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ORIGINAL ARTICLE

TERM

STX0119 Ameliorates Arthritis in SKG Mice via Inhibiting T Helper 17

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Rheumatoid arthritis (RA) is an autoimmune disease with chronic and excessive inflammation. Upregulation of interleukin (IL)-17 is involved in the pathogenesis of RA. STX0119 is a specific inhibitor of signal transducer and activator of transcription 3 (STAT3) as a potential target for the treatment of RA. STAT3 is a member of DNA-binding molecules that regulates the expression of proinflammatory cytokines involved in the pathogenesis of RA. The objective of this study was to determine whether STX0119 could inhibit STAT3 and IL-17. We demonstrated that STX0119 decreased T helper (Th) 17 differentiation and IL-17 expression *in vitro*. STX0119 also improved the severity of zymosan induced arthritis and reduced joint inflammation. STX0119 reduced the proliferation of Th17 and phosphorylated STAT3 expression while increasing Treg differentiation and phosphorylated STAT5 expression. Moreover, STX0119 decreased the expression of IL-6 and -17 but not IL-10. These findings suggest that STX0119 can be used to treat autoimmune RA through inhibiting the activation of STAT3.

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Key Words: Rheumatoid arthritis; Signal transducer and activator of transcription 3 inhibitor; T helper 17; Treg; Immune modualtion

INTRODUCTION

Rheumatoid arthritis (RA) is a clinically inflammatory autoimmune disease that causes chronic inflammation in joints, resulting in cartilage damage and disability. The exact pathogenesis of RA is currently unclear. However, proinflammatory cytokines such as interleukin (IL)-17 is known to be associated with RA pathogenesis. It has been demonstrated that chronic inflammation and immune response in RA patients is caused by IL-17 [1]. In addition, the expression of IL-17 is increased in RA patients compared to normal subjects [2]. IL-17 secreted by T helper (Th) 17 cells also plays an important role in RA development. It is well established that Th17 is increased in peripheral blood of RA patients [3].

In RA mice model, SKG strain of mice spontaneously progress $CD4^+$ T cell-mediated autoimmune arthritis clinically and immunologically paralleling RA [4]. The SKG arthritis is related with Th17 expression. It is well reported that the SKG mice is highly dependent on Th17 development [5]. Additionally, Th17 differentiation can be triggered by extrinsic or intrinsic stimuli in the SKG mice [6].

Signal transducer and activator of transcription (STAT) 3 is a member of DNA binding molecules that regulates the expression of several cytokines. It plays an important role in inflammatory immune response. The activation of STAT3 induces IL-17 production [7,8]. In addition, STAT3 directly regulates the proliferation of Th17 and induces inflammatory $CD4^+$ T cells such as Th17 [8-10]. It has been demonstrated that STAT3 is a potential target for the treatment of RA because the inhibition of STAT3 can alleviate the severity of experimental autoimmune arthritis by reducing Th17 [11,12].

STX0119 is a small molecule primarily used for cancer ther-

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apy. Oral administration of STX0119 could inhibit cell growth of human lymphoma [13]. STX0119 displays anti-tumor effect through inhibiting the expression of STAT3 target genes [14]. Interestingly, STX0119 biologically regulates the activity of STAT3. It has been demonstrated the expression of STAT3 target genes is downregulated significantly after STX0119 treatment [15].

We hypothesized that STX0119 treatment could improve experimental autoimmune arthritis. The objective of this study was to determine whether STX0119 could improve experimental autoimmune arthritis through inhibiting the activation of STAT3. Thus, we performed *in vitro* and *in vivo* tests to determine whether STX0119 could be used as a STAT3 inhibitor to alleviate the severity of arthritis. We examined the inhibitor activity of STX0119 on STAT3 activation and Th17 differentiation in splenocytes obtained from SKG mice. We also measured the therapeutic efficacy of STX0119 in a mouse model of zymosan induced arthritis (ZIA).

MATERIALS AND METHODS

Animals

SKG mice with BALB/c background were kindly provided by Professor Shimon Sakaguchi (Department of Experimental Immunology, World Premier International Immunology Frontier Research Center, Osaka University). These mice were maintained in a specific pathogen-free environment under climate-controlled conditions with a 12h:12h light:dark cycle at Catholic University of Korea. They were provided standard mouse chow (Ralston Purina, St. Louis, MO, USA) and water ad libitum. Male SKG mice at 7 to 8 weeks old were used in this study. Surgeries were conducted under isoflurane anesthesia with all efforts to minimize suffering. Experimental procedures were approved by the Institutional Animal Care and Use Committee at the School of Medicine, Animal Research Ethics Committee of The Catholic University of Korea. They were conducted in accordance with the Laboratory Animals Welfare Act, Guide for the Care and Use of Laboratory Animals.

