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ORIGINAL ARTICLE



Suppressor of TCR signaling-2 (STS-2) suppresses arthritis development in mice

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ABSTRACT

Objectives: Suppressor of TCR signaling-2 (STS-2) is one of the RA susceptibility genes identified in genome-wide association studies (GWAS). We tried to verify the involvement of STS-2 on the development of autoimmune arthritis in a mouse model.

Methods: STS-2 knock-out (KO) and wild type (WT) mice were immunized with chicken type II collagen (CII). For CD4⁺ helper T cell (Th) subset analysis, intracellular cytokines in splenocytes and lymph node cells were stained and analyzed by flow cytometry. Regulatory T cell (Treg) function was analyzed by co-culturing effector CD4⁺T cells and Tregs collected from non-immunized mice.

Results: CII-immunized STS-2 KO mice developed arthritis more frequently than WT mice. Although the T cell activation profile and Th subset in spleen and LNs were similar between STS-2 KO and WT mice, STS-2 KO mice showed increased IL-2-producing CD4⁺T cells in spleen when compared with WT mice. Accordingly, STS-2 KO CD4⁺T cells promoted IL-2 production by TCR stimulation. However, STS-2 KO Tregs normally suppressed T cell proliferation.

Conclusion: We proved that STS-2 is involved in the arthritis development by collagen-induced arthritis. Higher IL-2 production from STS-2 KO T cells is suggested to have a main pathogenic role in arthritis development.

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KEYWORDS

Animal model; collagen induced arthritis; IL-2; suppressor of TCR signaling-2; rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic synovitis, which causes joint swelling, pain and, if untreated, joint deformity. Genome-wide association studies (GWAS) have revealed many RA susceptibility loci, including major histocompatibility complex (MHC) genes and >100 non-MHC genes. Suppressor of TCR signaling-2 (STS-2) is one of the non-MHC RA susceptibility candidate genes that was identified by a meta-analysis of GWAS in Caucasian and Japanese people [1]. STS-2 has also been reported to be associated with various autoimmune diseases, such as type-I diabetes [2–5], vitiligo vulgaris [6], and systemic lupus erythematosus [7]. However, the role of STS-2 in autoimmune diseases has not been clearly clarified.

STS-1 (also called UBASH3B or TULA2) and STS-2 (also called UBASH3A or TULA) belong to STS family and consist of the following three domains; the UBA domain, which can bind to ubiquitins; the SH3 domain, which is used in the interaction between STS-interacting proteins; and the PGM domain, which has the phosphatase ability [8]. It has been reported that both STS-1 and STS-2 negatively control T cell receptor (TCR) signaling through dephosphorylation

of phosphorylated ZAP-70 [9,10]. Therefore, ZAP-70 was hyperphosphorylated in STS-1/2 double knockout (KO) T cells upon TCR stimulation and double KO T cells hyperproliferated, but the phosphorylation level of ZAP-70 in STS-1 or STS-2 single KO T cells was restored, indicating STS-1 and STS-2 have overlapping role in regulating ZAP-70 signaling [10]. The difference between the abilities of STS-1 and STS-2 to dephosphorylate ZAP-70 and Syk was also reported [11,12]. Interestingly, only STS-2 was reported as a disease susceptibility gene, although many reports demonstrate that STS-1 has stronger ability as a regulator of intracellular signaling than STS-2.

Here, we report that STS-2 KO mice were more prone to develop arthritis than wild type (WT) mice when collagen-induced arthritis (CIA) was induced, consistent with human RA.

Materials and methods

Mice

C57BL/6Jdcl (B6) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). STS-1 KO and STS-2 KO mice on the B6 background were established in Stony Brook University

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(New York, NY) [13]. All mice were maintained under specific pathogen-free conditions at Kyoto University, which were approved by the Institutional Committee for Animal Care and Use.

Induction and assessment of collagen-induced arthritis (CIA)

Native chicken type II collagen (CII; Chondrex Inc., Redmond, WA) was solubilized in 50 mM acetic acid at 2 mg/ml and emulsified in equal volume of complete Freund's adjuvant (CFA) (Chondrex Inc.) containing 5 mg/ml of killed *Mycobacterium tuberculosis*. Eight-week-old male mice were immunized with 100 µg CII dissolved in CFA via subcutaneous injection into their shaved backs, at day 0 and day 21.

Mice were scored for arthritis severity in each limb using the following scale: 0, normal; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to metatarsal joints; 4, erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb, according to nature protocol [14]. Scores for four limbs were summed, and each mouse was given the resulting clinical score (between 0 and 16).

ELISA for anti-CII antibody titer

Anti-CII antibodies in the sera of mice at day 56 from the first immunization were assessed. 96-well MAXISORP NUNC-IMMUNO PLATES (Thermo Fisher, Waltham, MA), were coated with 20 ng/well of chicken CII dissolved in sodium hydrogen carbonate, blocked with 2% bovine serum albumin (BSA)-phosphate buffered saline (PBS) for 3 h at room temperature, and incubated with diluted test sera for 2 h. Then, 100 µl of diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam, Cambridge, MA) were applied per well and incubated for 1.5 h at room temperature, followed by reaction with 3,3',5,5'-tetramethylbenzidine (TMB; eBioscience, San Diego, CA) substrate. 5 min later, 1 M H₂SO₄ was applied as stop solution, and absorbance at 450 nm was measured with iMarkTM Microplate Reader (Bio Rad, Hercules, CA). Test sera were diluted at 1/1500, and HRP-conjugated antibodies was diluted at 1/5000. The pooled sera of CII-immunized DBA1/J mice were used as standard sera. 2% BSA-PBS was used for wash buffer.

Real-time PCR (RT-PCR)

Cells for RT-PCR were sorted by a flow cytometer Aria II (Becton Dickinson; BD, Franklin Lakes, NJ) as described below. mRNA were extracted using RNeasy Plus Mini (QIAGEN, Venlo, Netherlands), then reverse-transcribed to cDNA with High Capacity cDNA Reverse Transcription Kits (Applied Biosystem, Foster City, CA). Taqman probes (Takara Bio, Shiga, Japan) were used for RT-PCR.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used for a housekeeping gene.

Flow cytometry

For RT-PCR analysis, we obtained spleen and thymus from 10 week-old mice. Red blood cells in the spleen were destructed with blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO). Splenocytes and thymocytes were stained with phycoerythrin (PE)-CD3, Pacific Blue-CD4, allophycocyanin (APC)-CD8, PE-CD25 (Biolegend, San Diego, CA), APC-B220, fluorescein isothiocyanate (FITC)-CD11b, then sorted by Aria II.

For peripheral lymphocyte analysis, we obtained thymus, spleen and inguinal or axillary lymph node (LN). Red blood cells were lysed with blood cell lysis buffer (SIGMA). Hemolyzed splenocytes, thymocytes, and LN cells were suspended in 2% fetal calf serum (FCS)-PBS, and their surface markers were stained with fluorescent antibodies; APC-CD3 (Biolegend, San Diego, CA), PE-CD3 (Biolegend), APC-CD4 (Biolegend), FITC-CD4 (BD), APC-CD8 (Biolegend), APC-CD19 (Biolegend), PE-CD62L (Biolegend), FITC-CD69 (Biolegend), APC-NK1.1 (Biolegend), or FITC-PD-1 (Biolegend), PE-CD25 (Biolegend), followed by washing twice with 2% FCS-PBS, and analysis with FACS Caliber HG (BD).

