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A Novel Therapy of Murine Collagen-Induced Arthritis with Soluble T1/ST2[1]

Bernard P. Leung,[2]* Damo Xu,* Shauna Culshaw,* Iain B. McInnes,[†] and Foo Y. Liew[3]*

Rheumatoid arthritis is characterized by chronic inflammatory infiltration of the synovium, leading to eventual cartilage and bone destruction. Previously, we have reported that soluble T1/ST2 (sST2), a member of the IL-1R gene family, inhibits LPS-induced macrophage proinflammatory cytokine production. In this study, we report the therapeutic effect of sST2-Fc in the murine model of collagen-induced arthritis. A short term administration of sST2-Fc fusion protein significantly attenuated disease severity compared with controls treated with normal IgG. Histological examination revealed that while control IgG-treated mice developed severe cellular infiltration in the joints, synovial hyperplasia, and joint erosion, this pathology was profoundly reduced in sST2-Fc-treated animals. Treatment of sST2-Fc also down-regulated serum levels of IL-6, IL-12, and TNF-α. Spleen cells from the sST2-Fc-treated mice produced significantly less IFN-γ, TNF-α, IL-6, and IL-12 compared with cells from the control mice when cultured with collagen in vitro. Finally, pretreatment with ST2-Fc markedly inhibited the ability of human monocytic THP1 cells to release TNF-α when cocultured with peripheral blood T cells from rheumatoid patients. Together these results demonstrate that sST2-Fc may provide a novel approach in treating chronic autoimmune conditions by inhibiting the release of proinflammatory cytokines. *The Journal of Immunology,* 2004, 173: 145–150.

A serum-responsive gene, T1/ST2 was originally identified in fibroblasts (1–3). It was subsequently found to be expressed by mast cells (4), and by Th2, but not Th1, cells (5–7). Although T1/ST2 (henceforward called ST2 to avoid confusion with type 1 T cells) is a member of the IL-1R family, it does not bind IL-1α, IL-1β, or IL-1R antagonist (8, 9). It is an orphan receptor with no known functional ligand. In the mouse, differential mRNA processing within the *st2* gene leads to the production of both a membrane-bound form of ST2 expressed primarily by hemopoietic cells (ST2L) and a soluble form of ST2 predominantly expressed by fibroblasts (sST2)[4] (10). sST2 is identical to the extracellular region of ST2L except for an additional nine amino acids present at the C terminus of ST2. More recently, another alternatively spliced ST2 transcript has been described in humans, the functional consequences of which are unclear (11). Transcription of the *st2* gene is controlled by two distinct promoters; an upstream promoter directs transcription in hemopoietic cells such as mast cells, while a promoter 10.5 kb downstream directs fibroblast-specific expression (10). Regulation of sST2/ST2L expression between mice and humans appears to be conserved, because two promoters also control ST2/ST2L expression in human cells (12). Studies using either anti-ST2 Abs or a recombinant soluble form of ST2 have demonstrated important roles for this molecule in regulating Th1/Th2-associated immune responses in vitro and in experimental disease models in vivo (6, 13). Such data are supported by studies using ST2-deficient mice (14, 15).

Apart from regulation of disease outcome through modulation of Th1/Th2 bias, there is indirect evidence to suggest that ST2 may also be involved in inflammatory responses. We have previously reported that the sST2-Fc fusion protein suppressed inflammatory responses induced by LPS both in vitro and in vivo (16). We now report here a therapeutic role of sST2 in inflammatory arthritis.

Elevated levels of proinflammatory cytokine production characterize rheumatoid arthritis (RA) synovial inflammation (17). Moreover, successful therapeutic targeting of cytokines in RA, particularly TNF-α, has demonstrated their critical pathogenic importance. Th1 responses predominate within RA synovial T cell subsets and contribute significantly to dysregulated cytokine production (18–20). Synovial Th1 cells may drive macrophage cytokine release through secretion of IFN-γ or IL-17 (21), or may act through direct cognate cell-cell contact involving ligand pairs including LFA-1/ICAM-1 and CD69 (22, 23). In the present study, we describe a novel antiarthritic effect of sST2. sST2-Fc effectively suppressed murine collagen-induced arthritis (CIA) even administered after clinically evident onset of disease via specific suppression of the pathogenic proinflammatory responses. The clinical relevance of these observations is illustrated by parallel studies in RA-derived human cells in which the sST2-Fc fusion protein suppressed cytokine release by macrophages following cell contact-dependent interaction with activated T cells.

