Inhibition of Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase Substrate-1 Reduces the Severity of Collagen-Induced Arthritis

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ABSTRACT. *Objective.* To investigate whether the blockade of Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) has any therapeutic effects on rheumatoid arthritis.

Methods. A functional blocking monoclonal antibody for SHPS-1 (anti-SHPS-1 mAb) was administered at various doses to collagen-induced arthritis (CIA) mice, and severity of the arthritis was evaluated by clinical and histological scores of the limbs. To clarify the mechanisms of action of the antibody, the serum concentration of anti-type II collagen antibody was measured in those mice, and *in vitro* experiments were conducted to determine the effects of the antibody on the induction of osteoclasts and the release of cytokines from mouse spleen cells.

Results. Compared with mice given control IgG, the administration of anti-SHPS-1 mAb significantly reduced the severity of inflammation and destruction of bone and cartilage in CIA mice. This therapeutic effect was observed even when the antibody treatment was started after the onset of arthritis. The appearance of anti-type II collagen antibody in CIA mice was not altered by the antibody treatment. In *in vitro* experiments, the anti-SHPS-1 mAb significantly inhibited osteoclastogenesis of bone marrow cells, and significantly reduced the release of interleukin 1ß (IL-1ß), IL-2, IL-12, interferon-γ, and tumor necrosis factor-α, but not that of IL-4 or IL-10, from the spleen cells after stimulation with concanavalin A.

Conclusion. Administration of a monoclonal antibody for SHPS-1 reduced the severity of arthritis in CIA mice. Regulation of biological functions of SHPS-1 may be a novel and potent strategy to treat patients with rheumatoid arthritis. (First Release Nov 1 2008; J Rheumatol 2008;35:2316–24; doi:10.3899/jrheum.080369)

Key Indexing Terms:
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Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects synovial joints systemically[1]. In spite of numerous investigations, there is still no fundamental therapy to treat RA. The hallmarks of this disease are synovial inflammation and destruction of articular cartilage and subchondral bone. Synovial tissue in rheumatoid joints is characterized by a marked intimal-lining hyperplasia due to increased numbers of macrophages and fibroblast-like synoviocytes. Accumulation of T cells, plasma cells, and other types of inflammatory cells in the synovial lining is also obvious[1,2]. Those cells produce cytokines such as interleukin 1ß (IL-1ß), tumor necrosis factor-α (TNF-α), and IL-6, which promote the expression of proteinases that cause tissue degradation in the joints. As well, those cytokines are responsible for the destruction of bone in the disease through the induction of osteoclasts. In joints involved in RA, osteoclasts are derived from precursor cells of the monocyte-macrophage lineage in the presence of several cytokines such as macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-κB ligand (RANKL), IL-1ß, and

TNF-α[3,4]. Therefore, the synovial hyperplasia and bone absorption around the joints in RA is closely correlated through the activity of those cytokines.

Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1), also known as signal regulatory protein α1 (SIRPα1)[5], a brain Ig-like molecule with tyrosine-based activation motifs[6], macrophage fusion receptor[7], and p84 neural adhesion molecule[8], is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. The extracellular domain of SHPS-1 consists of 3 parts, the amino-terminal Ig variable (IgV) region and 2 Ig constant (IgC) regions, although the latter may be removed by alternative splicing. The intracellular domain of SHPS-1 contains 2 immunoreceptor tyrosine-based inhibitory motifs, suggesting that it transmits signals for inactivation[9]. SHPS-1 is expressed by macrophages, dendritic cells (DC), neutrophils, and neurons[10]. CD47, another transmembrane glycoprotein belonging to the immunoglobulin superfamily, is a known ligand for SHPS-1[11-14]. CD47 is present on virtually all kinds of hemopoietic cells, including T cells, B cells, and neutrophils, as well as endothelial cells. SHPS-1 binds to CD47 via the IgV domain[11,15-18], which causes its various biological activities[19]. Interactions between SHPS-1 and CD47 are important for cellular fusion or multinucleation, processes necessary for osteoclast formation[7,11,20]. At this time, it is controversial whether CD47-SHPS-1 interaction plays a key role in the activation of T cells and the acquisition of cell-mediated immunity[21,22], or downregulates the activation of T cells by DC[16,17,23,24]. Considering that T cell activation and osteoclast formation are critical events in the pathology of RA, we speculated that inhibiting the interaction between SHIP-1 and CD47 might be beneficial in the treatment of RA.