For detection of regulatory T cells (Treg), surface markers were stained with FITC-CD4 (BD), PE-CD25 (Biolegend). After washing twice, cells were fixed in 75% ethanol at -30 °C over night, and stained with APC-Foxp3 (eBioscience). Washed twice again, cells were analyzed by flow cytometry.

For analysis of intracellular cytokines, hemolyzed splenocytes and LN cells were suspended in RPMI-1640 medium containing 10% FCS, penicillin, and streptomycin, and were stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA), 500 ng/ml of ionomycin, and monensin (BD) in a 48-well plate for 4 h under conditions of 5% CO₂ at 37 °C. Then, cells were harvested, washed twice with 2% FCS-PBS, and stained with APC-CD4. After Washing twice again, cells were fixed with Fix/Perm solution (BD), washed twice; then, intracellular cytokines were stained with PE- or FITC- IL-2 (Biolegend), PE-IL-17A (BD) PE-IFN-γ (Biolegend), PE-IL-4 (Biolegend), or PE-IL-10 (Biolegend), followed by washing twice and analysis by a flowcytometer Calibur HG.

Treg suppression assay

Spleens were obtained from 8- to 10-week-old mice. Hemolyzed splenocytes were suspended in 2% FCS-PBS. Then, CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells, CD4⁻ cells were isolated with CD4⁺CD25⁺ Regulatory T cell kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purities of the CD4⁺CD25⁺ cells and CD4⁺CD25⁻ cells were >90% by flow cytometry. CD4⁻ cells were irradiated at 20 Gy and used as antigen presenting cells (APCs). CD4⁺CD25⁺ cells and CD4⁺CD25⁻ cells were used as regulatory T cells

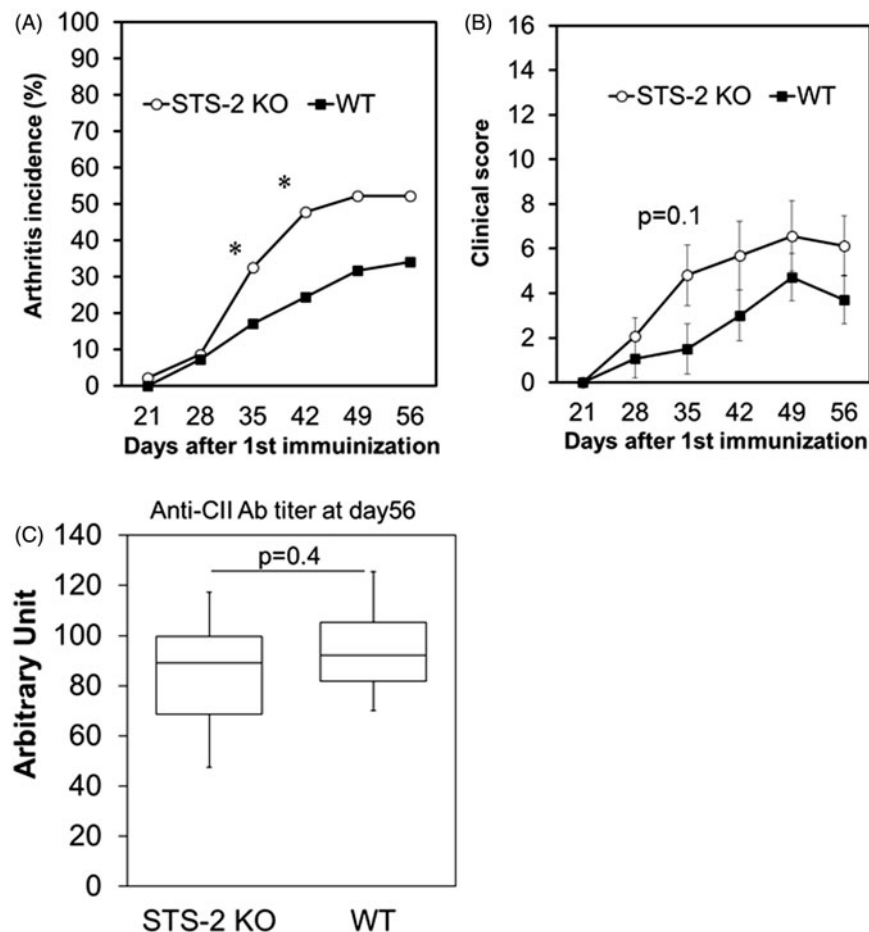


Figure 1. STS-2 KO mice show higher incidence of autoimmune arthritis than WT mice. STS-2 KO and WT mice were immunized with chicken type II collagen (CII) at day 0 and day 21 subcutaneously. (A) CIA incidence was recorded for 56 d from the first immunization (STS-2 KO $n = 46$, WT $n = 41$). (B) Clinical scores were evaluated in STS-2 KO and WT arthritic mice over time (STS-2 KO $n = 16$, WT $n = 14$). (C) Anti-CII IgG titer in sera was measured by ELISA (STS-2 KO; $n = 8$, WT; $n = 7$). * $p < .05$.

(Treg) and effector T cells (Teff), respectively. Teff were labeled by carboxyfluorescein succinimidyl ester (CFSE). T cells were resuspended in 1 ml 2 μ M CFSE buffer (DOJINDO, Kumamoto, Japan) and incubated for 10 min at room temperature; then, 1 ml FCS was applied, and incubated for 2 min at room temperature. Next, 10 ml RPMI was added to the cells, and they were centrifuged for 10 min at 4°C. After that, cells were washed twice with RPMI1640 (containing 10% FCS, penicillin, streptomycin, and 50 μ M 2-mercaptoethanol). The numbers of Treg was 2.5×10^4 (at the ratio Teff: Treg = 1:1), Teff was 2.5×10^4 , and APCs was 5×10^4 per well, and they were stimulated in a 96-well round-bottom plate with anti-CD3 antibodies (clone; 145-2C11, BD) for 72 h under conditions of 5% CO₂ at 37°C. The cells were analyzed by flow cytometry.

CD4⁺T cell in vitro stimulation assay for IL-2 production

The spleen and LNs were removed from 8- to 10-week-old male mice. Hemolyzed splenocytes and LN cells were suspended in 2% FCS-PBS. Then, CD4⁺ T cells were isolated by MACS (Miltenyi Biotec). The purity of CD4⁺ cells was checked to >90% by flow cytometry. CD4⁺ cells were irradiated at 20 Gy and used as APCs. 2.5×10^4 CD4⁺ cells and 5×10^4 APCs per well were stimulated in a 96 well

round-bottom plate with anti-CD3 antibodies (BD) for 72 h under conditions of 5% CO₂ at 37°C. The supernatant were collected. ELISA kits were used for IL-2 detection (Diaclone, Besancon, France).