Materials and Methods

Animals and reagents

Male DBA/1 mice obtained from Harlan Olac (Bicester, U.K.) were used at 8-10 wk old and maintained at the Joint Animal Facilities, University of Glasgow (Glasgow, U.K.). All animal experiments conducted in this study were conducted in accordance to the Home Office, U.K. animal guidelines. Murine bone marrow-derived macrophages (BMMs) were derived from cells of the femurs of adult BALB/c mice (Harlan Olac) and maintained as previously described (16). For in vitro experiments, LPS from *Salmonella minnesota* (Sigma-Aldrich, Poole, U.K.) was used at a concentration of 100

ng/ml in some cell culture experiments. The sST2-Fc fusion protein was prepared as previously described (16). Briefly, a mammalian expression plasmid containing the sST2 cDNA linked to the human IgG1 constant region and containing the C5 signal sequence was transfected into Chinese hamster ovary cells. The sST2-Fc or human IgG control protein (Sigma-Aldrich) was purified using protein A affinity chromatography. Both purified reagents were found to contain <0.1 ng/μg LPS as assessed by the *Limulus* amoebocyte assay (Sigma-Aldrich). In some experiments, a recombinant human ST2-Fc chimera (R&D Systems, Oxon, U.K.) was used with similar results.

Induction of CIA and assessment of arthritis

CIA was elicited in mice as previously described (24). Briefly, mice were immunized by intradermal injection of 200 μg of acidified bovine type II collagen (CII; Sigma-Aldrich) emulsified in CFA (Difco, Detroit, MI). Mice were boosted i.p. with CII (200 μg in PBS) on day 21. Mice were monitored for signs of arthritis as previously described (24). Scores were assigned based on erythema, swelling or loss of function present in each paw on a scale of 0–3, giving a maximum score of 12 per mouse. Paw thickness was measured with a dial caliper (Kroeplin, Munich, Germany). For histological assessment, mice were sacrificed and the hind limbs removed, fixed in 10% neutral-buffered formalin, and 5-μm sections were stained with H&E or toluidine blue (both Sigma-Aldrich). The quantification of arthritis was by a "treatment-blind" observer and a score was assigned to each joint based on the degree of inflammation, synovial hyperplasia, and erosion as described previously (25).

Administration of sST2-Fc

To investigate the effect of sST2-Fc in murine CIA, DBA/1 mice received daily i.p. injections of recombinant sST2-Fc at doses of 100 μg/mouse from days 25–27 and then once every 3 days until day 42. Control mice received similar amounts of purified human IgG (hIgG) only.

Collagen-specific in vitro culture

Spleens were removed on day 42 after primary immunization. Single cell suspensions were prepared and cultured in triplicate at 2×10^6 cells/ml in RPMI 1640 supplement with 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES buffer, and 10% FCS (all Life Technologies, Paisley, U.K.) at 37°C in 5% CO_2. Cells were cultured with graded concentrations of CII in flat-bottom 96-well plates (Nunc, Roskilde, Denmark). Supernatants were collected after 72 or 96 h and stored at −20°C until assayed for cytokine concentration. Proliferation assays were performed in parallel cultures in U-bottom 96-well plates (Nunc) for 96 h and were pulsed with [³H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) during the last 6 h of culture. Plates were then harvested and measured for incorporation of radioactivity as previously described (24).