We previously reported that an antibody against SHPS-1 effectively inhibits the migration of epidermal DC and Langerhans cells, resulting in decreased development of the delayed-type hypersensitivity response[23,24]. Using this SHPS-1 antibody, we conducted a series of *in vivo* and *in vitro* experiments to clarify the role of SHPS-1 in the pathology of RA. The results not only suggested the significance of SHPS-1 in RA, but also indicated a possibility that the administration of the anti-SHPS-1 antibody could be an effective strategy to treat patients with RA.

MATERIALS AND METHODS

Collagen-induced arthritis (CIA). Our study was performed under the approval of the Institutional Review Board of the National Hospital Organization, Sagamihara Hospital. The induction of arthritis in mice was based on a described method[25,26]. Briefly, bovine type II collagen (CII; Collagen Research Center, Tokyo, Japan) was dissolved at 2 mg/ml in 10 mM acetic acid, and was emulsified by mixing with an equal volume of Freund's complete adjuvant (Nippon BD, Tokyo, Japan). Five-week-old male DBA/1JN mice (Charles River Japan, Yokohama, Japan) were immunized by intradermal injection of the emulsion (100 μl) at the base of their tails. Twenty-one days later, the same volume of emulsion was injected

again in the same manner as a booster. With this protocol, arthritis developed in 100% of mice at around 4 weeks after the initial immunization.

Treatment with anti-SHPS-1 monoclonal antibody. Hybridoma cells producing anti-mouse SHPS-1 (P84) monoclonal antibody (anti-SHPS-1 mAb) was a generous gift from Dr. C.F. Lagenaur (Pittsburgh University, Pittsburgh, PA, USA)[27]. Ascites fluid was collected from BALB/c nu/nu mice that had been injected intraperitoneally with hybridoma cells, and the p84 antibody was purified from the ascites using a protein A column[23,24]. Rat IgG1 (Sigma Diagnostics, St. Louis, MO, USA) was used as a control immunoglobulin (control IgG). The experiments were performed according to either of the following 2 protocols. In Protocol A, CIA was induced as described, and anti-SHPS-1 mAb, control IgG, or methotrexate (MTX; Wyeth, Tokyo, Japan) were given to the mice every other day from Day 21 (the day of second immunization) until Day 31, 6 times in total. The antibody or control IgG was dissolved in 200 μl phosphate buffered saline (PBS) and injected intraperitoneally. MTX was administered orally. In Protocol B, the administration of the SHPS-1 antibody, control IgG, or MTX was started on Day 29 (8 days after the second immunization) and was repeated 6 times until Day 39, on every other day.

Evaluation of arthritis. The development of arthritis was determined by the presence of redness or swelling in any of the 4 limbs. If these signs were observed in at least 1 limb, the mouse was determined to be positive for arthritis. The incidence of arthritis was defined in each treatment group by the ratio of the number of positive mice to the total number of mice in the group. The occurrence and severity of arthritis were evaluated macroscopically on each hind limb in each mouse by scores from 0 (normal) to 3 (joint deformity or rigidity). The sum of scores for both hind limbs (0 to 6) was used as the clinical score for that animal. The severity of arthritis was also evaluated by the average thickness of footpads of the right and left hind limbs, which was measured using a caliper. The body weight was recorded daily throughout the experimental period.

Histological evaluation. For histology, the mice were sacrificed and their hind limbs were amputated, fixed with 10% formaldehyde, decalcified with EDTA, and embedded in paraffin. Four-micron-thick sections of the ankle and toe joints were prepared in a sagittal plane and were stained with hematoxylin and eosin (H&E). Using a light microscope, the severity of inflammation and joint destruction was assessed semiquantitatively based on a described procedure[28]. The severity of inflammatory change was assessed as a score from 0 to 4, considering the extent of inflammatory cell infiltration, synovial lining-cell hyperplasia, and pannus formation. Further, the severity of bone destruction was evaluated by scores from 0 to 5, according to the following criteria: 0 = normal; 1 = minimal loss of cortical bone at a few sites; 2 = mild loss of cortical and trabecular bone at a few sites; 3 = moderate bone loss at multiple sites; 4 = marked bone loss at multiple sites; and 5 = marked bone loss with distortion of the profile of the remaining cortical surface.

