Original Article

# Elevated Levels of Soluble ST2 were Associated with Rheumatoid Arthritis Disease Activity and Ameliorated Inflammation in Synovial Fibroblasts

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## Abstract

**Background:** Much evidence has demonstrated that interleukin (IL)-33 plays an important role in rheumatoid arthritis (RA). However, there have been limited studies about soluble ST2, a receptor for IL-33, in RA. The aims of this study were to detect the levels of ST2 in the serum and synovial fluid of RA patients and to reveal the association of these levels with disease activity and the function of ST2 in RA.
**Methods:** A total of 56 RA patients and 38 age-matched healthy controls were enrolled in this study. Synovial fluid samples were collected from another 30 RA patients and 20 osteoarthritis patients. Serum and synovial fluid levels of ST2 were measured by ELISA. In addition, the levels of ST2 in the serum of RA patients before and after therapy were detected. The function of ST2 in RA was revealed by the results of an *in vitro* cell assay, where recombinant ST2 proteins were used to treat peripheral blood mononuclear cells (PBMCs) and RA synovial fibroblasts (RASFs).
**Results:** Serum-soluble ST2 levels were significantly higher in RA patients (127.14 ± 61.43 pg/ml) than those in healthy controls (78.37 ± 41.93 pg/ml, $P < 0.01$). Synovial fluid-soluble ST2 levels (41.90 ± 33.58 pg/ml) were much higher in RA patients than those in osteoarthritis patients (19.71 ± 16.72 pg/ml, $P < 0.05$). RA patients who received effective therapy for 6 months showed decreased serum-soluble ST2 levels (113.01 ± 53.90 pg/ml) compared to baseline (139.59 ± 68.36 pg/ml) ($P = 0.01$). RA patients with high disease activity had higher serum-soluble ST2 levels (162.02 ± 56.78 pg/ml) than those with low disease activity (94.67 ± 40.27 pg/ml, $P = 0.001$). Soluble ST2 did not affect IL-1β, IL-6, IL-8, or tumor necrosis factor-α (TNF-α) expression in PBMCs from RA patients. However, soluble ST2 ameliorated the expressions of IL-33 and IL-1β but not that of IL-6, IL-8, or TNF-α in resting RASFs. Interestingly, in the RASFs stimulated by TNF-α plus IL-1β, soluble ST2 showed extensive suppressive effects on the expression of IL-6, IL-8, and TNF-α.
**Conclusion:** Elevated levels of ST2 in the serum and synovial fluid were associated with disease activity and ameliorated IL-33 expression and IL-33-induced inflammation in RASFs, suggesting that soluble ST2 might be a potential therapeutic candidate for RA.

**Key words:** Inflammatory Cytokines; Interleukin-33; Rheumatoid Arthritis; Soluble ST2

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation in the synovial membranes of multiple affected joints.[1] Interleukin-1 (IL-1) and IL-1 receptor families play important roles in inflammatory and immunological responses.[2,3] IL-33 and its receptor ST2 belong to the IL-1

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and IL-1 receptor families, respectively.[3] The pathogenic role of IL-33 in RA has been widely studied,[4-6] but there are few studies about its receptor ST2, especially the soluble form, in RA.

The *ST2* gene produces two forms: soluble ST2 and transmembrane ST2 (ST2L).[7] Soluble ST2 lacks the intracellular and transmembrane domains, but both isoforms have the same extracellular domain. ST2L is selectively expressed on Th2 but not on Th1 T-cells,[8] which indicates that it might be involved in Th2 cell-mediated immunological responses. Evidence has been provided that treatment with an anti-ST2 antibody attenuates the severity of joint arthritis in mice with collagen-induced arthritis (CIA).[4] This finding might be due to the blocking of the IL-33/ST2L axis by an anti-ST2 antibody.