Induction of arthritis and STX0119 treatment

Zimosan A (Sigma, St Louis, MO, USA) was suspended in phosphate-buffered saline (PBS) and incubated for 10 min in boiling water. Zymosan A solution (2 mg/mice) was injected intraperitoneally into 7- or 8-week-old SKG mice (n=10) to induce ZIA. ZIA SKG mice were orally fed STX0119 (5 mg/kg) or PBS control once daily for 9 weeks starting from day 7 after the first immunization. Arthritis in these mice was examined visually twice per week for the appearance of arthritis in peripheral joints.

Assessment of arthritis

Mice were observed twice per week for the onset, duration, and severity of joint inflammation for 9 weeks after primary immunization. The severity of arthritis was recorded using mean arthritis index with a scale of 0−4 as described previously [16]. Score of 0 indicated no evidence of erythema or swelling. Score of 1 indicated erythema or mild swelling confined to the mid foot (tarsals) or ankle joint. Score of 2 indicated erythema and mild swelling extending from the ankle to the mid foot. Score of 3 was used for erythema and moderate swelling extending from the ankle to the metatarsal joints. Score of 4 indicated erythema and severe swelling encompassing the ankle, foot, and digits. The severity of arthritis was indicated by the sum of scores from all legs and assessed by two independent observers without any knowledge of our experimental groups.

Immunoglobulin measurement

Mice were bled from the eye. Individual sera were analyzed for IgG. Total IgG was measured using Mouse Total IgG enzyme-linked immunosorbent assay (ELISA) Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA).

Cell culture

Total splenocytes were isolated from the spleen of SKG mice. Splenocytes were stimulated with plate-bound anti-CD3 (0.5 μg/mL) and treated with STX0119. Culture supernatant and cells were collected three days after the STX0119 treatment.

Western blot analysis

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Western blot was performed by SNAP i.d. protein detection system (Millipore). Protein bands were visualized using enhanced chemiluminescence detection kit (Thermo Scientific brand of Thermo Fisher Scientific, Inc., MA, USA). The following antibodies were used: anti-pSTAT3 Y705, anti-pSTAT3 S727, anti-STAT3 (all from Cell Signaling, CA, USA), and anti-β-actin (Santa Cruz, TX, USA).

Enzyme-linked immunosorbent assay

Concentrations of IL-17 in culture supernatant were measured using sandwich ELISA. Briefly, specific anti-mIL-17 monoclonal Ab was incubated with culture supernatant in 96-well plate overnight at 4°C. After the incubation, the plate was blocked with PBS containing 1% bovine serum albumin and 0.05% Tween 20 for 2 hours at room temperature. Culture supernatant and target specific mIL-17 recombinant were added to the plate and incubated at room temperature for 2 hours. Biotinylated specific anti-mIL-17 polyclonal Ab was then add-

ed to the plate and incubated at room temperature for another 2 hours. Extravidin-alkaline phosphate (Sigma Aldrich, MO, USA) was then added and the absorbance at 405 nm was measured using an ELISA microplate reader (Molecular Devices).

Immunohistochemistry

Mouse joint tissues were fixed in 4% paraformaldehyde, decalcified in EDTA bone decalcifer, and embedded in paraffin. Tissue sections (7 μm) were prepared and stained with hematoxylin and eosin (H&E) to detect proteoglycans. These sections were dewaxed using xylene and dehydrated in a gradient of alcohols. Endogenous peroxidase activity was quenched with methanol and 3% H_2O_2. Immunohistochemistry was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Tissues were incubated with primary anti-IL-17, anti-IL-21 (R&D systems, NY, USA), and anti-IL-6 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C, biotinylated secondary Ab, and a streptavidin-peroxidase complex for 1 h. The final color product was developed using DAB chromogen (DAKO, Carpinteria, CA, USA). These sections were counterstained with hematoxylin and photographed with an Olympus photomicroscope (Tokyo, Japan).

Histological assessment of arthritis

Joints of each mouse were fixed in 10% formalin, decalcified in 10% EDTA, and embedded in paraffin wax. H&E stained sections were scored for inflammation and cartilage damage. Inflammation was scored according to published criteria [17] with a score of 1 to 4. Score of 0 indicated no inflammation. Score of 1 suggested slight thickening of lining layer or some infiltrating cells in sublining layer. Score of 2 was used for slight thickening of lining layer plus some infiltrating cells in sublining layer. Score of 3 indicated thickening of lining layer, influx of cells in sublining layer, and presence of cells in the synovial space. Score of 4 suggested that synovium was highly infiltrated with many inflammatory cells. For cartilage erosion, five scores were used. Score of 0 indicated no destruction. Score of 1 was for minimal erosion limited to single spots. Score of 2 indicated slight to moderate erosion in a limited area. Score of 3 was used for more extended erosions. Score of 4 indicated general destruction. Neutrophil quantification was performed for three adjacent sections.