Growth factor withdrawal cell death assay

Spleens were obtained from 8- to 10-week old mice. Hemolyzed splenocytes were suspended in 2% FCS-PBS. Then, CD4⁺ T cells were isolated by MACS (Miltenyi Biotec). The purity of CD4⁺ cells was checked to >95% by flow cytometry. 5×10^5 CD4⁺T cells per well were stimulated in 48 well plate with plate-bound anti-CD3 antibodies (BD) and soluble anti-CD28 antibodies (BD) (2 μ g/ml, each) for 72 h in RPMI1640 (containing 10% FCS, penicillin, streptomycin, and 50 μ M 2-mercaptoethanol). After stimulation, cells were harvested and washed twice with fresh medium, incubated for 3 h at 37°C in fresh medium, and washed again to completely remove IL-2 from the medium and bound to IL-2R. Then, 5×10^4 CD4⁺T cells per well were incubated with or without 100 U/ml of recombinant murine IL-2 (PeproTech Inc., Rocky Hill, NJ), or 5 μ M etoposide (SIGMA) for 24 h. For apoptosis detection, cells were stained with Alexa Fluor 647 Annexin V (Biolegend) and Propidium Iodide (PI) (Biolegend).

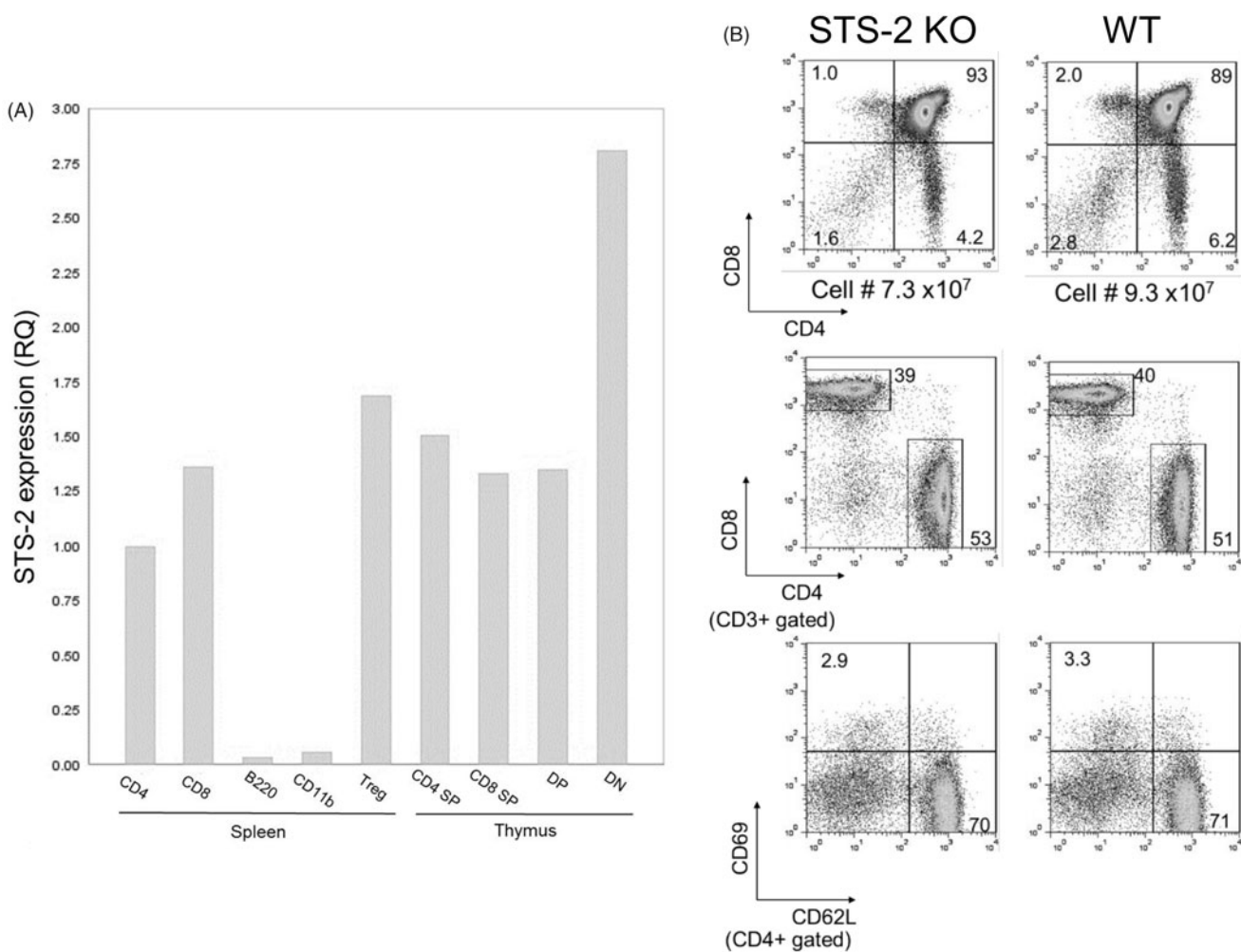


Figure 2. STS-2 is expressed in T cells but does not affect T cell differentiation. (A) RT-PCR analysis for STS-2 was performed using the sorted splenocytes and thymocytes indicated in the figure; Treg, CD4⁺CD25^{hi}, SP, single positive, DP, CD4⁺CD8⁺ double positive, DN, CD4⁻CD8⁻ double negative cells. (B) Thymocytes (upper panel) and splenocytes (middle and lower panel) of unimmunized STS-2 KO and WT mice were stained with indicated antibodies. Representative profiles of two independent experiments are shown. Numbers in plots are the percentage of cells in indicated gated area.

Statistics

Arthritis incidence was tested by log-rank test. Arthritis score was tested by Wilcoxon rank sum test. Other results were tested by two-tailed student *t* test.

Results

STS-2 KO mice develop collagen-induced arthritis more frequently

To clarify whether STS-2 is involved in the pathogenesis of autoimmune arthritis, we induced CIA, a mouse model of human RA, to STS-2 KO mice, and WT mice. As shown in Figure 1(A), the incidence of arthritis development in STS-2 KO mice was higher than in WT mice (51 vs. 34%, $p < .05$). The clinical score of arthritic mice tended to be higher ($p = .109$) in STS-2 KO mice, but did not differ significantly (Figure 1(B)). Since the background of mice was B6, the incidence of CIA was not as high as the case of DBA/1. We also examined CIA incidence by using STS-1 KO mice, but the arthritis incidence in STS-1 KO mice was similar to that

in WT mice (Supplemental Figure 1). To investigate the reasons of the difference in arthritis incidence between STS-2 KO and WT, we measured anti-CII antibody titer. We collected sera from CII-immunized STS-2 KO and WT mice, which include both arthritic and non-arthritic mice, at day 56 after immunization and measured antibody titer by ELISA. STS-2 KO and WT mice showed similar anti-CII antibody titer (Figure 1(C)). These data indicated that STS-2 is engaged in the pathogenesis of autoimmune arthritis, but does not have so much impact on the severity.