Elevated levels of soluble ST2 have been found in many diseases, such as asthma, dengue virus infection,[9] sepsis, and RA.[10] Our previous studies have found elevated soluble ST2 in the serum of RA patients, which is consistent with other studies. However, to date, only one study has reported an association between soluble ST2 and RA disease activity, but it is limited in early RA.[11] In addition, the results of the former studies were limited in terms of information about the function of soluble ST2 in RA. Most of the studies focused on other diseases instead of RA. A recent study on allergic airway inflammation demonstrated that soluble ST2 blocked IL-33 signaling.[12] In addition, an *in vitro* study demonstrated that soluble ST2 could suppress lipopolysaccharide (LPS)-induced IL-6 production in THP-1 cells,[7] and whether this function was restricted to the inhibition of the IL-33/ST2 axis was unknown. Furthermore, there was no report of the function of soluble ST2 in RA.

In this study, we therefore investigated the correlation between soluble ST2 levels and disease activity and explored the function of soluble ST2 in RA.

## Methods

### Ethical approval

The study protocols were approved by the Institutional and Medical Ethics Review Board of Peking University International Hospital. Informed consent was obtained from all patients.

### Patients and healthy controls

From 2014 to 2015, a total of 56 RA patients (51 women and 5 men) were enrolled in this study; all of them met *the 1987 revised classification criteria of the American College of Rheumatology*. All patients had active disease, which was defined by a Disease Activity Score with C-reactive protein (DAS28-CRP) in 28 joints of >2.6. Thirty-eight age-matched healthy controls (HCs) were recruited from staff of our hospital, and all of them did not have any rheumatologic conditions.

The 56 enrolled RA patients who received disease-modifying antirheumatic drugs (at least two of methotrexate, sulfasalazine, hydroxychloroquine, and leflunomide) were evaluated for 24 weeks. The sera from the patients who had a lower disease activity than at baseline were collected and soluble ST2 levels were evaluated.

Synovial fluid (SF) samples were collected from another 30 RA patients and 20 osteoarthritis (OA) patients and were stored at −70°C for <6 months.

### Clinical and laboratory assessments

All the 56 RA patients underwent clinical assessments at baseline. The patients who had a lower disease activity after treatment also underwent clinical assessments at the end of the follow-up period. The assessment consisted of the number of swollen joints (28 joints were evaluated) and the number of tender joints (28 joints were evaluated). Cut points of DAS28 for low (<3.2), moderate (3.2–5.1), and high (>5.1) disease activity were used.

Laboratory assessments included complete blood counts, the levels of rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPAs), CRP, and the erythrocyte sedimentation rate (ESR) of the patients.

### Cell culture and cell treatment

The synovial tissue specimens were minced into small pieces and incubated with 1 mg/ml Type I collagenase (Sigma-Aldrich, Bornem, Belgium, Germany) in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C for 2 h. The cells were collected by filtering the suspension through nylon mesh (70 μm), followed by extensive washing with phosphate-buffered saline. Then, RA synovial fibroblasts (RASFs) were cultured in complete high-glucose DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics in 6-well plates for 24 h. The concentration of the cells was $5 \times 10^4$ cells per well in 1 ml medium.

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 RA patients and then cultured in RPMI 1640 (HyClone, Logan, UT, USA) with 10% FBS and 1% antibiotics. PBMCs were cultured in 24-well plates at a concentration of $1 \times 10^6$/well with 1 ml medium (or medium with soluble ST2 1 μg/ml) for 24 h. Every sample was divided into two groups: control or soluble ST2 treated. After 24 h of treatment, we collected the PBMCs and the supernatants and then determined the protein or mRNA levels of cytokines, including IL-1β, IL-33, IL-6, IL-8, and tumor necrosis factor-α (TNF-α). We detected the above cytokines in supernatants with ELISA, and if the cytokine was undetectable, we then evaluated the cytokine mRNA levels by real-time-quantitative polymerase chain reaction (RT-qPCR).

