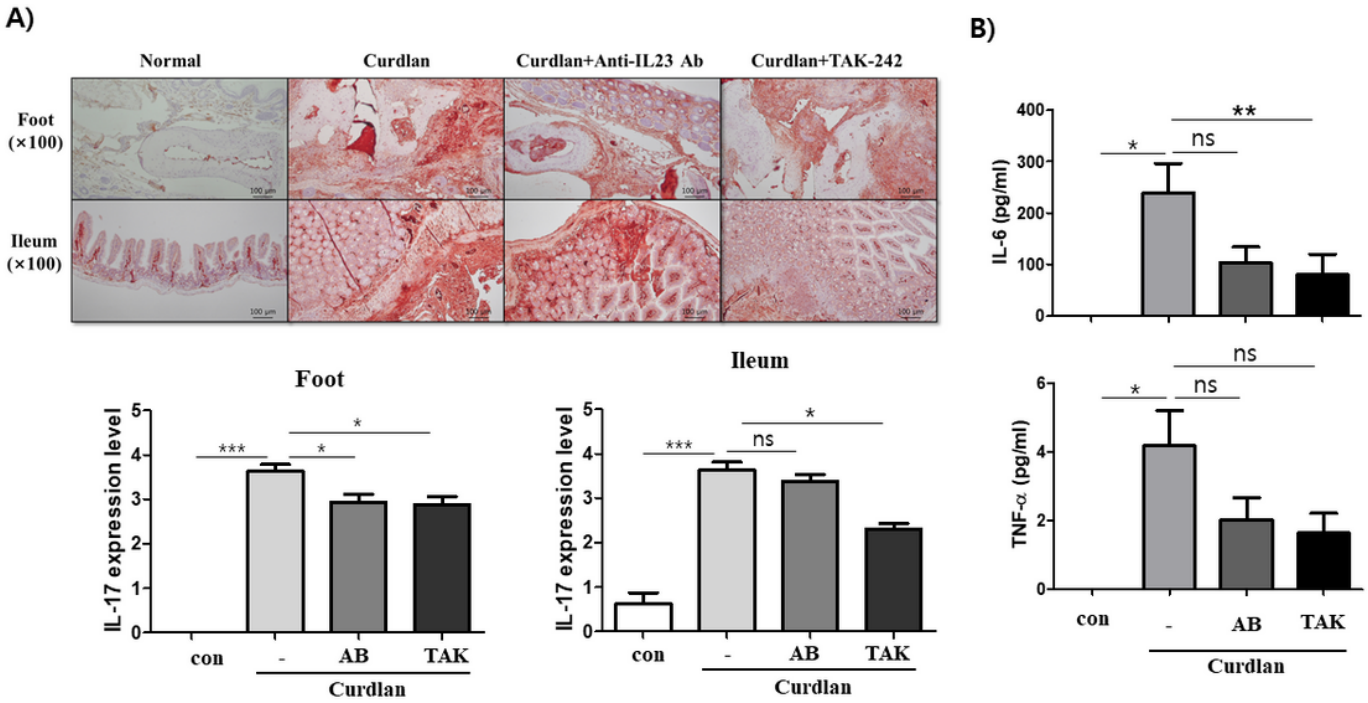

Figure 3

Anti-inflammatory effect of TAK-242 was measured by level of pro-inflammatory cytokines in tissues and serum of SKG mice with spondyloarthritis. Inflammation level was measured by (A) immunostaining of IL-17A in foot and ileum and (B) level of pro-inflammatory cytokines TNF-α and IL-6 in serum. Differences between the four groups were considered statistically significant at $P < 0.05$. *P or #P < 0.05; **P or ##P < 0.01; ***P or ###P < 0.001; ns, not significant. Con, control; AB, anti-IL-23 antibody treatment; TAK, TAK242 treatment.