STS-2 does not affect T cell development

To narrow down the target cells of STS-2, we examined which cell subset expresses STS-2 by RT-PCR. As shown in Figure 2(A), only T cells but not B cells or myeloid cells expressed STS-2 in the spleen, and all fractions of thymocytes expressed STS-2. Because STS-2 expresses only in T cells, it suggests that STS-2 influences on T cell differentiation and activation. However, CD4/CD8 patterns and cell number of thymocytes and splenocytes were similar between

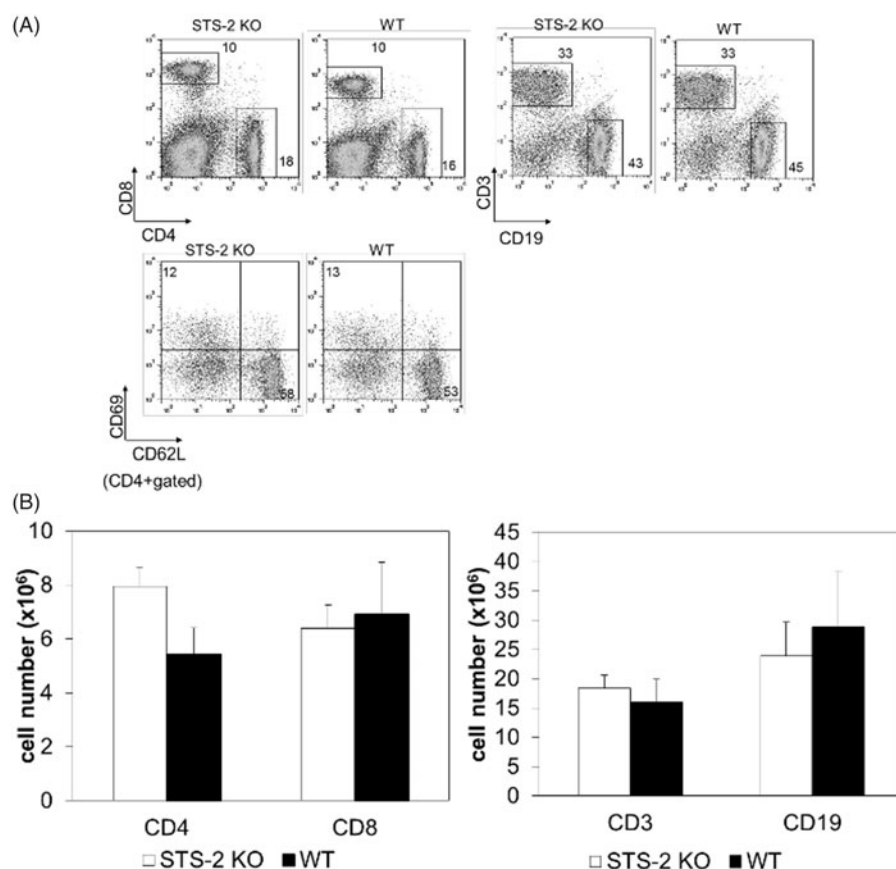


Figure 3. CII-immunized STS-2 KO and WT mice have similar peripheral immune cell numbers. Splenocytes were obtained from STS-2 KO and WT mice at day 56 from the first immunization. Cells were stained with surface markers shown in Methods. (A) Representative plots of splenocytes are shown. Numbers in plots are the percentage of cells in indicated gated area. (B) Average cell numbers and SE of each fraction of STS-2 KO and WT spleens are shown. 4 independent experiments were performed.

STS-2 KO and WT mice (Figure 2(B)), indicating STS-2 is not essential in thymocyte selection and T cell development.

Next, we analyzed lymphocyte profile in arthritic mice of STS-2 KO and WT mice immunized with CII. We obtained spleen and inguinal and axillary LNs from arthritic CIA mice at 56 d after the first immunization, and the FACS profiles of CD4/CD8, CD3/CD19, and CD69/CD62L (gated on CD4⁺ cells of splenocytes) are shown in Figure 3(A). The average cell numbers of CD4⁺, CD8⁺, CD3⁺, and CD19⁺ cells in the STS-2 KO and WT spleens are shown in Figure 3(B). There were no substantial differences in CD4/CD8 T cell numbers and activation statuses of CD4⁺ T cells between STS-2 KO and WT mice.

We also investigated CD4⁺CD25⁺FoxP3⁺ Treg proportion and function. The proportion of Treg cells was not changed in the spleen and LNs from immunized mice (Figure 4(A)), and Treg suppression assay revealed there were no significant differences in the suppressive function of Tregs between STS-2 KO and WT mice (Figure 4(B,C)), indicating STS-2 is not essential in Treg function.

STS-2 KO T cells from arthritic mice produce more IL-2 in the spleen than WT

We further analyzed the T cell subsets in CII-immunized STS-2 KO and WT mice. Splenocytes and LN cells were

collected from CII-immunized mice at day 35, when arthritis was not evident yet, and at day 56, when the arthritis is around its peak. Splenocytes or LN cells were stimulated with PMA and ionomycin and were stained with antibodies against surface CD4 and intracellular cytokines, followed by flowcytometric analysis. There was significant difference in the proportion of IL-2-producing CD4⁺ T cells between STS-2 KO and WT mice in the spleen (38.7 vs. 22.4%, $p < .05$) but not in the LNs (19.4% vs. WT 18.1%). However, there were no significant differences between STS-2 KO and WT mice in the proportion of Th1 (IFN- γ ⁺CD4⁺), Th2 (IL-4⁺CD4⁺), or Th17 (IL-17⁺CD4⁺) subsets at day 35 (data not shown) and day 56 (Figure 5(A,B)). These results suggest STS-2 does not influence the differentiation of CD4⁺ T cells into specific effector subsets. We also tested other inflammatory cytokines, such as TNF- α and IL-6, but there were no significant differences (data not shown).

To verify that STS-2 KO CD4⁺ T cells promote IL-2 production, we examined whether STS-2 KO CD4⁺ T cells promote IL-2 production by anti-CD3 antibody stimulation. As shown in Figure 5(C), STS-2 KO CD4⁺ T cells obtained from the spleen showed higher IL-2 production than WT, but not CD4⁺ T cells from the LNs. We also examined IL-2 production from CII-specific CD4⁺ T cells from immunized mice at day 35 (Supplemental Figure 2). In accord with the results of TCR stimulation, our results showed that STS-2