Measurement of anti-type II collagen antibodies. The concentration of anti-CII antibodies in the sera of mice was determined by ELISA[29]. For this, 96-well flat-bottom plates (Iwaki, Tokyo, Japan) were coated with 50 μl CII (2 μg/ml in PBS) overnight at 4°C. Prior to use, the wells were blocked with PBS containing 1% (w/v) bovine serum albumin at 37°C for 1 h. Sera were then diluted appropriately in PBS containing 0.05% (v/v) Tween-20, and were added to the wells. After incubation at 37°C for 2 h, levels of CII-specific IgG2a were measured using biotin-labeled rat anti-mouse IgG2a (R&D Systems, Minneapolis, MN, USA). The amount of biotin-labeled antibody bound was determined by color reaction using streptavidin-peroxidase coupled with peroxidase substrate (Substrate Reagent Pack, Stop Solution; R&D Systems). All measurements were performed in triplicate and averages were calculated.

Effect of antibodies on osteoclast formation from murine bone marrow cells. Bone marrow cells were obtained from 6-week male Balb/c mice, and were plated in wells of 24-well plates at 1 × 10^6 cells per well. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Invitrogen), glut-

amine, streptomycin, penicillin, macrophage colony-stimulating factor (M-CSF, 50 ng/ml; R&D Systems), and RANKL (30 ng/ml; R&D Systems). In this experiment, the effect of anti-SHPS-1 mAb was compared with that of control IgG. Immediately after plating, anti-SHPS-1 mAb or control IgG was added to the media at graded concentrations, and the cells were cultured for 5 days. The medium was then removed and the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a commercial kit (Sigma Diagnostics Acid Phosphatase Kit; Sigma Diagnostics). TRAP-positive multinuclear cells that had more than 3 nuclei were counted as osteoclasts. The experiment was then repeated with an anti-CD47 monoclonal antibody, and the results were compared. The antibody against CD47 (miap301; anti-CD47 mAb) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Effect of antibodies on cytokine production by murine spleen cells. Spleen cells were obtained from 6-week male Balb/c mice, and were plated at a density of 5×10^5 cells per well in 96-well plates. Cells were maintained in RPMI-1640 medium (Invitrogen) containing 10% FBS, glutamine, streptomycin, and penicillin. One hour after plating, anti-SHPS-1 mAb, anti-CD47 mAb, or control IgG were added to the media at the indicated concentrations. One hour after the addition of antibody or control IgG, the cells were stimulated by 5 μg/ml concanavalin A (ConA; Wako, Osaka, Japan). After 24 h, the supernatants were collected and the concentrations of IL-1ß, IL-2, IL-12, interferon-γ (IFN-γ), TNF-α, IL-4, and IL-10 in the media were determined by ELISA (R&D Systems).

Statistical analysis. For parametric data, statistical significance was determined by 2-way analysis of variance and contrast as a post hoc test. Nonparametric data were analyzed using the Kruskal-Wallis test, and the Dunn procedure was used as a post-hoc test when necessary. Log-rank test was used to determine the difference in the incidence of arthritis. The level of significance was set at $p < 0.05$.

RESULTS

Anti-SHPS-1 antibody reduces incidence and severity of CIA. Six groups of mice, each consisting of 10 animals, were prepared, and CIA was induced in 5 of those groups. The other group was maintained without any treatment and served as a non-CIA control. Each of the 5 CIA-induced groups received 6 consecutive administrations of either anti-SHPS-1 mAb (1, 10, or 100 μg), control IgG (100 μg), or MTX (3 mg), following Protocol A, in which the treatments were started on the day of the second immunization. The incidence of arthritis was significantly reduced by the SHPS-1 antibody treatment (Figure 1A). While arthritis developed in all mice treated with the control IgG, the administration of 10 μg or 100 μg anti-SHPS-1 mAb reduced the incidence by 20% and 30%, respectively, although no significant reduction was observed with 1 μg anti-SHPS-1 mAb. The decline in the incidence was significant in the 10 and 100 μg antibody-treated groups (both at $p < 0.05$). The incidence of arthritis was dramatically reduced by MTX, indicating that the immune response was profoundly involved in the development of arthritis.