### Real time-quantitative polymerase chain reaction assays

Total RNA was extracted from the RASF cells with an RNeasy mini kit (QIAGEN, USA). Reverse transcription was performed with the RevertAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA) according

to the manufacturer's instructions. The resulting cDNA was subjected to PCR and real-time PCR analyses. RT-qPCR analyses were then performed with SYBR Green Master mix kit (Applied Biosystems, Foster City, CA, USA) and primers for GAPDH, IL-1β, TNF-α, IL-6, IL-8, and IL-33. The primer sequences were as follows: GAPDH forward, 5'-AAGGTGAAGGTCGG AGTCAA-3', GAPDH reverse, 5'-AATGAAGGGGTCATTGATGG-3'; IL-1β forward, 5'-GCTGAGGAAGATGCTGGTTC-3', IL-1β reverse, 5'-GTGATCGTACAGGTGCATCG-3'; TNF-α forward, 5'-CGAGTGACAAGCCTGTAGC-3', TNF-α reverse, 5'-GGTGTGG GTGAGGAGCACAT-3'; IL-6 forward, 5'-GCCCAGCTATGAACTCCTTCT-3', IL-6 reverse, 5'-GAAGGCAGCAGGCAACAC-3'; IL-8 forward, 5'-AACTTCTCCAC AACCCTCTG-3', IL-8 reverse, 5'-TTGGCAGCCTTCCTGATTTC-3'; and IL-33 forward, 5'-GGTGTTACTGAGTTACTATGAA-3', IL-33 reverse, 5'-GGAGCTCCACAGAGTGTTCCTTG-3'.

### ELISA assay

Serum samples and SF were obtained and stored at −70°C until analysis. Soluble ST2 was quantified with an R and D Systems human IL-1R4/ST2 DuoSet ELISA. IL-1β was measured with a human IL-1 beta ELISA Ready-SET-Go! kit (eBioscience, USA). IL-6 and IL-8 ELISA kits were purchased from Neobioscience Technology Co, Ltd., Beijing, China. The ELISA procedure was performed according to the instructions of the manufacturer.

### Statistical analysis

Statistical analyses were performed with SPSS 17.0 (SPSS, Chicago, Illinois, USA). Continuous variables from the study were analyzed by the Student's *t* test with a parametric distribution or the Mann-Whitney *U* test with a nonparametric distribution. The association between clinical and laboratory parameters was evaluated with Spearman's correlation coefficients. Differences between cell groups were evaluated by the Mann-Whitney *U* test or the Wilcoxon signed-rank test, when appropriate. A value of $P < 0.05$ was considered statistically significant.

## Results

### Soluble ST2 levels in serum of rheumatoid arthritis patients and healthy controls and in synovial fluid of rheumatoid arthritis and osteoarthritis patients

We first assessed the soluble ST2 levels in the serum of 56 RA patients and 38 HCs. The characteristics of RA patients are shown in Table 1. RA patients were found to have significantly higher serum levels of soluble ST2 (127.14 ± 61.43 pg/ml) than that of HCs (78.37 ± 41.93 pg/ml, $P < 0.01$; Figure 1a). To extend the study, we then measured the soluble ST2 levels in the SF of another thirty RA patients and twenty OA patients. SF levels of soluble ST2 tended to be higher in RA patients (41.90 ± 33.58 pg/ml) than that in OA patients (19.71 ± 16.72 pg/ml, $P < 0.05$; Figure 1b).

**Table 1: Clinical characteristics of RA patients**

| Characteristics | RA patients ($n$ = 56) |
|---|---|
| Age (years), mean (range) | 56.4 (34–75) |
| Sex, *n* | |
| Female | 51 |
| Male | 5 |
| Duration (years), mean (range) | 8.4 (0.5–30.0) |
| TJC, mean (range) of 28 joints | 10.6 (2.0–25.0) |
| SJC, mean (range) of 28 joints | 7.5 (3.0–20.0) |
| CRP (mg/L), mean (range) | 23.20 (1.99–78.90) |
| DAS28, *n* | |
| HDA | 15 |
| MDA | 27 |
| LDA | 14 |
| ESR (mm/h), mean (range) | 42.9 (3.0–94.0) |
| RF, *n* | |
| Positive | 44 |
| Negative | 12 |
| ACPA, *n* | |
| Positive | 53 |
| Negative | 3 |

TJC: Tender joint count; SJC: Swollen joint count; CRP: C-reactive protein; DAS28: Disease Activity Score in 28 joints; HDA: High disease activity (DAS28 >5.1); MDA: Moderate disease activity (3.2< DAS28 ≤5.1); LDA: Low disease activity (2.6< DAS28 ≤3.2); ESR: Erythrocyte sedimentation rate; RF: Rheumatoid factor; ACPA: Anti-citrullinated protein antibodies; RA: Rheumatoid arthritis.