Confocal microscopy of immunostaining

Spleen tissues were obtained on day 35 after the first immunization. Tissues were stained with PE-conjugated anti-CD4, FITC-conjugated anti-forkhead box P3 (Foxp3), APC-conjugated anti-CD25, FITC-conjugated anti-IL-17, FITC-conjugated anti-pSTAT3 (Y705), and FITC-conjugated anti-pSTAT3 (S727) (all from eBiosciences, San Diego, CA, USA). Stained sections were visualized using a Zeiss microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).

Flow cytometry

Splenocytes were immunostained with various combinations of fluorescing antibodies against CD4, interferon-gamma (IFN-γ), IL-4, IL-6, IL-10, and IL-17. Cells were incubated with antibodies against IFN-γ (BD Biosciences, MA, USA), IL-17, and Foxp3 (eBioscience). To analyze intracellular cytokines, cells were re-stimulated with phorbol myristate acetate (25 ng/mL) and ionomycin (250 ng/mL) in the presence of GolgiSTOP (BD Biosciences, MA, USA) for 4 h. Intracellular staining was conducted using a kit (eBioscience, CA, USA) following the manufacturer's protocol. Flow cytometry was performed on a FACSCalibur apparatus (BD Biosciences, MA, USA). All data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

All data were expressed as mean±standard deviation. Statistical analysis was performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance followed by a post hoc pairwise comparison adjusted with Student-Newman-Keuls or unpaired Student's t-test assuming equal variances. Statistical significance was considered when p value was less than 0.05.

RESULTS

STX0119 inhibits STAT3 activation and Th17 differentiation *in vitro*

To identify whether STX0119 can decrease STAT3 activation, splenocytes were pretreated by vehicle or STX0119 (20 μM, 24 hours) and stimulated by IL-6 (20 ng, 1 hour). The expression of p-STAT3 was significantly decreased in STX0119 treated mouse cells than in vehicle-treated control cells. However, the expression level of STAT3 protein was not affected by the STX0119 treatment (Fig. 1A). Since the expression of IL-17 can be regulated by STAT3 activation [7], we measured the expression of IL-17 in culture medium of mouse cells treated by STX0119. The expression of IL-17 was decreased significantly by STX0119 (Fig. 1B). We used MTT assays to determine whether such effect was due to the toxicity of STX0119. As a result, no significant change in cell viability was observed after the treatment with STX0119 (Fig. 1C). Treatment with STX0119 in these *in vitro* mouse splenocytes significantly reduced the populations of Th17. However, STX0119 treatment did not induce variations in Th1 or Th2 differentiation (Fig. 1D).