In untreated mice, the body weight increased by 1.8 g on average between Day 21 and Day 41 (Figure 1B). Among the 5 experimental groups, the mice treated with control IgG lost 1.0 g in weight during that period, likely due to the general exhaustion associated with the arthritis. This decline in
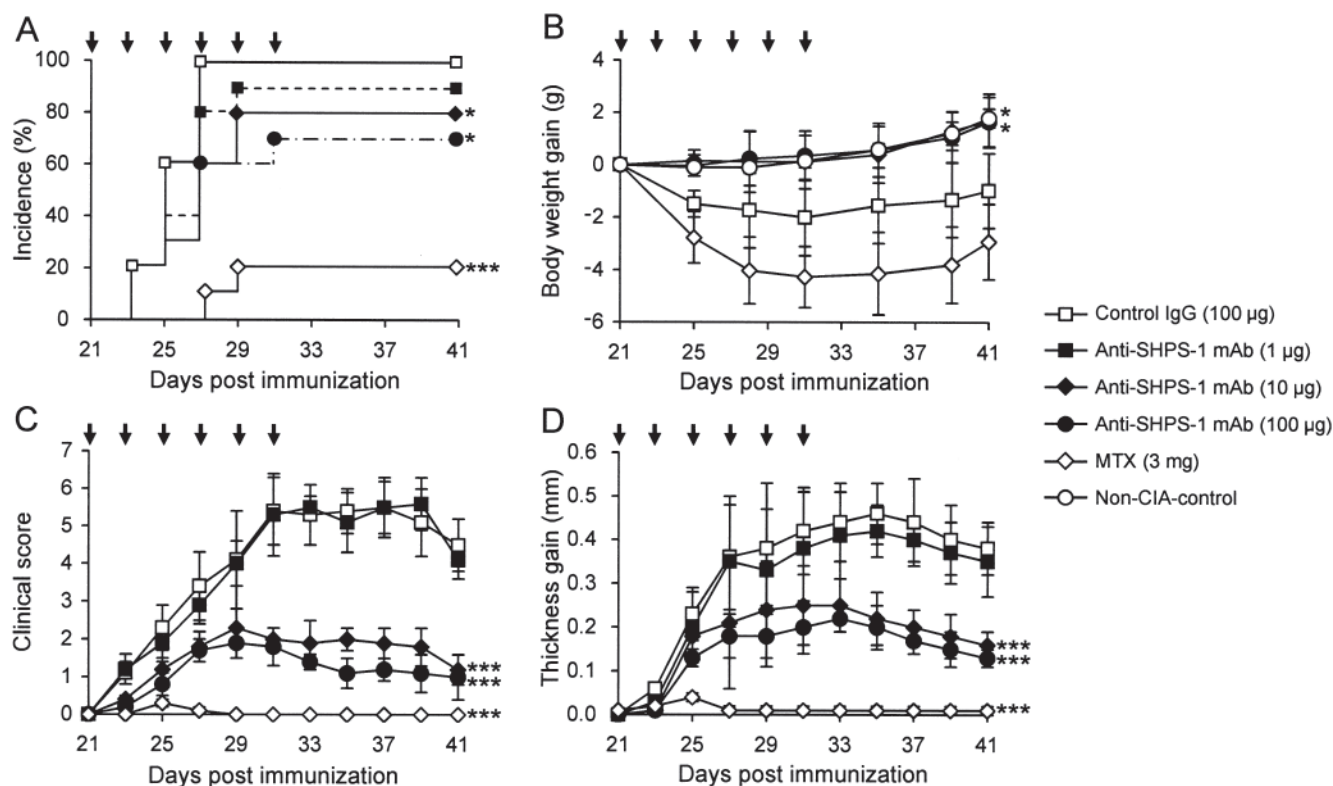

Figure 1. Incidence and severity of arthritis in CIA mice treated with anti-SHPS-1 mAb. Mice were given graded doses of antibody, and the incidence and severity of arthritis were compared with those in control IgG- or MTX-treated mice. Incidence (A), gain of body weight (B), clinical score (C), and increase of footpad thickness (D) from Day 21 to Day 41 are shown. Black arrows indicate the timing of antibody administration. Data are mean ± SD. *$p < 0.05$ and ***$p < 0.001$ compared with control IgG.

body weight was reversed by the anti-SHPS-1 mAb treatment. The administration of 10 or 100 μg anti-SHPS-1 mAb recovered the body weight almost completely to the level of non-CIA mice. The increase of body weights in those 2 groups was significantly greater than that of the control IgG group (both at $p < 0.05$). Although the development of arthritis was strongly inhibited by MTX, the mice treated with MTX lost approximately 2.9 g in body weight during the experimental period, which might be ascribed to the toxic effects of the immunosuppressant.