Cell contact experiments

Peripheral blood (PB) was collected from healthy individuals or RA patients who satisfied the America College of Rheumatology 1987 diagnostic criteria (26), following approval from the Glasgow Royal Infirmary Ethical Committee (Glasgow, U.K.). PB T cells were purified and cell contact experiments were performed as previously described (22, 23). Briefly, PB T cells were activated with PHA (1 μg/ml; Murex Diagnostics, Dartford, U.K.) and PMA (1 nM; Sigma-Aldrich) for 72 h and then fixed in 1% paraformaldehyde to preserve membrane structure. Control nonactivated T cells were also fixed for comparison. Fixed PB T cells were cocultured with the monocyte cell line THP-1 (American Type Culture Collection, Manassas, VA) that were pretreated with sST2-Ig fusion protein or hIgG (both at 10 μg/ml; R&D Systems) for 1 h. Supernatants were harvested at 48 h for TNF-α estimation.

Measurement of cytokines, sST2, and anti-collagen Ab levels

All cytokines, sST2, and anti-collagen Ab levels were detected by ELISA. The Ab pairs for TNF-α, IFN-γ, IL-5, IL-6, and IL-10 were obtained from BD Pharmingen (San Diego, CA) and assays were performed according to manufacturer's instructions. Detection limits were as follows: IL-5, IL-6, and TNF-α all at 10 pg/ml; IL-10 and IFN-γ both at 20 pg/ml. sST2 was determined by coating a 96-well plate with a purified rabbit anti-ST2 Ab (6) and detected by monoclonal anti-ST2 Ab (Morewell Diagnostics, Geneva, Switzerland). Sensitivity of the assay was 50 pg/ml. Serum anti-collagen II Ab titers of individual serum were detected with biotin-conjugated anti-mouse IgG1 or IgG2a (BD Pharmingen), followed by conjugated avidin peroxidase (Sigma-Aldrich) and developed with tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Statistical analysis

Clinical and histological scores were analyzed with the nonparametric Mann-Whitney *U* test. Differences between cumulative incidences at a given time point were analyzed by the χ^2 contingency analysis. Cytokine and collagen-specific IgG levels were compared using the Student *t* test.

Results

Soluble ST2-Fc reduces LPS-induced BMM cytokine production

To determine the bioactivity of the recombinant sST2-Fc fusion protein, we examined its effect on LPS-induced proinflammatory cytokine production from macrophages. Murine-derived BMMs were pretreated for 1 h with sST2-Fc or hIgG (both at 50 μg/ml) or medium alone and then stimulated with LPS (100 ng/ml). BMMs activated with LPS produced significant amounts of IL-6, IL-12, and TNF-α, and this production was markedly inhibited by pretreatment with sST2-Fc but not with the control hIgG (Fig. 1). In contrast, IL-10 synthesis was not modified by the sST2-Fc treatment (data not shown).

Treatment with sST2-Fc inhibits disease progress of murine CIA

We next tested the ability of sST2-Fc to inhibit the progression of murine CIA. DBA/1 mice were immunized with CII/CFA as described in *Materials and Methods*. Mice began to show clinical sign of arthritis on day 25 (4 days after boosting on day 21) after
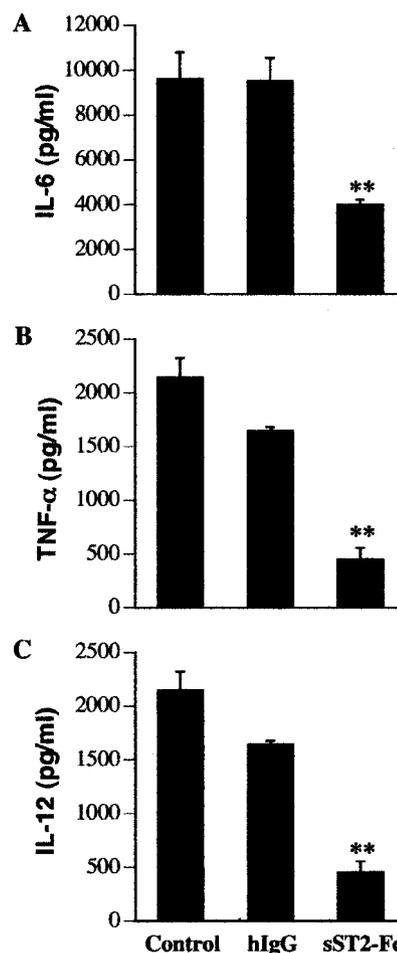