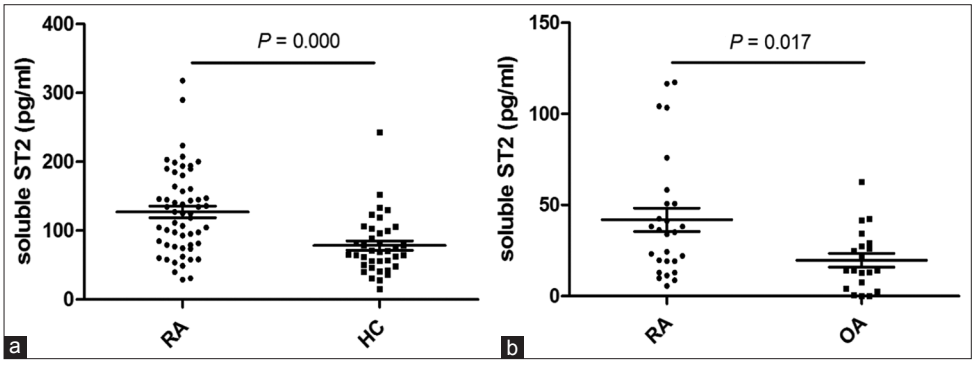


**Figure 1:** Soluble ST2 was elevated in serum and synovial fluid. Levels of soluble ST2 were assessed by ELISA in the serum of patients with RA and of HC participants (a), as well as in synovial fluid of patients with RA and OA (b). The results were showed as individual values (symbols) and means (line) for each group of patients. The differences between the two groups were assessed via Student's *t* test (a) and Mann-Whitney *U* test (b). RA: Rheumatoid arthritis; HC: Healthy control; OA: Osteoarthritis.

### Association of soluble ST2 with laboratory parameters and disease activity

The 56 RA patients were included in a follow-up study. Of all the 56 RA patients who received DMARD therapy, 24 patients had a lower disease activity than at baseline. We detected soluble ST2 levels at the end of the follow-up period and compared these with baseline values. We found that soluble ST2 levels (113.01 ± 53.90 pg/ml) were lower than baseline values (139.59 ± 68.36 pg/ml) and the difference was statistically significant ($P = 0.01$, Figure 2a).

To further confirm the result, we compared the soluble ST2 levels in patients with different disease activities. We found that RA patients with high disease activity had higher soluble serum ST2 levels (162.02 ± 56.78 pg/ml) than those with low disease activity (94.67 ± 40.27 pg/ml, $P = 0.001$; Figure 2b).

However, interestingly, we found that the serum levels of soluble ST2 had no correlation with CRP, ESR, DAS28-CRP, or antibodies, including anti-keratin antibody (AKA), RF, and ACPA (data not shown).

### Soluble ST2 did not affect interleukin-1β, interleukin-6, interleukin-8, or tumor necrosis factor-α expression in peripheral blood mononuclear cells from rheumatoid factor patients

IL-33 was undetectable at both the mRNA and protein levels, and IL-1β and TNF-α were detectable only at the mRNA level. IL-6 and IL-8 were detectable in the culture supernatants. In some patients, soluble ST2 showed negative effects on IL-1β, TNF-α, IL-6, and IL-8 expressions, but in other patients, there was no suppressive function on these cytokines. There were no significant differences in IL-1β [Figure 3a], TNF-α [Figure 3b], IL-6 [Figure 3c], and IL-8 [Figure 3d] levels before and after soluble ST2 treatment.