In the control mice, the clinical score started to increase on Day 23 and reached a maximum on Day 31 (Figure 1C). The administration of 10 or 100 μg anti-SHPS-1 mAb significantly improved the clinical score on Day 31 and later. Since improvement was not observed in mice treated with 1 μg anti-SHPS-1 mAb, the critical dose of antibody treatment for the mice was considered to be between 1 and 10 μg per injection. Interestingly, the improvement in clinical score was maintained until Day 41, 10 days after the last antibody administration. In mice treated with MTX, the development of arthritis was completely inhibited.

Consistent results were obtained by the measurement of footpad thickness (Figure 1D). In control mice, the footpad thickness started to increase on Day 25, and it continued to increase until Day 35, while no increase was observed in the MTX-treated mice. In mice treated with 10 or 100 μg anti-SHPS-1 mAb, the increase of footpad thickness was inhibited on Day 27 and later. In accord with the clinical score, 1 μg anti-SHPS-1 mAb was not enough to show the effect.

Anti-SHPS-1 antibody ameliorated the severity of established arthritis. We then investigated whether the administration of anti-SHPS-1 mAb could reduce the severity of established arthritis. In this experiment, the antibody treatment was commenced after the onset of arthritis (on Day 29), and the severity of arthritis was evaluated by the clinical score and footpad thickness of the hind limbs. The clinical score was reduced significantly as early as 2 days after the first injection of 100 μg anti-SHPS-1 mAb (Figure 2A). The reduction in clinical score became more obvious, and this was maintained until Day 45, 6 days after the completion of antibody administration.

In accord with the change of clinical score, the antibody treatment reduced the footpad thickness at 2 days and later after the initiation of antibody treatment (Figure 2B). Similar to the clinical score, the reduction in footpad thickness was maintained until Day 45.

Histological evaluation. Next, the effect of the antibody treatment was evaluated by histology. The mice were given 6 injections of 10 or 100 μg anti-SHPS-1 mAb after the onset of arthritis following Protocol B, and sacrificed 6 days after the last antibody administration. H&E-stained sections of ankle and tarsal joints were prepared, and the severity of arthritic change was evaluated by scores that were compared with those of mice treated with control IgG.
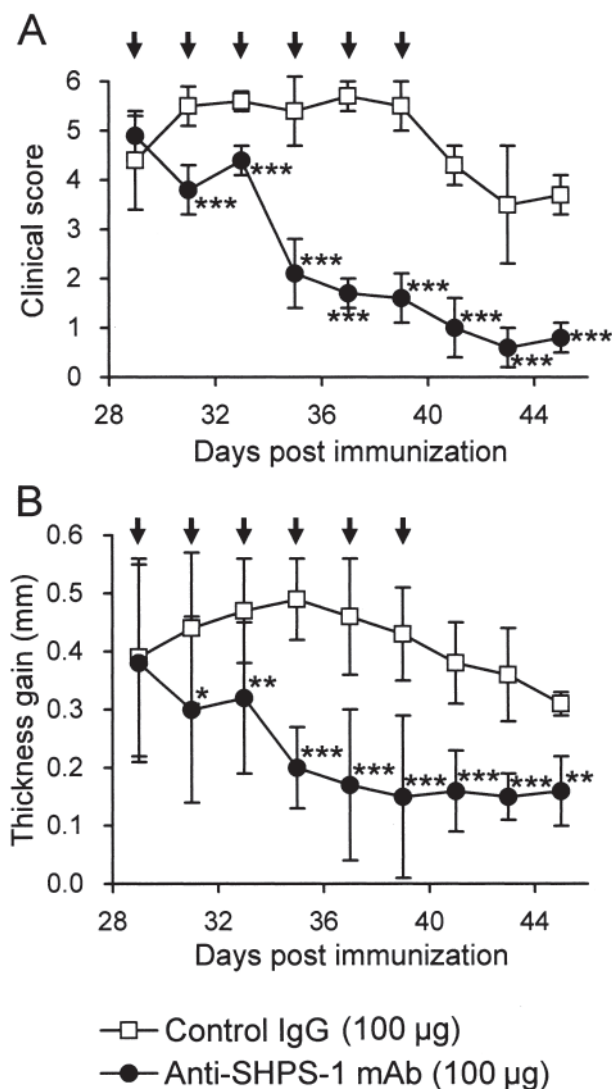

Figure 2. Effect of anti-SHPS-1 mAb treatment on severity of established arthritis. 100 μg of anti-SHPS-1 mAb or control IgG was given to mice after onset of arthritis, and severity of arthritis was evaluated by clinical score (A) and gain of footpad thickness (B), from the beginning of the treatment until 2 weeks after its end. Black arrows indicate the timing of antibody administration. Data are mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control IgG.