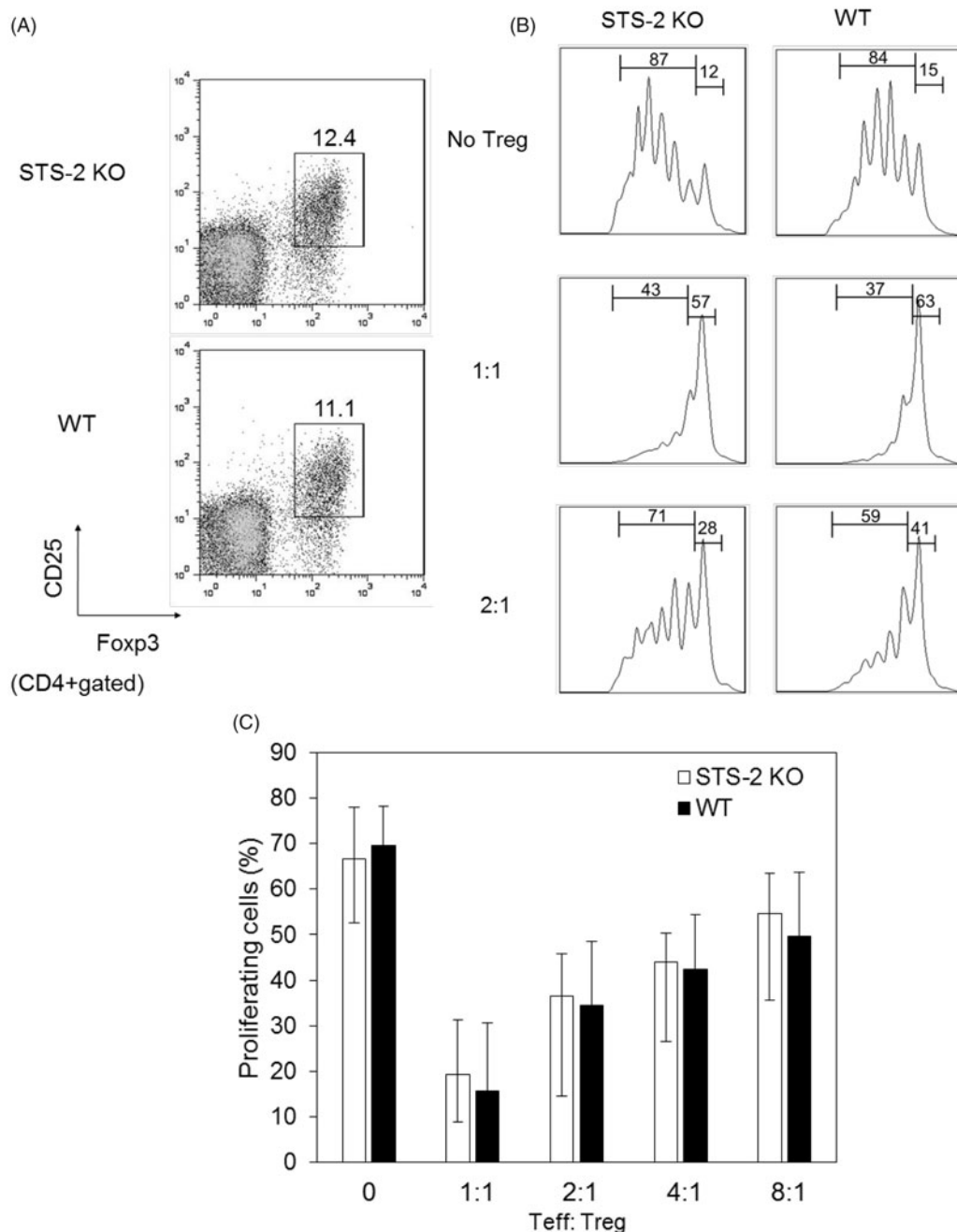


Figure 4. Treg number and function in STS-2 KO mice. (A) The proportion of CD4⁺CD25⁺Fop3⁺ Treg cells in the spleen of CII immunized STS-2 KO and WT mice at day 56. Treg suppression assay were performed as described in Methods. (B) Histogram of CFSE in 7AAD⁺CD4⁺ cells. (C) Percentage of proliferating Teff cells. Mean and SE from four independent experiments are shown.

KO CD4⁺T cells tended to produce more IL-2 by CII stimulation, when the CD4⁺T cells were obtained from spleens but not from LN.

To identify which T cell subset produces IL-2 in STS-2 KO mice, we counter-stained intracellular IL-2 with several surface markers on CD4⁺T cells in the spleens from CII-immunized mice. As shown in Figure 6, follicular helper T cells (Tfh, CD4⁺PD-1⁺) or Treg cells (CD4⁺CD25⁺) in STS-2 KO mice do not substantially produce IL-2. There were only a few NKT cells (CD4⁺NK1.1⁺) which produce IL-2 in the spleen, but there were no differences between in STS-2 KO and WT mice.

STS-2 deficient CD4⁺T cells are resistant to growth factor withdrawal cell death

To explain the higher incidence of CIA in STS-2 KO mice, we hypothesized that autoreactive T cells in STS-2 KO mice survive longer by escaping apoptosis. STS-2 was reported to bind apoptosis-inducing factor (AIF) and promote apoptosis *in vitro* [15]. So, we examined whether STS-2 KO CD4⁺T cells are more resistant to cell death. We tested growth factor withdrawal cell death of STS-2 KO and WT CD4⁺T cells. As expected, STS-2 KO CD4⁺T cells were more resistant to apoptosis when IL-2 was deprived (Figure 7(A,B)).