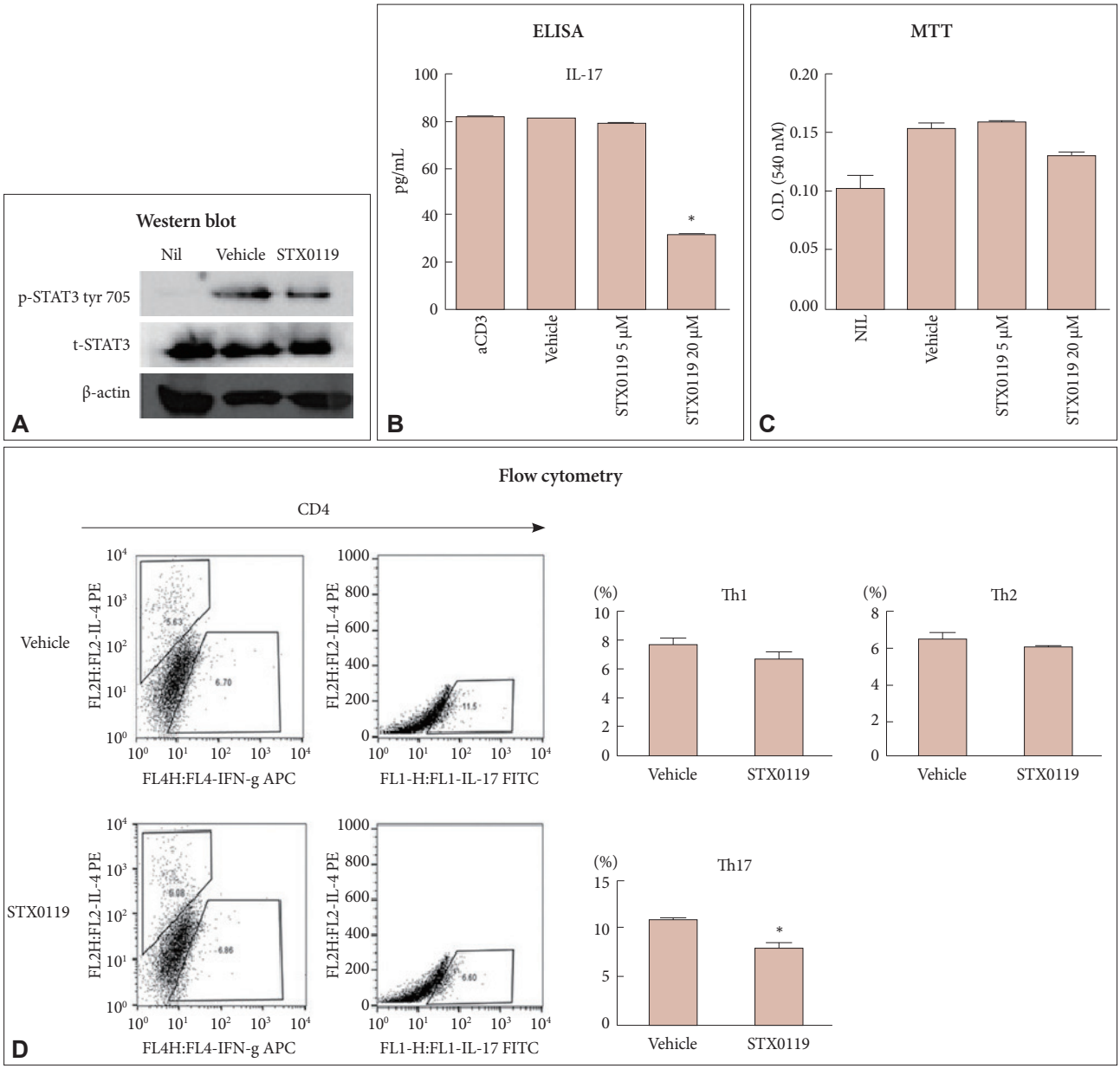


Figure 1. STX0119 treatment reduces Th17 cell differentiation in splenocytes isolated from SKG mice. (A) Western blot of p-STAT3 tyr 705, t-STAT3, and β-actin in splenocytes stimulated by IL-6 (20 ng/mL, 1 hour). (B) The production of IL-17 induced by stimulation with anti-CD3 in the presence or absence of STX0119. (C) After treated by different concentration of STX0119, cell viability was measured using MTT assay. (D) The number of IL-17 producing $CD4^+$ T cells stimulated by anti-CD3 in the presence or absence of STX0119 was determined by using antibodies specific for CD4 and IL-17 by intracellular flow cytometry. Data are presented as mean±SD of three independent experiments. *p<0.05 compared to vehicle control. Th17: T helper 17, STAT3: signal transducer and activator of transcription 3, IL: interleukin, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, ELISA: enzyme-linked immunosorbent assay.

STX0119 reveals therapeutic effect in ZIA induced SKG *in vivo* model

To determine whether STX0119 had anti-arthritic effect, ZIA SKG mice were orally fed daily with either STX0119 or PBS once daily from day 7 after the first immunization. STX0119 significantly decreased the severity of arthritis in ZIA SKG mice (Fig. 2A). Total IgG antibody was significantly decreased in ZIA SKG mice treated with STX0119 compared to that in mice treated by vehicle control (Fig. 2B). Histological scores based on inflammatory cell infiltration were significantly lower in STX0119 treated mice compared to those treated by vehicle control (Fig. 3A). Immunohistochemical analysis showed that the treatment with STX0119 suppressed the expression of proinflammatory cytokines such as IL-6, IL-17, and IL-21 (Fig. 3B).