In control mice, severe arthritic change with obvious inflammatory cell infiltration and bone erosion was observed within and around the ankle and tarsal joints (Figure 3A-3C). Although the anti-SHPS-1 antibody was given after the onset of arthritis, the severity of arthritic change was considerably reduced in mice treated with anti-SHPS-1 mAb (Figure 3D-3F). Thus, the scores for inflammatory cell infiltration and those for bone destruction were significantly reduced in the antibody-treated mice (Figure 3G and 3H, respectively).

Anti-SHPS-1 antibody did not affect induction of anti-type II collagen antibodies. In CIA mice, arthritis is caused by
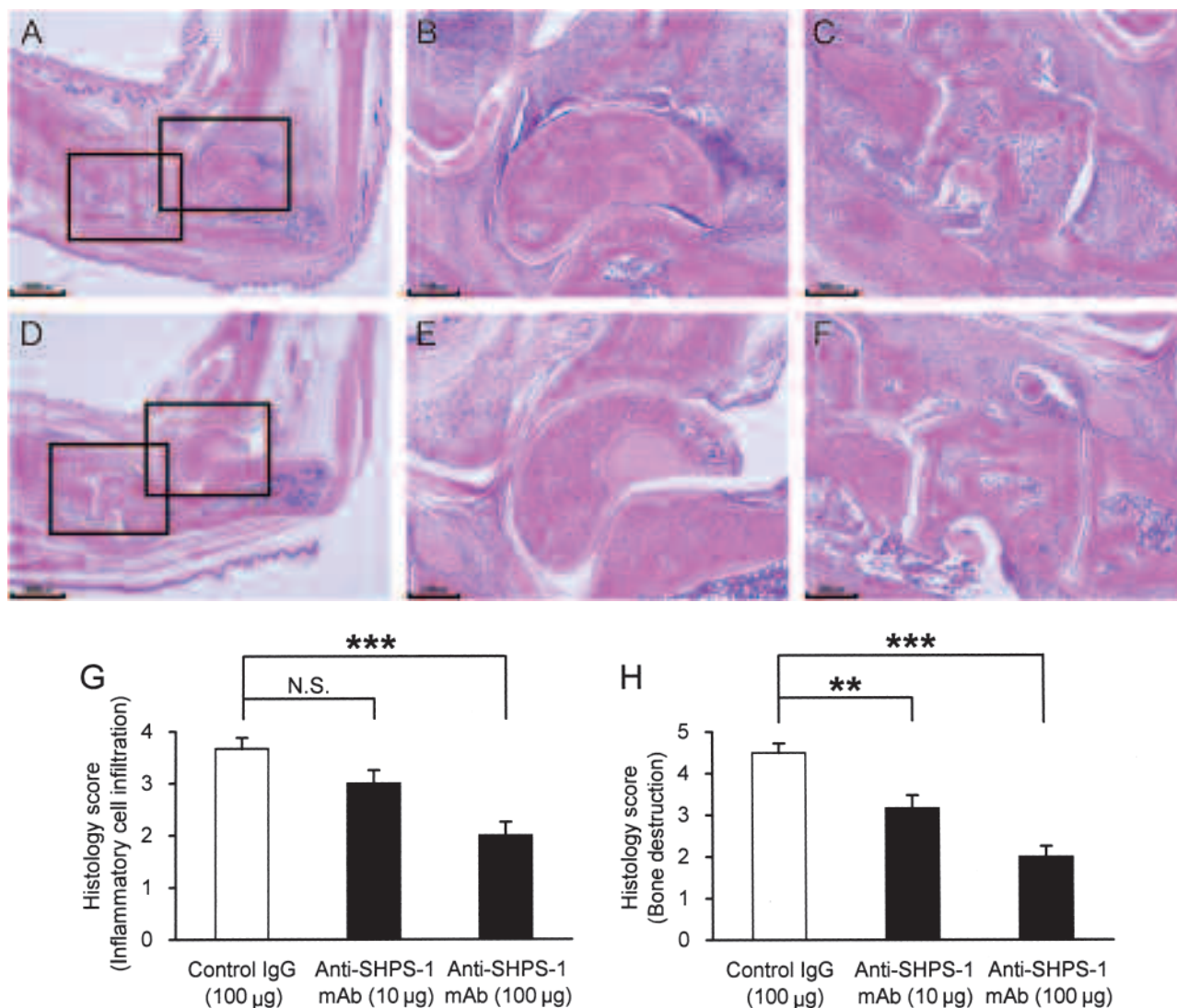
Figure 3. Histological evaluation of ankle and tarsal joints in antibody-treated and control mice. (A-C) In control mice given 100 μg control IgG, obvious synovial thickening and pannus formation was observed, together with marked inflammatory cell infiltration. Bone erosion occurred at multiple sites, which often extended deep into the subchondral bone. (D-F) In mice treated with 100 μg anti-SHPS-1 mAb, severity of synovial thickening and extent of inflammatory cell infiltration were considerably reduced. Area of bone erosion was also reduced, and rarely extended into the subchondral bone. Higher magnification images of inset areas in A and D are shown in B and C, and E and F, respectively. Scale bars are 1000 μm in A and D, and 300 μm in B, C, E, and F. H&E staining. (G and H) Histological scores for inflammatory cell infiltration (G) and bone destruction (H) in mice treated with 10 or 100 μg anti-SHPS-1 mAb are shown together with those for mice given 100 μg control IgG. Data are mean ± SD. **$p < 0.01$ and ***$p < 0.001$ compared with control IgG.