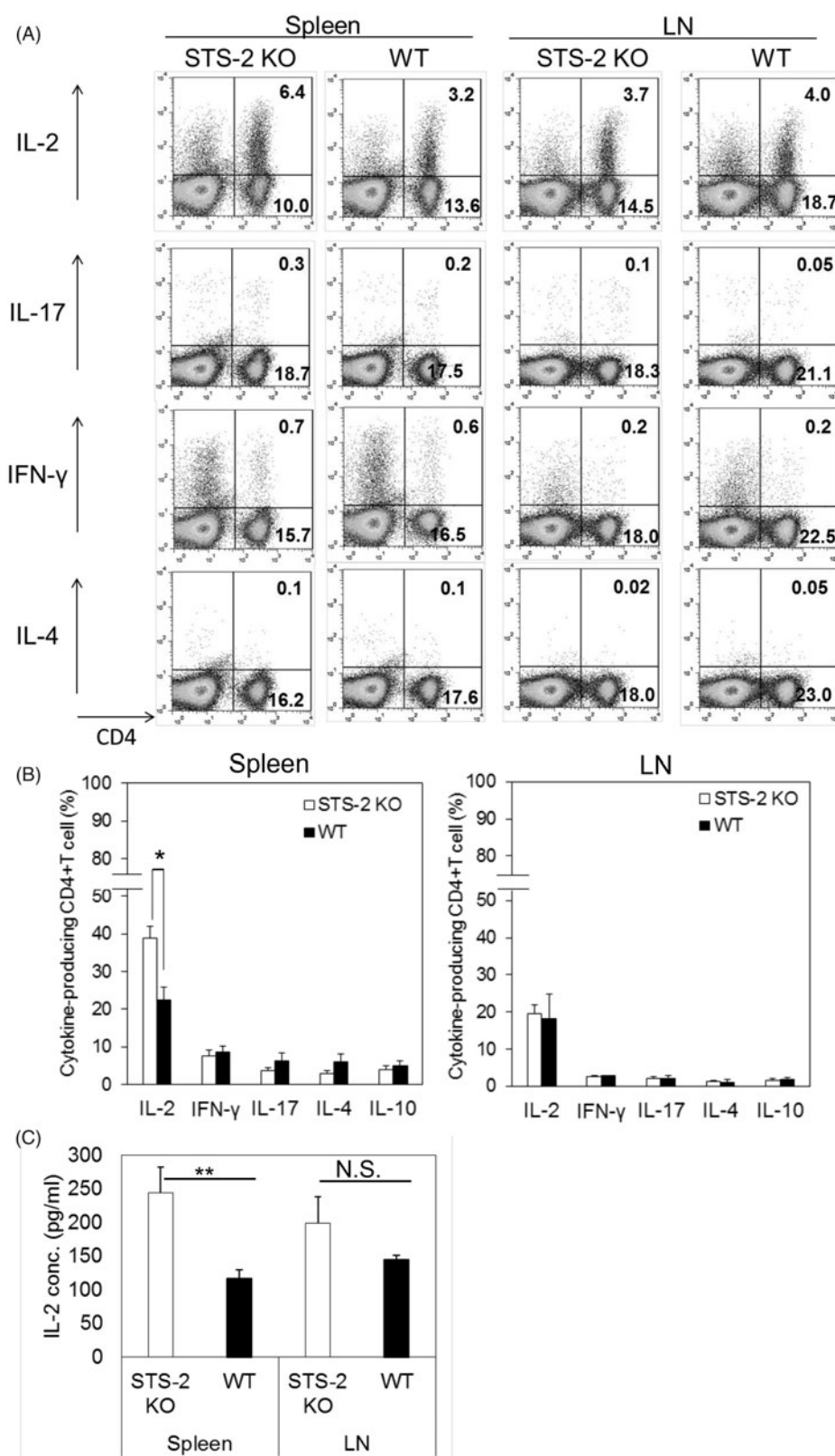


Figure 5. STS-2 KO CD4⁺ splenic T cells produce more IL-2 but not other cytokines. Splenocytes and LN cells were prepared from STS-2 KO and WT mice at day 56 from the first immunization. (A) Representative plots of splenocytes and LN cells after stimulation with PMA/ionomycin, followed by staining with anti-CD4 antibodies and antibodies against various intracellular cytokines. (B) Percentage of cytokine-producing CD4⁺T cells in spleen and LNs. Mean and SEM from five independent experiments are shown. (C) CD4⁺T cells from non-immunized STS-2 KO and WT mice were incubated with irradiated splenocytes and anti-CD3 antibody for 3 d. IL-2 in the supernatant was measured by ELISA. Mean and SE are shown from seven independent experiments. * $p < .05$, ** $p < .01$.

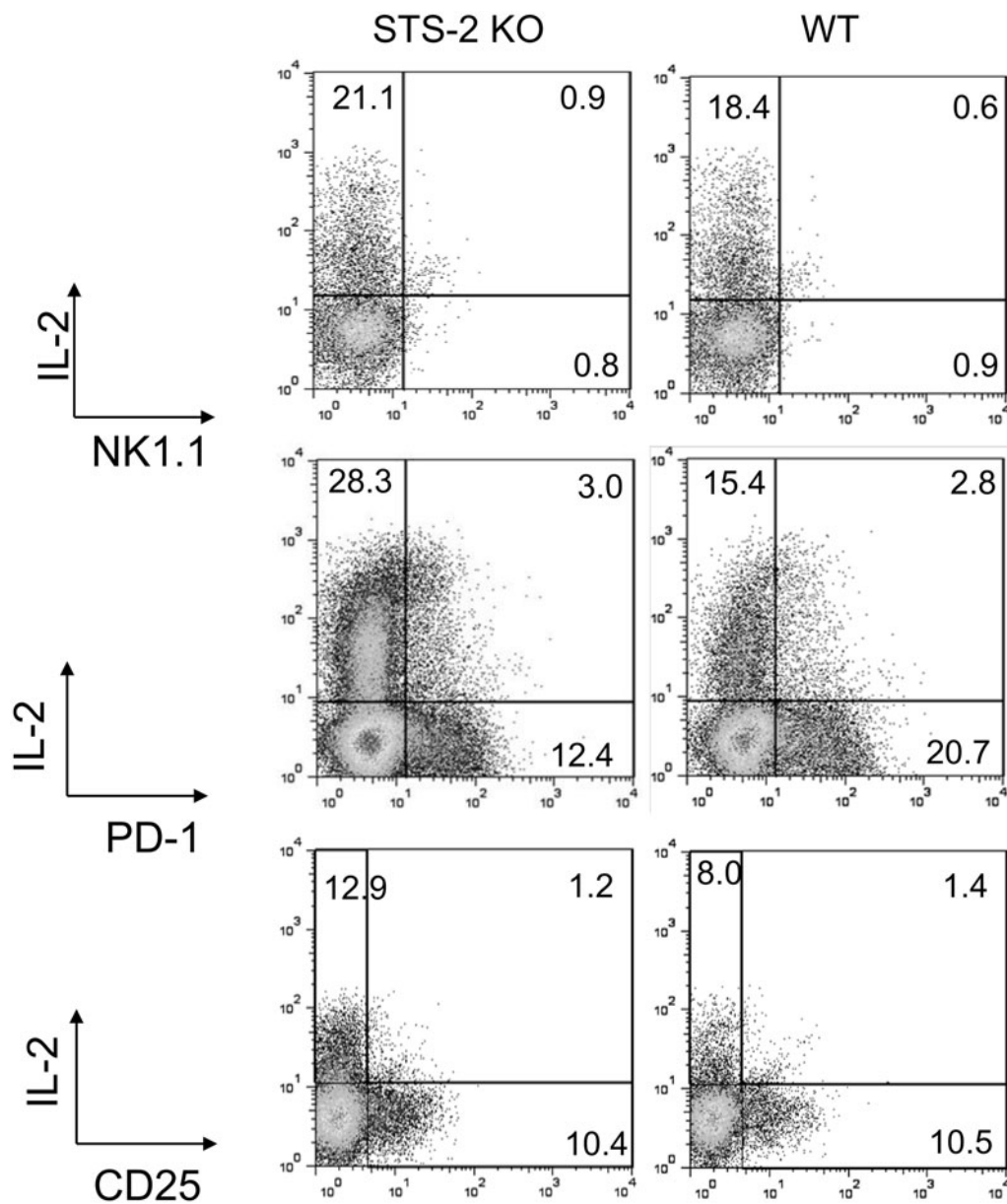


Figure 6. IL-2 production from NKT cells, Tfh, or Treg in STS-2 KO and WT mice. Splenocytes were prepared from STS-2 KO and WT mice at day 56 from the first immunization. Splenocytes were stimulated with PMA/ionomycin, followed by staining with antibodies against CD4, other surface markers and intracellular cytokines.

Therefore, STS-2 was found to be partially involved in promoting T cell apoptosis.

Discussion

It has been reported that the genetic polymorphism in the STS-2 locus is associated with RA development, and we validated the involvement of STS-2 in the pathogenesis of autoimmune arthritis by using an animal model. We showed STS-2 KO mice developed CIA with higher incidence than WT mice. Since STS-2 is expressed only in T cells, we examined the difference of T cell subset and activation status for STS-2 KO mice and WT mice. We found IL-2-producing CD4⁺ T cells are more abundant in STS-2 KO mice than WT mice in the spleen but not in the draining LNs.

Such higher incidence of CIA and IL-2 production was not observed in STS-1 KO mice (Supplemental Figure 1), suggesting that STS-2, but not STS-1, is crucial in the pathogenesis of arthritis development.