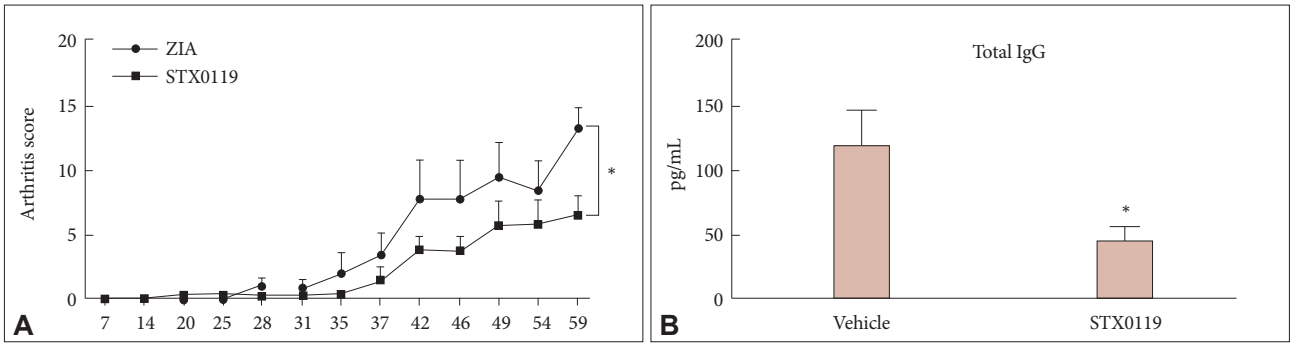
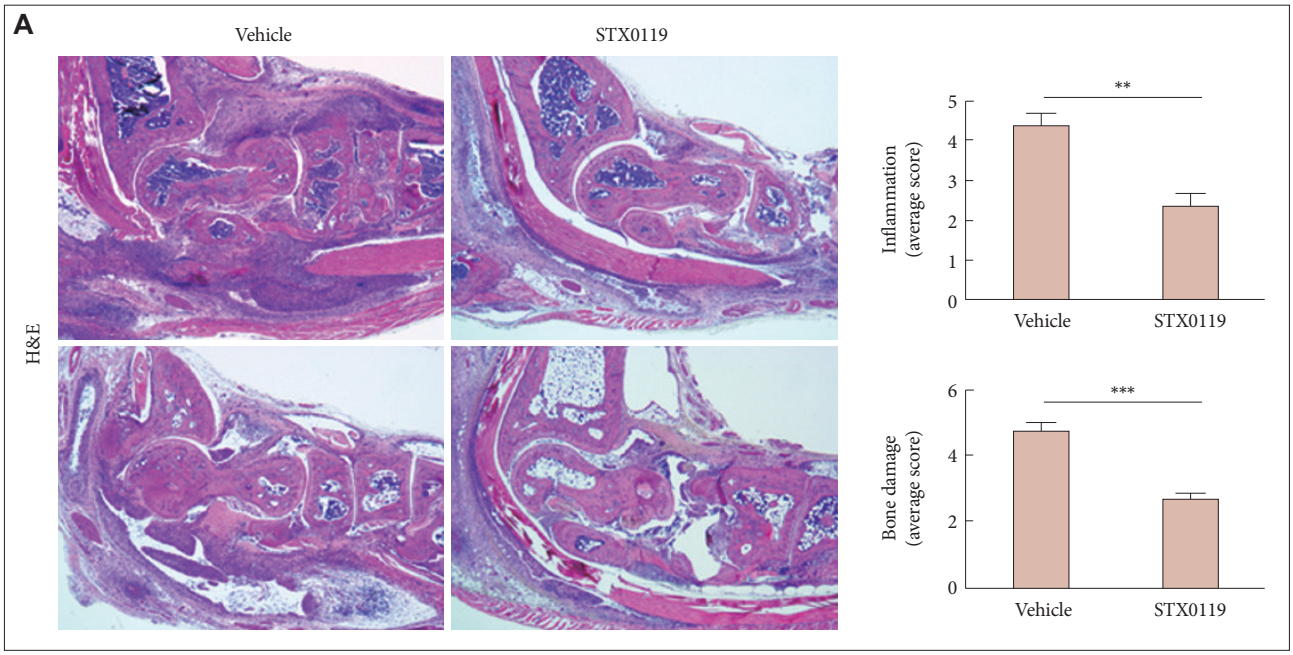


Figure 2. Therapeutic activity of STX0119 in ZIA mice model. ZIA was induced in SKG mice. STX0119 (5 mg/kg) or PBS was orally fed once daily. Mice were sacrificed on day 59 after the first immunization. (A) Clinical scores in ZIA SKG mice (n=8). (B) IgG concentration in serum of ZIA SKG mice (n=8). *p<0.05, compared to vehicle control. ZIA: zymosan induced arthritis, PBS: phosphate-buffered saline, IgG: immunoglobulin G.