FIGURE 1. Effect of sST2-Fc on LPS-induced proinflammatory cytokine production. BMMs were pretreated with medium (control) alone, hIgG, or sST2-Fc (both at 50 μg/ml) for 1 h before stimulation with 100 ng/ml LPS for 8 h. Levels of IL-6, IL-12, and TNF-α were measured in the supernatant by ELISA. Data are mean ± SD; ∗, $p < 0.01$ by Student's *t* test.

immunization. Mice were injected i.p. daily with sST2-Fc or hIgG (both at 100 μg/dose) from days 25–27, and then once every 3 days until day 42, for a total of eight administrations. As expected, untreated mice (data not shown) and those injected with control human IgG developed arthritis reaching a peak score on day 32. In contrast, mice treated with sST2-Fc developed significantly attenuated disease ($p < 0.05$) in the mean arthritic index (Fig. 2A) and mean number of arthritic paw (Fig. 2B). There was no difference in arthritic incidence (Fig. 2C) because the treatment was initiated after the onset of disease. To examine whether sST2-Fc administration modified articular destruction, we evaluated cartilage and bone integrity histologically. Mononuclear and polymorphonuclear cell infiltration into the joint compartment, synovial hyperplasia, and adjacent cartilage, and bone erosion, were clearly evident in the IgG-treated control mice (Fig. 3A). Each of these parameters was markedly suppressed in the mice treated with
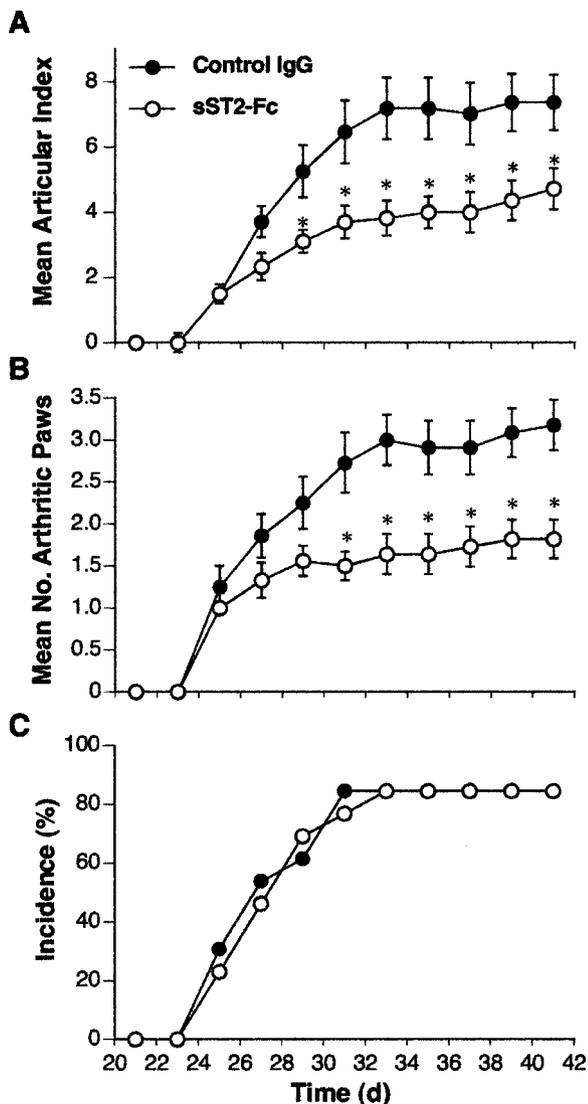