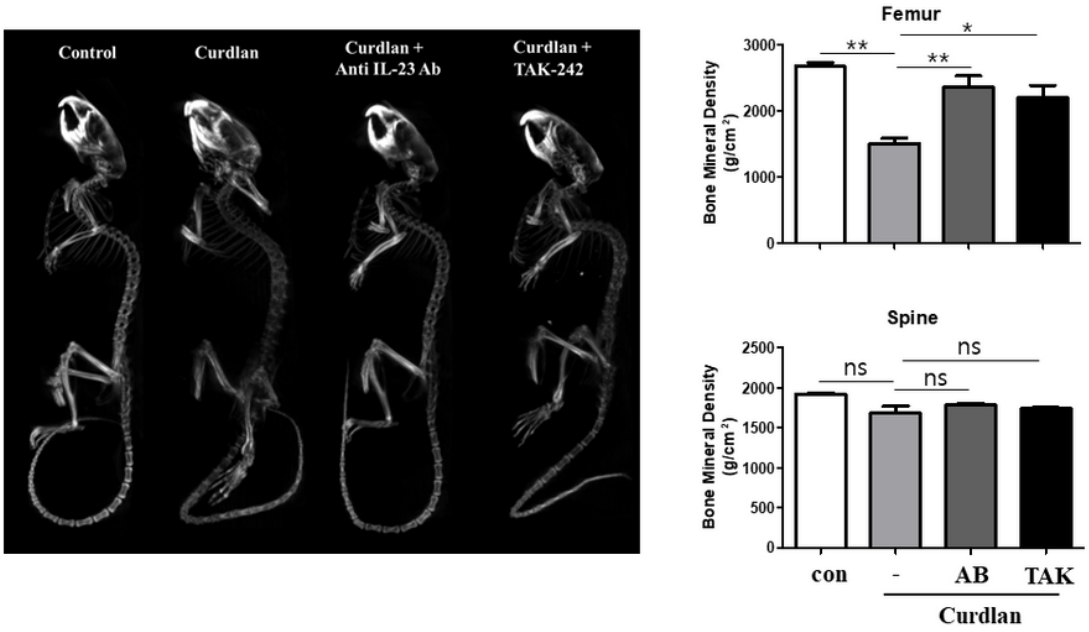

Figure 4

TAK242 treatment prevented the loss of bone mineral density in femurs of SKG mice with spondyloarthritis. BMDs of femur and spine were measured by micro-CT as described in Materials and Methods. Differences between the four groups were considered statistically significant at P < 0.05. *P or #P < 0.05; **P or ##P < 0.01; ***P or ###P < 0.001; ns, not significant. Con, control; AB, anti-IL-23 antibody treatment; TAK, TAK242 treatment.

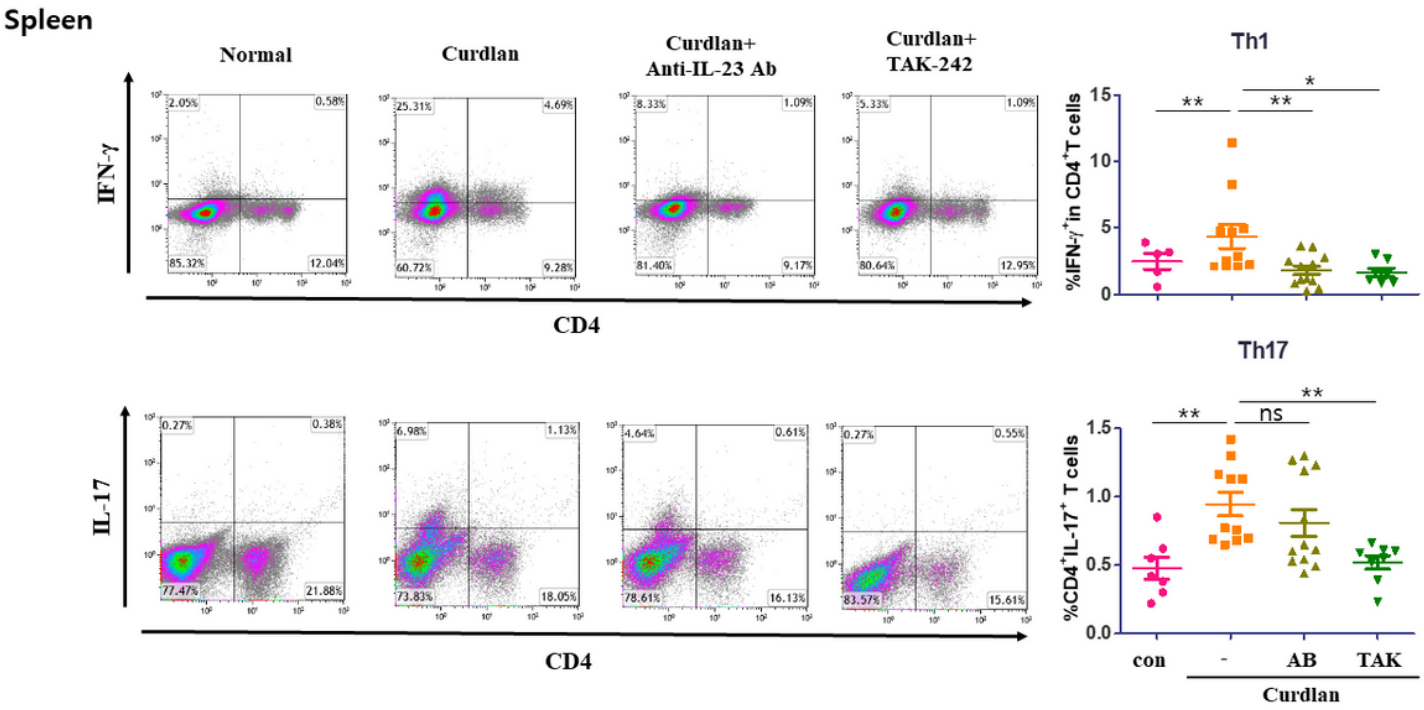

Figure 5

TAK242 inhibited the proliferation of inflammatory subtype T cells Th1 and Th17 in spleen of SKG mice with spondyloarthritis. T cell subtypes Th1 and Th17 were analyzed by fluorescence-activated cell sorter (FACS) using IFN-γ-FITC+CD4-PE-Cy™5 and IL-17-PE/Cy7+CD4-PE-Cy™5, respectively. Differences between the four groups were considered statistically significant at $P < 0.05$. *P or #P < 0.05; **P or ##P < 0.01; ***P or ###P < 0.001; ns, not significant. Con, control; AB, anti-IL-23 antibody treatment; TAK, TAK242 treatment.