### Soluble ST2 suppressed interleukin-1β and interleukin-33 expressions in resting rheumatoid arthritis synovial fibroblasts

Soluble ST2 was higher in synovial fluid from RA patients than in that from patients with OA, but there was no information about the function of ST2 on RASFs. Therefore, we obtained RASF cells from three RA patients. Every

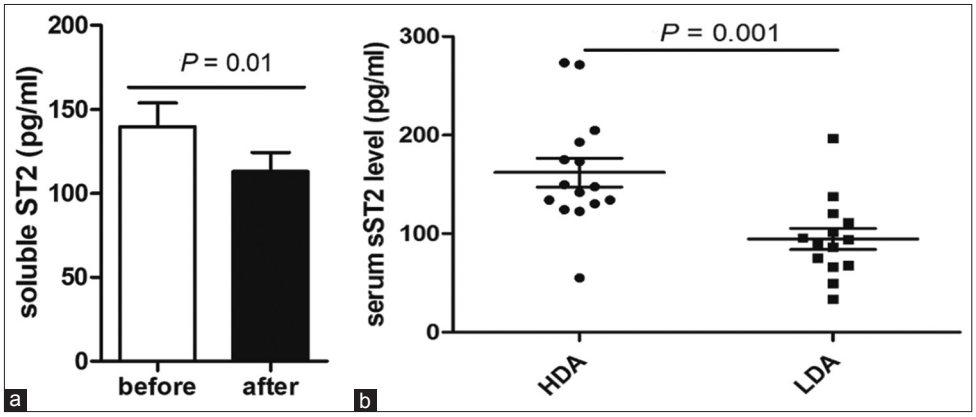


**Figure 2:** Serum levels of soluble ST2 were associated with disease activity. (a) The serum levels of soluble ST2 were decreased in patients who received effective DMARD therapy. (b) The serum levels of soluble ST2 were higher in patients with HDA (DAS28 >5.1) compared to patients with LDA (DAS28 ≤3.2). HDA: High disease activity; LDA: Low disease activity; DAS28: Disease Activity Score in 28 joints.

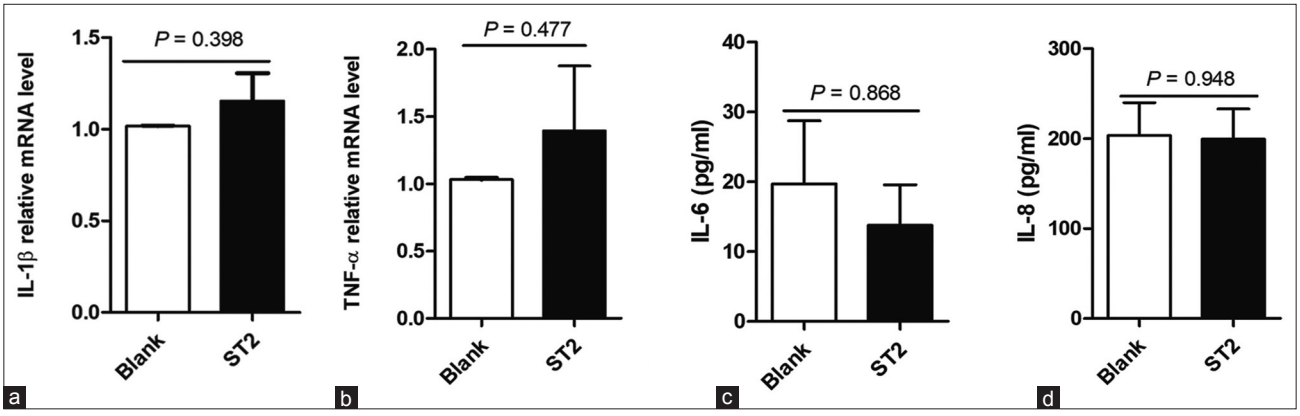


**Figure 3:** Function of soluble ST2 in PBMCs. PBMCs were treated with 1 μg/ml soluble ST2 for 24 h, the TNF-α and IL-1β transcriptional levels were determined via real-time PCR (a and b) and the IL-6 and IL-8 protein levels were assessed with ELISA (c and d). Soluble ST2 had no effects on the inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8. The results are presented as the mean ± standard deviation. The differences between the two groups were assessed by paired *t*-tests. PBMCs: Peripheral blood mononuclear cells; TNF-α: Tumor necrosis factor-α; IL: Interleukin; PCR: Polymerase chain reaction.

sample was divided into two groups: control and soluble ST2 treated. After 24 h of culture, we collected the cells and the supernatants. We detected IL-1β, IL-33, IL-6, IL-8, and TNF-α. At the protein level, all of them were undetectable. At the mRNA level, IL-1β and IL-33 were suppressed by soluble ST2 [Figure 4a and 4b], but no suppression of IL-6, IL-8, or TNF-α was found.