autoimmune mechanisms that involve both humoral and cellular immune responses to CII[30,31]. Thus, we next determined the effect of the anti-SHPS-1 antibody treatment on the humoral response by measuring the concentration of anti-CII antibodies in sera. In this experiment, administration of anti-SHPS-1 mAb was started on the day of the second immunization, following Protocol A, and blood was obtained 2 weeks after the end of treatment. Comparison of the results between the antibody-treated mice and those of mice given control IgG or MTX revealed that the induction of anti-CII antibodies was not affected by the antibody treatment. This implies that the therapeutic effect of anti-SHPS-

1 mAb is likely through the suppression of T cell responses, rather than via the modulation of B cell function.

Anti-SHPS-1 antibody and anti-CD47 antibody inhibited osteoclast formation. The observation that bone destruction was significantly reduced by the administration of anti-SHPS-1 mAb led us to hypothesize that the antibody could inhibit osteoclast formation. We then tested this hypothesis by an *in vitro* experiment. We also examined the effect of anti-CD47 mAb on osteoclast formation. In this experiment, murine bone marrow cells were obtained and osteoclast formation was induced in the presence of anti-SHPS-1 mAb or anti-CD47 mAb. The results clearly indicated that those

antibodies both inhibited the formation of osteoclasts in a dose-dependent manner (Figure 4). The inhibition was more obvious with anti-SHPS-1 mAb. With this antibody, the number of osteoclasts was significantly reduced with as little as 0.05 µg/ml of the antibody, and osteoclast formation was almost completely abrogated at the concentration of 2.5 µg/ml. Compared with this, the effect of anti-CD47 mAb was considerably lower. The ratio of inhibition did not reach 50% even with 10 µg/ml of this antibody.