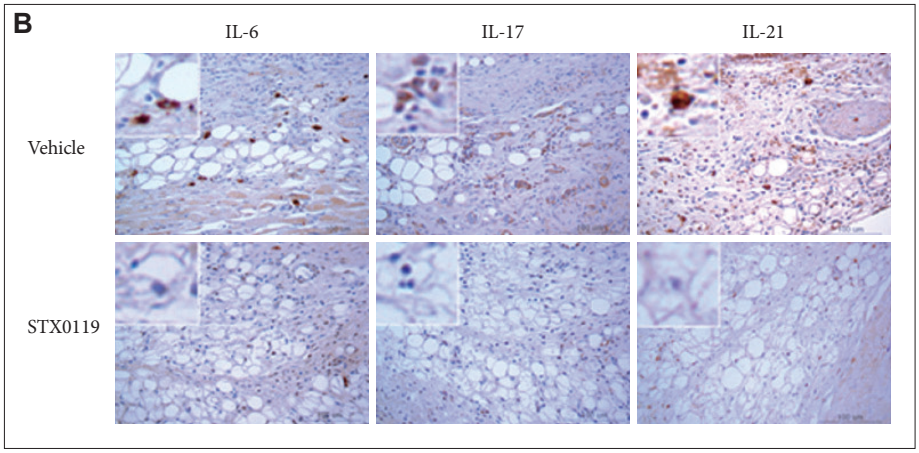


Figure 3. (A) The joint tissues from ZIA. STX0119 treated ZIA mice were stained with H&E and histological score in ZIA SKG mice (original magnification, 40×, n=6, *p<0.05, compared to vehicle control). (B) Immunohistochemical detection of IL-6, IL-17, and IL-21 in the synovium of ZIA and STX0119 treated ZIA. All tissues were counterstained with hematoxylin (n=6) (×40). All histological analyses were conducted more than three times. Representative images are shown. **p<0.03, ***p<0.01 compared to vehicle control. ZIA: zymosan induced arthritis, H&E: hematoxylin and eosin, IL: interleukin.