### Soluble ST2 suppressed interleukin-33, interleukin-1β, tumor necrosis factor-α, interleukin-6, and interleukin-8 expressions in activated rheumatoid arthritis synovial fibroblasts

Soluble ST2 exerted a suppressing role on IL-1β in RASFs but not in PBMCs, which may be attributed to the expression of IL-33 in RASFs. Therefore, if we increase the level of IL-33 in RASFs, soluble ST2 may play a negative role on more cytokines, such as IL-6, IL-8, and TNF-α. To examine this hypothesis, we activated RASF cells by exposure to TNF-α plus IL-1β for 24 h, and then RASFs showed higher IL-33 expression [Figure 5a]. The activated RASFs were treated with 1 μg/ml ST2 for 24 h. Our findings demonstrated that soluble ST2 suppressed IL-33 [Figure 5b], IL-1β [Figure 5c], TNF-α [Figure 5d], IL-6 [Figure 5e], and IL-8 [Figure 5f] expressions in RASFs.

## Discussion

In the present study, we demonstrated elevated levels of soluble ST2 both in serum and synovial fluid of RA patients and found that soluble ST2 negatively affected both IL-1β and IL-33 in RASF cells. The soluble ST2 levels in serum and synovial fluid were markedly elevated in RA patients compared to those in HCs and OA patients, respectively. The latter finding was not consistent with a recent study[13] but was consistent with a former study.[14] We thought that there might be two possible reasons for this discrepancy: (1) all our synovial fluid samples were fresh samples that had been acquired in the last 6 months and (2) the sample size was larger than in the recent study.

According to our knowledge, studies on the correlation between soluble ST2 and disease activity in RA were limited. Very recently, Shen *et al*. demonstrated that soluble ST2 was decreased after treatment and lower baseline soluble ST2 was an independent predictor of remission of early RA.[11] However, their finding was limited in the early RA patients. Another study found that soluble ST2 was correlated with disease activity in patients with systemic juvenile idiopathic arthritis.[15] We found that all patients in our follow-up who had a good response to the therapy had lower levels of soluble ST2 than at baseline. This result to a certain extent was consistent with a recent study[16] that utilized seven naïve RA patients and compared the soluble ST2 levels before and after treatment. In our current study, there was no significant correlation between soluble ST2 and disease activity or laboratory parameters (including CRP, ESR, RF, ACPA, and AKA). Our findings indicated that the soluble ST2 was only an index associated with RA disease activity, but not directly correlated with the disease activity.

We also investigated the function of soluble ST2 in RA. As soluble ST2 is a decoy receptor for IL-33,[17] several studies have focused on the function of IL-33 in heart disease[18] or allergic airway inflammation.[12] Hayakawa *et al*.[12] have demonstrated that soluble ST2 can block IL-33 signaling in allergic airway inflammation. Ohto-Ozaki *et al*.[19] demonstrated *in vivo* that ST2 can protect against the IL-33-induced Th2-type immune response in ST2 transgenic