FIGURE 2. sST2-Fc attenuated the progression of murine CIA. Collagen-primed DBA/1 mice were injected i.p. with sST2-Fc or hIgG (both at 100 μg/dose, $n = 13$ mice/group) from days 25–27 and then once every 3 days until day 42 for a total of eight administrations. Mice were monitored for disease progression as indicated by (A) mean articular index, (B) mean number of arthritic paws, and (C) incidence. sST2-Fc-treated mice developed significantly less severe disease compared with control hIgG-treated mice, while incidence of arthritis remains indistinguishable. Data are mean ± SEM; *, $p < 0.05$ by Mann-Whitney U test.
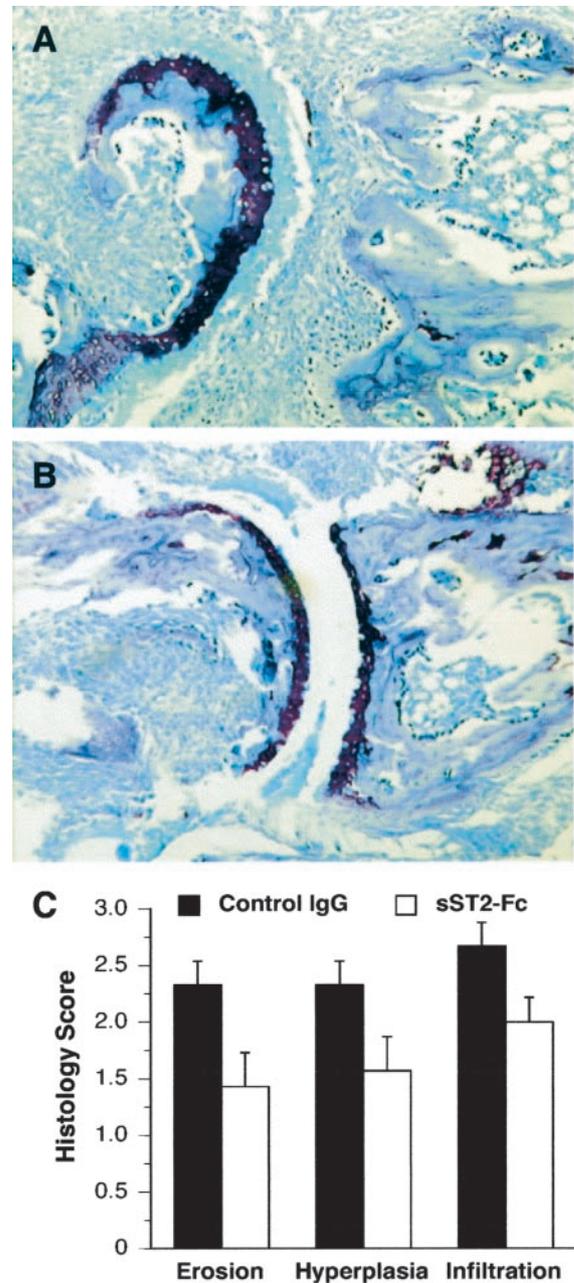
FIGURE 3. sST2-Fc treatment of mice with CIA resulted in reduced joint pathology. Mice were administered with sST2-Fc or control hIgG as described in Fig. 2. On day 42, mice were sacrificed and arthritic paws removed and stained with H&E or toluidine blue. Profound cartilage surface erosion and loss of proteoglycan was observed in the control hIgG-treated mice (A). sST2-Fc recipients exhibited reduced histologic evidence of destruction (B). Histological appearances were scored for the presence of synovial bone erosion, hyperplasia, and cellular infiltration (C). Data are mean ± SEM ($n = 6$). Original magnification (A and B), ×50.

sST2-Fc (Fig. 3B). The histological scores were summarized in Fig. 3C. Together these data clearly indicate that sST2-Fc potently suppressed the development of CIA and such activity can prevent progression of articular damage.