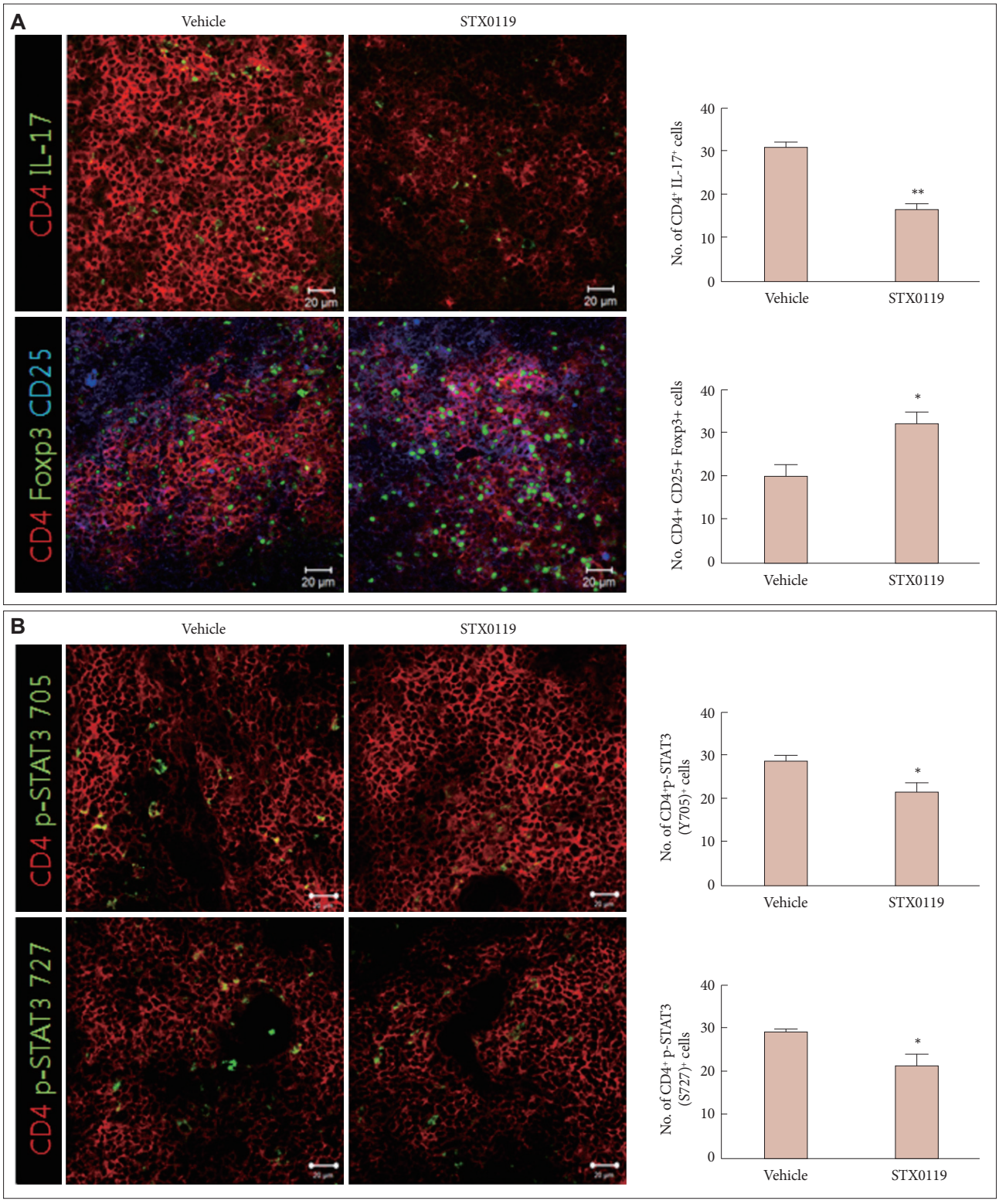


Figure 4. STX0119 treatment reduces STAT3 phosphorylation in $CD4^+$ T cells but increases Treg cells in ZIA mice. (A) Spleen tissues of STX0119-treated ZIA mice or PBS-treated ZIA mice were subjected to immunostaining for $CD4^+IL\text{-}17^+$ or $CD4^+CD25^+Foxp3^+$ cells (*p<0.05, n=6). (B) Spleen tissues of STX0119-treated ZIA mice or PBS-treated ZIA mice were subjected to confocal staining for $CD4^+pSTAT3y705^+$, $CD4^+pSTAT3s727^+$, or $CD4^+pSTAT5^+$ cells. The number of cells was counted in four independent quadrants. Data are presented as mean±SD of three independent experiments (*p<0.05, n=6). ZIA: zymosan induced arthritis, PBS: phosphate-buffered saline.

STX0119 reduces IL-17 expression but induces Foxp3 expression

The expression of IL-17 was decreased in the spleen tissues of mice treated with STX0119. However, the expression of Treg cell-related molecules such as Foxp3 was enhanced in ZIA SKG mice treated with STX0119 (Fig. 4A). In addition, the STX0119 treatment reduced the number of IL-17 producing $CD4^+$p-$STAT705^+$ or p-$STAT727^+$ T cells in the spleen tissues of ZIA SKG mice based on immunofluorescence confocal microscopy. However, the number of $CD4^+$p-$STAT5^+$ T cells in the spleen tissues of STAT0119 treated mice was significantly increased compared to that in the spleen tissues of ice treated with vehicle control based on immunofluorescence confocal microscopy (Fig. 4B).

STX0119 reduces inflammation in the spleen of ZIA SKG mice

In flow cytometry, the number of cells expressing IL-6 and IL-17 in the spleen of ZIA SKG mice treated with STX0119 was significantly lower compared to that in the spleen of mice treated with vehicle control (Fig. 5A). However, the STX0119 treatment did not change the expression of IL-10 compared to that in the mice treated with vehicle control based on flow cytometry (Fig. 5B).

DISCUSSION

STX0119 has been mainly used as a therapeutic to overcome cancer [13,14]. Up to date, there is no evidence that STX0119 could be used for inflammatory diseases or T cell-mediated immune responses. Little has been reported about the therapeutic activity of STX0119 in RA. Therefore, we investigated the therapeutic function of STX0119 for RA in this study. The most significant observation in this study was that STX0119 suppressed Th17 differentiation and improved ZIA development. To our knowledge, this is the first study to provide documentation suggesting that STX0119 could be used as an anti-arthritic factor by inhibiting Th17 differentiation. Previously, several reports have suggested that the activation of STAT3 can increase the proliferation of Th17 while the inhibition of STAT3 can improve arthritic severity by reducing Th17 differentiation [9-11]. As Th17 differentiation is involved in the activation of B cells in autoantibody production [18], the inhibition of STAT3 can decrease IgG concentration. It has been demonstrated that the inhibition of STAT3 activation results in downregulation of IgG concentration in experimental autoimmune arthritis [11, 19]. In this study, we found that STX0119 decreased Th17 differentiation and IL-17 expression. STX0119 also downregulated the activation of STAT3. The inhibitory function of STX0119 attenuated the severity of ZIA reducing IgG concentration. Our results suggest that STX0119 could be used as a novel therapeutic to improve RA by reducing the proliferation of Th17 and IL-17 expression.

The SKG strain derived from BALB/c mice can spontaneously reveal chronic and severe arthritis because a point mutation in ZAP-70 (a significant signaling molecular in T cells) is