It was of note that the overexpression of IL-2 from CD4⁺ T cells resulted in higher incidences of arthritis. This is consistent with the fact that GWAS for RA revealed *IL-2*, *IL-2RA*, and *IL-2RB* are the RA susceptibility genes [16]. The role of IL-2 in arthritis has been investigated in human and animal models. IL-2 mRNA level in synovial fluid and synovial tissue increased in the acute phase of RA [17,18]. In a mouse model of RA, IL-2 mRNA level dramatically increased at the onset of CIA [19], and anti-IL-2 antibody administration just before the onset of CIA resulted in decreased CIA incidence [20]. While administration of

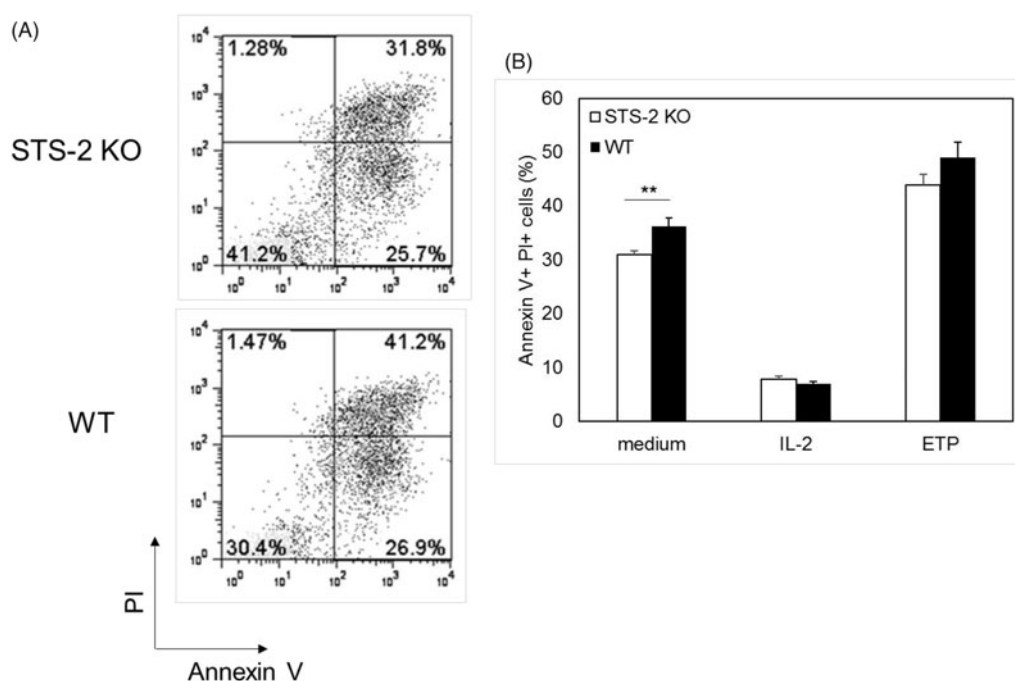


Figure 7. STS-2 deficiency affects growth factor withdrawal cell death. $CD4^+$ T cells were prepared from STS-2 KO and WT mice and were stimulated with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody for 3 d. Then, IL-2 was deprived from cell suspension and cells were incubated with culture medium with or without IL-2, or with etoposide (ETP). After 24 h, cells were stained with Annexin V and Propidium Iodide (PI). Representative plots of flowcytometry are shown in A and percentage of Annexin V⁺/PI⁺ cells (mean and SE) from four independent experiments are shown.

recombinant human IL-2 (rhIL-2) before the onset of CIA inhibited disease severity and incidence, rhIL-2 treatment at the established stage of CIA exacerbated CIA [21]. These reports support the idea that IL-2 has distinct role at the different stage of arthritis and that STS-2 downregulation is involved in IL-2 production from T cells, which results in the development of autoimmune arthritis.

It was somewhat surprising that IL-2 was overexpressed in T cells from spleen but not from LNs in the STS-2 KO mice. To identify which $CD4^+$ T cell subsets produce IL-2 more in the spleen of CII-immunized STS-2 KO mice than that of WT mice, we co-stained IL-2 with other cytokines or some representative surface markers of $CD4^+$ T cell subsets. In STS-2 KO mice, IL-17⁺IL-2⁺ or IFN- γ ⁺IL-2⁺ T cells were increased, while there were no IL-2 productions from NK1.1⁺ cells, PD-1⁺ cells (Tfh), and CD25^{hi} cells (Treg) in either STS-2 KO or WT mice. These data indicate that IL-2 production from Th1, Th17, or undifferentiated $CD4^+$ T cells are the responsible effector cells (data not shown). It seems the slight increase of these cell types in the spleen of STS-2 KO mice resulted in the difference of incidence of arthritis development. In experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, it was reported that when lymphocytes obtained from the spleen or LNs in EAE-developing mice were transferred into healthy mice, the recipient mice with transferred LN cells dramatically developed EAE and died, while recipient mice transferred splenocytes showed a more delayed EAE development, and they relapsed into EAE later. This phenomenon did not seem to be caused by the difference of transferred $CD4^+$ T cell number in spleen and LN cells [22]. Analysis of mice with transferred splenocytes showed

that they increased $CD4^+$ T cells which produce IL-2, IFN- γ , IL-10 only in LNs [23]. These data indicate that T cells in spleen and LN have different functions for disease pathogenesis.

Because IL-2 is a major T cell growth factor, it was expected that STS-2 KO $CD4^+$ T cells proliferate more by TCR stimulation. However, preliminary data indicated that STS-2 KO and WT $CD4^+$ T cells proliferated in a similar manner by anti-CD3/anti-CD28 stimulation *in vitro* (data not shown). Furthermore, similar numbers of $CD4^+$ T cells in CII-immunized STS-2 KO and WT mice suggest that STS-2 deficiency does not influence antigen specific T cell proliferation. It is possible that IL-2 production from STS-2 KO $CD4^+$ T cells is high enough to activate themselves, but too little to promote proliferation.

STS-1 and STS-2 have been reported to have similar functions and compensate each other [9,10]. However, in human GWAS of some autoimmune diseases, only the SNPs in the STS-2 locus were reported to be associated with disease development. This could be explained by the fact that the reported SNP (rs1893592) in STS-2 locus influences STS-2 expression (<http://genenetwork.nl/bloodeqtlbrowser/>), but none of the SNPs in STS-1 locus do. Our preliminary data showed that STS-1 KO mice developed arthritis as frequently as the WT mice did (Supplementary Figure 1). Because we moved animal facility between STS-2 KO and STS-1 KO experiments, CIA incidence of the WT mice in each facility was different. Therefore, we cannot directly compare the result of STS-2 KO mice with that of STS-1 KO mice. However, our results showed that only STS-2 KO mice, but not STS-1 KO mice, increased the incidence of

arthritis, suggesting STS-2 is playing more important role in the development of autoimmune arthritis than STS-1.