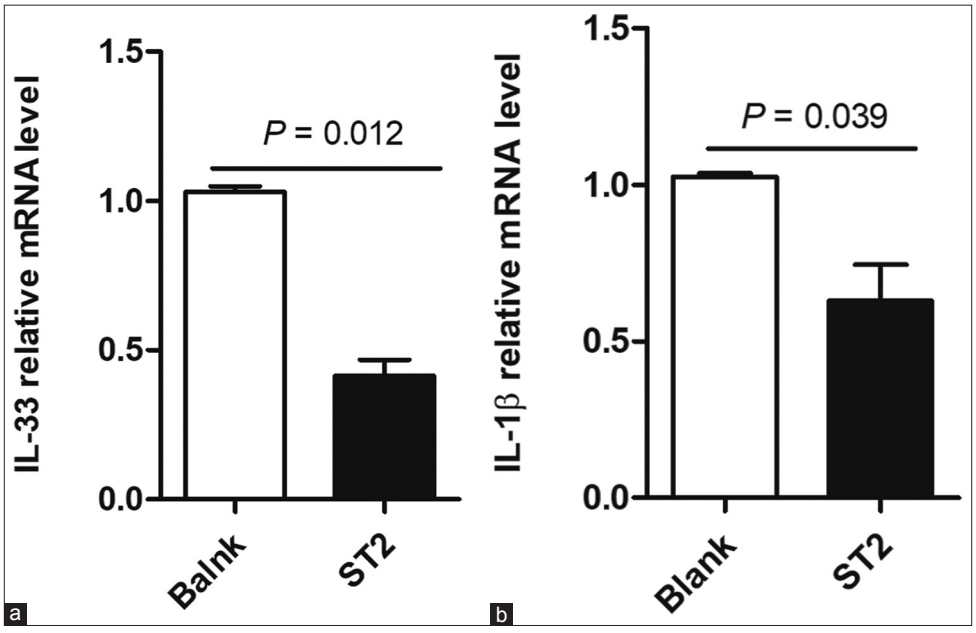


**Figure 4:** Function of ST2 in resting RASFs. RASFs were treated with 1 μg/ml soluble ST2 for 24 h, and the TNF-α and IL-1β transcriptional levels were determined via real-time PCR. Soluble ST2 ameliorated IL-33 (a) and IL-1β (b) expressions in RASFs. RASFs: Rheumatoid arthritis synovial fibroblasts; TNF-α: Tumor necrosis factor-α; PCR: Polymerase chain reaction; IL: Interleukin.