Serum cytokines and anti-collagen Ab production in vivo

A potential mechanism by which sST2-Fc could suppress CIA pathology is by blocking proinflammatory cytokine expression in vivo. To compare the extent of such modulation, serum cytokine

concentrations from arthritic mice were measured by ELISA at the end of the treatment period (day 42). High concentrations of IL-12, TNF-α, and IFN-γ were detected in hIgG-treated control mice. These were present at significantly ($p < 0.05$) reduced levels in the sST2-Fc-treated group (Fig. 4). IL-4, IL-5, and IL-10 were not detected (data not shown). CII-specific IgG1 and IgG2a levels were also analyzed by ELISA and found to be similar in the two groups of mice (data not shown). sST2 was not detected in the serum or the joints of untreated CIA mice (data not shown).

sST2-Fc suppresses CII-specific proinflammatory immune response in vitro

We next investigated the mechanisms of sST2-Fc-mediated suppression of articular inflammation. CIA is associated with a Th1-polarized immune response, rendering it an excellent model to explore the effect of sST2 upon functional T cell maturation in vivo. CII-specific immune responses were examined in vitro in spleen cells obtained at day 42. Cells from sST2-Fc-treated mice produced significantly less IFN-γ, TNF-α, IL-6, and IL-12 compared with cells from control animals (Fig. 5). However, T cell proliferation and IL-10 production remained similar between the two groups. IL-4 and IL-5 were not detectable in the culture supernatant of both groups of mice (data not shown). Immune mod-
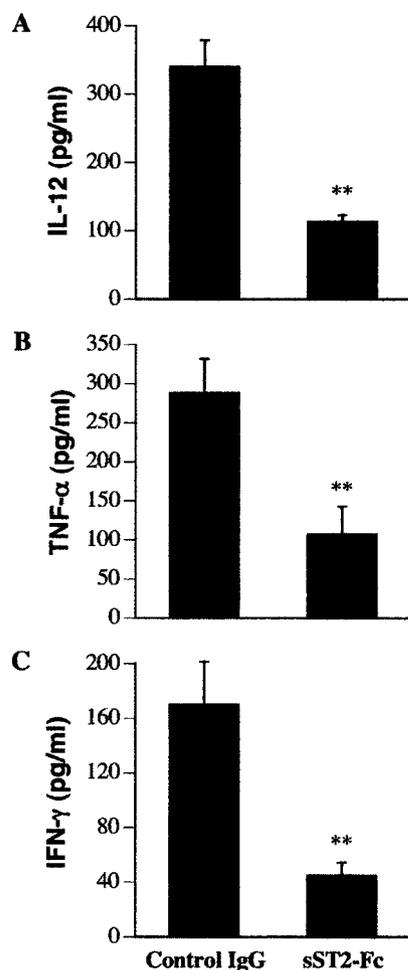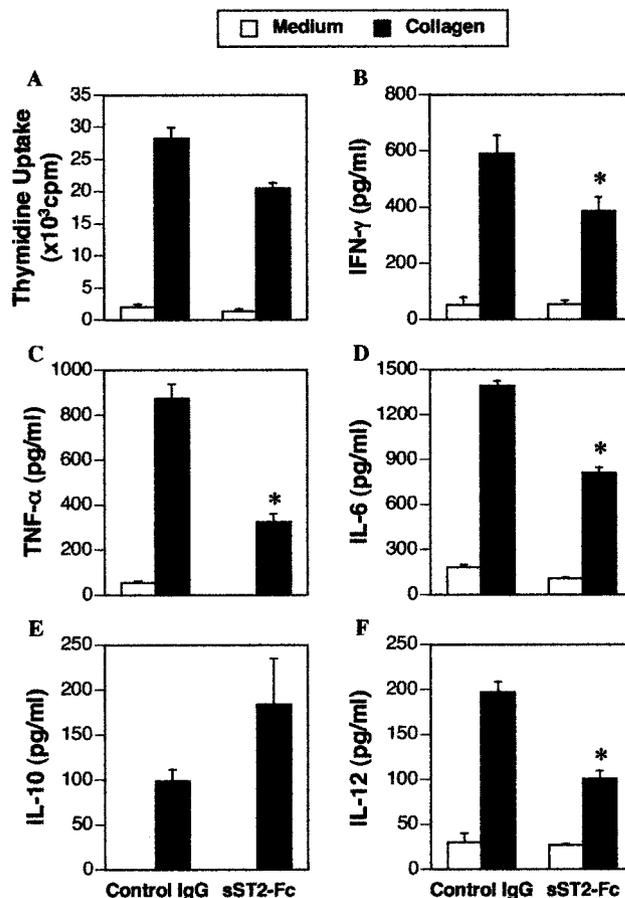