Although STS-1 and STS-2 share the functions of suppressing T cell activation through TCR, only STS-2 was reported to bind AIF and promote caspase-independent apoptosis *in vitro* [15]. AIF is a flavoprotein that acts as NADH oxidase and regulates caspase-independent apoptosis [24–26]. AIF is located in inner mitochondrial membrane at steady state. Once AIF receives an apoptosis signal, it is released from mitochondria and moves into nucleus, then binds to DNA and induces aggregation and degradation of chromatin, resulting in apoptosis [27]. STS-2 is thought to promote interaction between released AIF and other co-factors in cytoplasm, and to promote apoptosis [15]. Our results of apoptosis analysis are compatible with the previous results that STS-2 promoted growth factor withdrawal cell death by enhancing AIF function.

Our results may indicate that autoreactive T cells survive by escaping activation-induced cell death in CII-immunized STS-2 KO mice. So far, the role of STS-1 and STS-2 in autoimmune animal models was evaluated only in EAE [13]. In this report, STS-1/2 double KO mice were used and developed severer symptoms and showed higher mortality. The results of STS-1 or STS-2 single KO mice were not evaluated.

In this study, we first demonstrated STS-2 has an important role in developing autoimmune arthritis by using an animal model. Our results indicate IL-2 is one of the critical cytokines regulated by STS-2. The involvement and the compensation of STS-1 in arthritis have to be clarified, especially when clinical applications are considered. The effects of STS-1 single KO and STS-1/2 double KO mice on CIA were ought to be elucidated.

Conflict of interest

None.

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References

- Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. *Nature*. 2014;506:376–81.
- Concannon P, Onengut-Gumuscu S, Todd JA, Smyth DJ, Pociot F, Bergholdt R, et al. A human type 1 diabetes susceptibility locus maps to chromosome 21q22.3. *Diabetes*. 2008;57:2858–61.
- Grant SF, Qu HQ, Bradfield JP, Marchand L, Kim CE, Glessner JT, et al. Follow-up analysis of genome-wide association data identifies novel loci for type 1 diabetes. *Diabetes*. 2009;58:290–5.
- Johnson K, Wong R, Barriga KJ, Klingensmith G, Ziegler AG, Rewers MJ, et al. rs11203203 is associated with type 1 diabetes risk in population pre-screened for high-risk HLA-DR/DQ genotypes. *Pediatr Diabetes*. 2012;13:611–15.
- Plagnol V, Howson JM, Smyth DJ, Walker N, Hafler JP, Wallace C, et al. Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. *PLoS Genet*. 2011;7:e1002216.
- Jin Y, Birlea SA, Fain PR, Gowan K, Riccardi SL, Holland PJ, et al. Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. *N Engl J Med*. 2010;362:1686–97.
- Diaz-Gallo LM, Sánchez E, Ortego-Centeno N, Sabio JM, García-Hernández FJ, de Ramón E, et al. Evidence of new risk genetic factor to systemic lupus erythematosus: the UBASH3A gene. *PLoS One*. 2013;8:e60646.
- Feshchenko EA, Smirnova EV, Swaminathan G, Teckchandani AM, Agrawal R, Band H, et al. TULA: an SH3- and UBA-containing protein that binds to c-Cbl and ubiquitin. *Oncogene*. 2004;23:4690–706.
- Carpino N, Chen Y, Nassar N, Oh HW. The STS proteins target tyrosine phosphorylated, ubiquitinated proteins within TCR signaling pathways. *Mol Immunol*. 2009;46:3224–31.
- San Luis B, Sondgeroth B, Nassar N, Carpino N. Sts-2 is a phosphatase that negatively regulates zeta-associated protein (ZAP)-70 and T cell receptor signaling pathways. *J Biol Chem*. 2011;286:15943–54.
- Agrawal R, Carpino N, Tsygankov A. TULA proteins regulate activity of the protein tyrosine kinase Syk. *J Cell Biochem*. 2008;104:953–64.
- Thomas DH, Getz TM, Newman TN, Dangelmaier CA, Carpino N, Kunapuli SP, et al. A novel histidine tyrosine phosphatase, TULA-2, associates with Syk and negatively regulates GPVI signaling in platelets. *Blood*. 2010;116:2570–8.
- Carpino N, Turner S, Mekala D, Takahashi Y, Zang H, Geiger TL, et al. Regulation of ZAP-70 activation and TCR signaling by two related proteins, Sts-1 and Sts-2. *Immunity*. 2004;20:37–46.
- Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. *Nat Protoc*. 2007;2:1269–75.
- Collingwood TS, Smirnova EV, Bogush M, Carpino N, Annan RS, Tsygankov AY. T-cell ubiquitin ligand affects cell death through a functional interaction with apoptosis-inducing factor, a key factor of caspase-independent apoptosis. *J Biol Chem*. 2007;282:30920–8.
- Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. *Nat Genet*. 2010;42:508–14.
- Firestein GS, Xu WD, Townsend K, Broide D, Alvaro-Gracia J, Glasebrook AS, et al. Cytokines in chronic inflammatory arthritis. I. Failure to detect T cell lymphokines (interleukin 2 and interleukin 3) and presence of macrophage colony-stimulating factor (CSF-1) and a novel mast cell growth factor in rheumatoid synovitis. *J Exp Med*. 1988;168:1573–86.
- Buchan G, Barrett K, Fujita T, Taniguchi T, Maini R, Feldmann M. Detection of activated T cell products in the rheumatoid joint using cDNA probes to Interleukin-2 (IL-2) IL-2 receptor and IFN-gamma. *Clin Exp Immunol*. 1988;71:295–301.
- Thornton S, Duwel LE, Boivin GP, Ma Y, Hirsch R. Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression. *Arthritis Rheum*. 1999;42:1109–18.
- Banerjee S, Wei BY, Hillman K, Luthra HS, David CS. Immunosuppression of collagen-induced arthritis in mice with an anti-IL-2 receptor antibody. *J Immunol*. 1988;141(4):1150–4.
- Thornton S, Boivin GP, Kim KN, Finkelman FD, Hirsch R. Heterogeneous effects of IL-2 on collagen-induced arthritis. *J Immunol*. 2000;165:1557–63.
- Yang J, Kubera M, Zelek-Molik A, Nalepa I, Hukkanen V, Lindsberg PJ, et al. Splenectomy and adoptive cell transfer reveal

- a prominent role for splenic memory lymphocytes in the development of chronic relapsing experimental autoimmune encephalomyelitis. *Scand J Immunol.* 2000;52:356–61.
23. Yang J, Lindsberg PJ, Hukkanen V, Seljelid R, Gahmberg CG, Meri S. Differential expression of cytokines (IL-2, IFN- γ , IL-10) and adhesion molecules (VCAM-1, LFA-1, CD44) between spleen and lymph nodes associates with remission in chronic relapsing experimental autoimmune encephalomyelitis. *Scand J Immunol.* 2002;56:286–93.
24. Miramar MD, Costantini P, Ravagnan L, Saraiva LM, Haouzi D, Brothers G, et al. NADH oxidase activity of mitochondrial apoptosis-inducing factor. *J Biol Chem.* 2001;276:16391–8.
25. Modjtahedi N, Giordanetto F, Madeo F, Kroemer G. Apoptosis-inducing factor: vital and lethal. *Trends Cell Biol.* 2006;16:264–72.
26. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature.* 1999;397:441–6.
27. Lipton SA, Bossy-Wetzel E. Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. *Cell.* 2002;111:147–50.