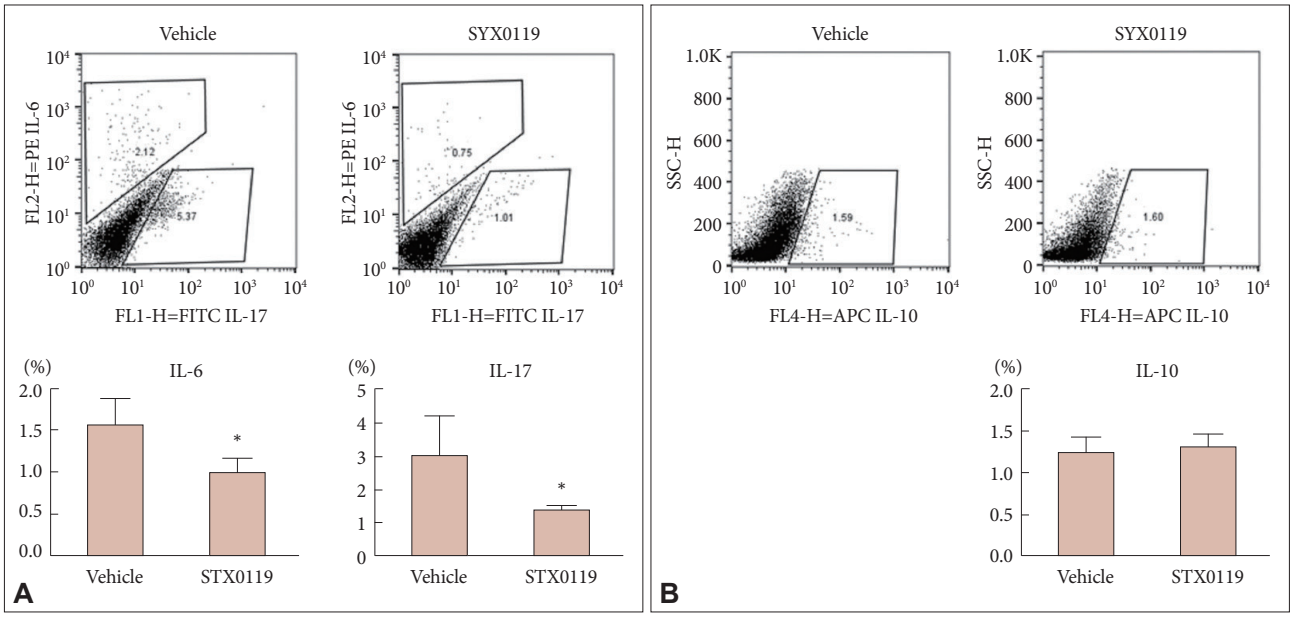


Figure 5. STX0119 treatment downregulates the expression of proinflammatory cytokines but not anti-inflammatory cytokines. (A and B) The populations of IL-6, IL-17, and IL-10 producing $CD4^+$ T cells in spleen of ZIA mice treated with STX0119 or PBS were analyzed by using antibodies specific for IL-6, IL-17, and IL-10 by intracellular flow cytometric analysis. Data are presented as mean±SD of three independent experiments (*p<0.05, n=10). ZIA: zymosan induced arthritis, IL: interleukin, PBS: phosphate-buffered saline.

generated in SKG mice [20]. It has been suggested that a mutation in ZAP-70 can induce autoimmune arthritis and impair T cell signaling in SKG mice [4]. ZIA caused SKG arthritis has severe synovitis with massive subsynovial infiltration of immune cells bone erosion [19]. In this study, we found that STX0119 decreased Th17 differentiation of SKG splenocytes and improved ZIA development. These results suggest that STX0119 might be used as a therapeutic to treat RA.

In the pathogenesis of RA, the proliferation of Th17 and Treg plays a key role. Th17/Treg imbalance is an important target for RA therapy. As Th17 induces inflammation and exacerbates autoimmune arthritis [21], Th17 is increased, resulting in imbalance of Th17/Treg in the peripheral blood of RA patients compared to healthy controls [3]. Recently, reciprocal modulation of Th17/Treg balance has been found to be able to attenuate autoimmune arthritis and reduce joint inflammation [22,23]. In this investigation, STX0119 improved ZIA severity by regulating Th17/Treg. These results suggest that the therapeutic function of STX0119 for RA might be related to Th17/Treg balance.

The activation of STAT3 induces the inflammatory response by upregulating proinflammatory cytokines such as IL-6 and IL-17 [24]. IL-6, IL-17, and IL-21 can initiate signals mediated by STAT3 [25-27]. On the other hand, IL-10 can result in anti-inflammatory response and reduce the activation of STAT3 [25, 28]. We observed that the administration of STX0119 into ZIA mice decreased the expression of IL-6, IL-17, and IL-21. However, the production of IL-10 was not affected by the STX0119 treatment. Thus, the therapeutic function of STX0119 in ZIA observed in this study might be due to the anti-inflammatory activity of STX0119.

Our observations of the inhibitory activity of STX0119 on the proliferation of Th17 and STAT3 activation indicate the possibility that STX0119 can be used to reduce inflammation. This study demonstrated that STX0119 could improve ZIA and decrease Th17 differentiation by inhibiting STAT3. Therefore, STX0119 might be used as a new therapeutic for T cell-mediated inflammatory response and RA treatment.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This study was approved by the Institutional Animal Care and Use Committee at the School of Medicine, Animal Research Ethics Committee of The Catholic University of Korea.

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