FIGURE 5. sST2-Fc reduces in vitro Ag-specific proinflammatory cytokine production. Spleen cells ($n = 5$ mice/group) were harvested from mice (as in Fig. 2) on day 42 and cultured with CII (50 μg/ml) for up to 96 h. Cytokine concentrations in the culture supernatant (72 h for IL-6, IL-12, and TNF-α; 96 h for IL-10 and IFN-γ) were determined by ELISA. T cell proliferation was assayed by uptake of [^{3}H]thymidine after 96 h. T cell proliferation was found to be similar between both groups of mice (A). Significant suppression of IFN-γ (B), TNF-α (C), IL-6 (D), and IL-12 (F) production was observed in cultures of spleen cells from sST2-Fc-treated mice compared with control animals. There was no difference in IL-10 production (E). Data are mean $\pm$ SEM of triplicate cultures; *, $p < 0.05$ by Student's t test.

ulation by sST2-Fc in vivo was Ag-specific because Con A-induced production of IFN-γ, TNF-α, and IL-6 in parallel cultures was not affected (data not shown).

sST2-Fc inhibits cell contact-induced TNF-α production

T cells from RA synovium or PB, particularly those activated by PHA/PMA, IL-15, or IL-18, are capable of driving macrophages to produce TNF-α in a cell contact-dependent manner (22, 23, 27). Furthermore, we have also shown that sST2-Fc suppressed proinflammatory cytokine production by macrophages in response to LPS (16). Therefore, we investigated the ability of sST2-Fc to influence the production of TNF-α by macrophages in response to activated T cells in the cell contact coculture system (23). Purified PB T cells from normal or RA individuals were cultured with PHA and PMA, fixed with paraformaldehyde, and then cocultured with human THP-1 monocytic cells, which had been pretreated for 1 h with sST2-Fc or normal IgG. As expected, activated T cells induced substantial TNF-α production by THP-1 cells treated with normal IgG. Such TNF-α synthesis was markedly reduced when the THP-1 cells were treated with sST2-Fc (Fig. 6). Therefore, these

FIGURE 4. Effect of sST2-Fc treatment on serum proinflammatory cytokine concentrations. DBA/1 mice treated with either purified sST2-Fc or control hIgG (as in Fig. 2 above) were sacrificed on day 42 and serum was collected from eight mice in each group. Levels of IL-12 (A), TNF-α (B), and IFN-γ (C) were determined by ELISA of individual samples. Data are mean $\pm$ SEM; *, $p < 0.01$ by Mann-Whitney U test.
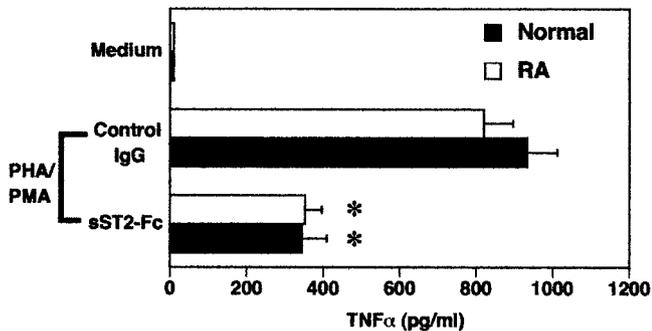
FIGURE 6. Effect of sST2-Fc on cell contact induction of TNF-α production by macrophages. Peripheral blood T cells purified from normal or RA (*n* = 3/group) individuals were stimulated with PHA and PMA for 72 h, fixed in paraformaldehyde, and cocultured with THP-1 monocytic cells, which were either pretreated with sST2-Fc or hIgG (both at 10 μg/ml) for 1 h. TNF-α concentrations in the supernatant after 48 h were measured by ELISA. sST2-Fc significantly suppressed TNF-α released by THP-1 cells. Data are mean ± SEM of a representative sample from each group; *, $p < 0.05$ by Student's *t* test.

results demonstrate that the suppressive effect of sST2-Fc extends to the proinflammatory cytokine synthesis in a system directly relevant to clinical arthritis. sST2 was not detected in the synovium of RA patients (data not shown).