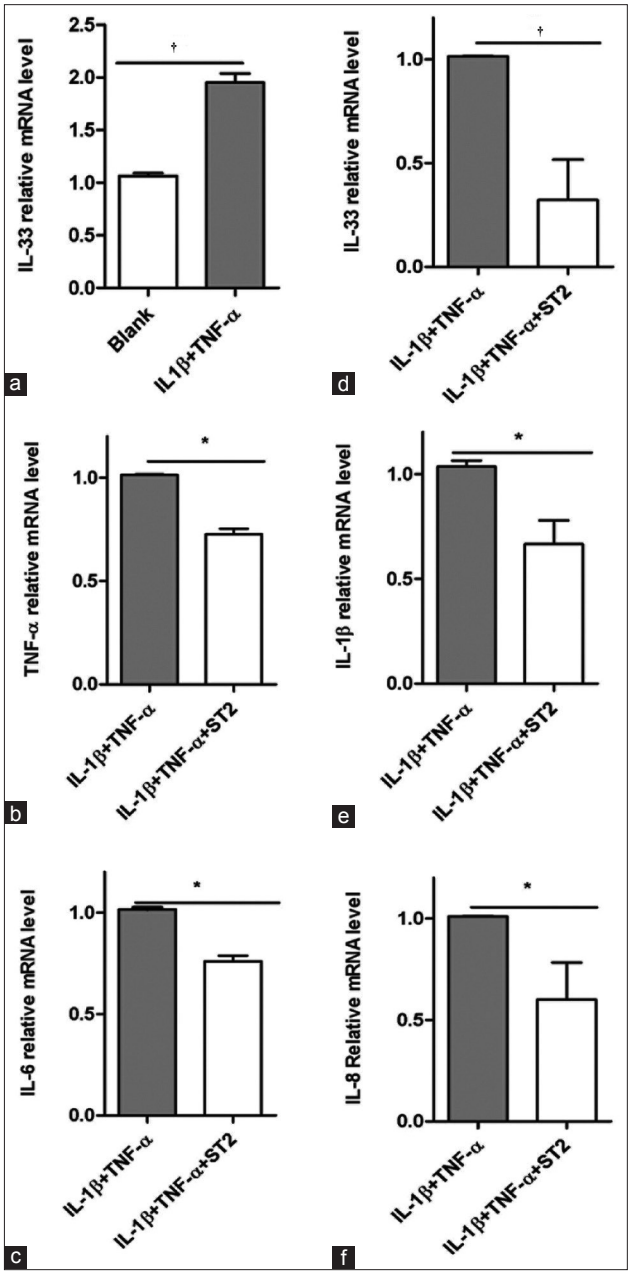


**Figure 5:** Function of ST2 in activated RASF cells. (a) RASFs were treated with TNF-α plus IL-1β for 24 h, and IL-33 expression increased. (b-f) After treatment with TNF-α and IL-1β for 24 h, the RASFs were exposed to new culture medium and treated with 1 μg/ml ST2. Soluble ST2 showed suppressive effects on IL-33, TNF-α, IL-1β, IL-6, and IL-8. The bar chart shows cumulative data as the mean ± standard deviation of three independent experiments. *$P < 0.05$, †$P < 0.01$. RASFs: Rheumatoid arthritis synovial fibroblasts; TNF-α: Tumor necrosis factor-α; IL: Interleukin.

mice. All studies have consistently indicated that ST2 can selectively block the IL-33 response. Another study found that soluble ST2 suppressed LPS-induced IL-6 production in THP-1 cells.[7] In our study, there were elevated ST2 levels in RA patients' serum and synovial fluid, and in the subsequent *in vitro* study, we found that soluble ST2 could suppress not only IL-33 but also IL-1β in resting RASFs. In activated RASFs, ST2 negatively affected IL-33, IL-1β, TNF-α, IL-6, and IL-8. In the PBMC culture study, soluble ST2 showed no effects on these inflammatory cytokines which might be attributed to the fact that there was no IL-33 expression in human PBMCs.[17] Therefore, we thought that the negative effects of soluble ST2 on the above inflammatory cytokines might depend on IL-33 levels and these inflammatory cytokines might be downstream of IL-33.

IL-33, the ligand of ST2, plays a very important role in RA.[4-6] The relationship between IL-1β and IL-33 has been explored in several studies.[20,21] The finding that IL-1β could increase IL-33 expression is consistent with a recent study. In the present study, we also demonstrated the role of soluble ST2 on cytokines, including IL-33, IL-1β, TNF-α, IL-6, and IL-8. After soluble ST2 treatment, the expressions of both IL-1β and IL-33 were downregulated in resting RASFs, but the expressions of TNF-α, IL-6, and IL-8 were not affected. However, in the RASFs activated by TNF-α plus IL-1β, the negative effects of soluble ST2 extended to TNF-α, IL-6, and IL-8. In addition, Hayakawa *et al*.[12] have reported that ST2 binds to IL-33, not IL-1β. It indicated that the extended effects of soluble ST2 on TNF-α, IL-6, and IL-8 might be attributed to the increased production of IL-33, not IL-1β, by TNF-α and IL-1β. Moreover, the findings also suggested that IL-33 could regulate TNF-α, IL-1β, and IL-6, as well as IL-8.

In an *in vivo* study, Leung *et al*.[22] have demonstrated that soluble ST2 could attenuate CIA. Furthermore, Matsuyama *et al*.[21] have found that patients with high levels of IL-33 in serum and synovial fluid are nonresponsive to antitumor necrosis factor therapy because of persistent IL-1β signaling. Based on our findings above, soluble ST2 could suppress not only IL-33 but also IL-33-induced inflammatory cytokines, so we speculated that ST2 might be a promising therapy choice in RA patients who had higher serum IL-33 level.

In summary, elevated and disease activity-associated soluble ST2 showed a negative regulatory function on IL-33 and inhibited expressions of IL-33-induced IL-1β, TNF-α, IL-6, and IL-8 in RASFs, which suggested that soluble ST2 might serve as a potential therapeutic candidate for RA.


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## Conflicts of interest

There are no conflicts of interest.

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