Discussion

There is currently considerable interest in the potential of immune modulatory therapies in the treatment of inflammatory diseases, particularly that targeting cytokine expression (28). Our data indicate that sST2 provides an effective novel therapy in treating murine CIA, a surrogate model for human RA. We have previously shown that sST2 reduced toxic-shock mortality in mice. We now show that sST2 can suppress the progression of acute to chronic inflammation in vivo. Importantly, sST2 administration was effective even after the onset of arthritis. Whether the treatment is effective against a fulminating CIA is currently unknown. These results indicate that sST2-Fc may find therapeutic use in a variety of autoimmune conditions. They further suggest that exploration of sST2-targeted pathways may reveal novel immune regulatory events.

Pathways that drive cytokine production in RA synovium are unclear but include a significant contribution by T cells, either through the direct release of IFN-γ or via cell contact (29). Our data indicate that sST2 suppresses at least the cell contact pathway by directly blocking macrophage activation. Whether sST2 also has a direct influence on T cell functions is currently unknown. The effect upon macrophage function was seen when the cells were cocultured with activated T cells from both RA and healthy donors. This study was not powered or designed for formal comparison of sST2 sensitivity in RA with healthy donor T cells, and our data should suggest only that sST2-sensitive pathways are maintained in the disease state. It is of interest to note that sST2 was not detected in the serum or joints of untreated CIA mice. Although this may reflect the relative sensitivity of the ELISA used, it could well be that the absence of sST2 contributed to the disease state of the mice.

The receptor on macrophages responsible for binding sST2 is at present unknown. Although sST2 has significant homology with IL-1R, it did not bind to known members of the IL-1 family (8, 9, 30). cDNA that encodes a ST2-binding protein has been identified, although signaling through ST2L-bearing 3T3 cells in response to this protein (9) has not been found. Given that the ST2-binding

protein contains a transmembrane sequence and a short 12 amino acid intracellular domain, the possibility that it is involved in recognition of sST2 by macrophages and subsequent signaling should be considered. We have detected expression of the ST2 binding protein in murine bone marrow macrophages by RT-PCR (M. J. Sweet, unpublished observation). Alternatively, sST2 may bind to an as yet unidentified member of the IL-1R family.

In a previous study, we demonstrated that sST2 suppressed the expression of TLR1 and 4, and that this may contribute, at least in part, to the anti-inflammatory effect of sST2 in macrophages (16). The role of TLRs in RA has now been consistently demonstrated (31–35). For example, the TLR2 signaling pathways have been reported to contribute to the pathogenesis of RA (32, 33), and is required for streptococcal cell wall-induced joint inflammation in mice (35). Furthermore, innate immune functions via TLR4 might perpetuate inflammatory mechanisms and bypass the need for IL-1 in chronic joint inflammation (31). TNF-blocking treatment ex vivo significantly inhibited TLR4 expression on dendritic cells from RA patients (34). Therefore, it is conceivable that sST2 inhibits CIA through its down-regulation of TLR4 and TLR1. TLR1 is required as a coreceptor for TLR2 signaling (36). It would be of considerable interest to define the sST2 receptor and the mechanism by which it transmits a signal leading to the inhibition of TLR4 and TLR1 expression.

In conclusion, we have demonstrated in this study a novel therapeutic role for sST2 that may be applicable to RA. ST2 has been shown to be selectively expressed on Th2, and not Th1, cells (6, 7), and modulation of ST2L expression regulated Th2 functions in infections (6) and in allergic reactions (13). In addition to its role in reduction of endotoxic shock (16), sST2 is now demonstrated in the present study to markedly attenuate Ag-specific inflammatory responses in vitro and in vivo. Therefore, it is possible that sST2 will also play a beneficial role in other Ag-specific inflammations. Appropriate clinical studies are now required to test this hypothesis in vivo in inflammatory arthritis.

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