Anti-SHPS-1 antibody and anti-CD47 antibody reduced secretion of proinflammatory cytokines from ConA-stimulated murine spleen cells. In order to determine the effects of anti-SHPS-1 mAb and anti-CD47 mAb on cytokine release from lymphatic cells, murine spleen cells were stimulated with ConA in the presence of anti-SHPS-1 mAb or anti-CD47 mAb, and cytokine concentrations in the media were determined. Upon stimulation with ConA, the spleen cells released all measured pro- and antiinflammatory cytokines to the media. The effect of anti-SHPS-1 mAb on cytokine release differed among the cytokines (Figure 5A-5G). The release of IL-1ß, IL-2, IL-12, IFN-γ, and TNF-α into the media was suppressed by the antibody, while that of IL-4 or IL-10 was almost unaffected. The suppression was most obvious for IFN-γ, with as little as 0.02 µg/ml of the antibody significantly reducing its release. The IC_{50} values of IL-1ß, IL-2, IL-12, IFN-γ, and TNF-α were 0.68, 0.50, 0.16,
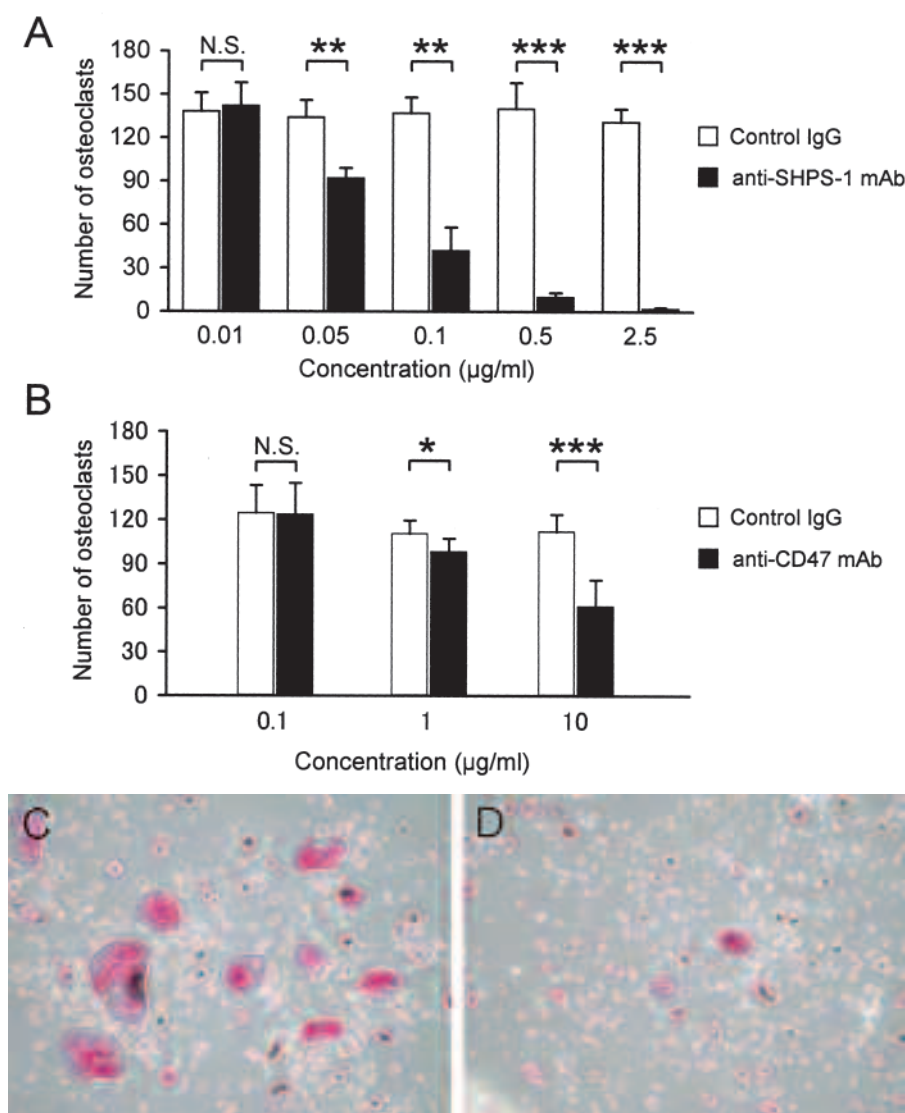

Figure 4. Effect of anti-SHPS-1 mAb and anti-CD47 mAb on osteoclast formation. (A and B) Bone marrow cells were obtained from Balb/c mice, and formation of osteoclasts was induced by M-CSF and RANKL for 5 days, in the presence of various concentrations of anti-SHPS-1 mAb or control IgG (A), and anti-CD47 mAb or control IgG (B). Number of TRAP-positive multinucleated cells in each well is shown. Data are mean ± SD. **$p < 0.01$ and ***$p < 0.001$ against control IgG. (C and D) Formation of osteoclasts in the presence of control IgG (2.5 µg/ml; panel C) or anti-SHPS-1 mAb (2.5 µg/ml; panel D). TRAP staining.
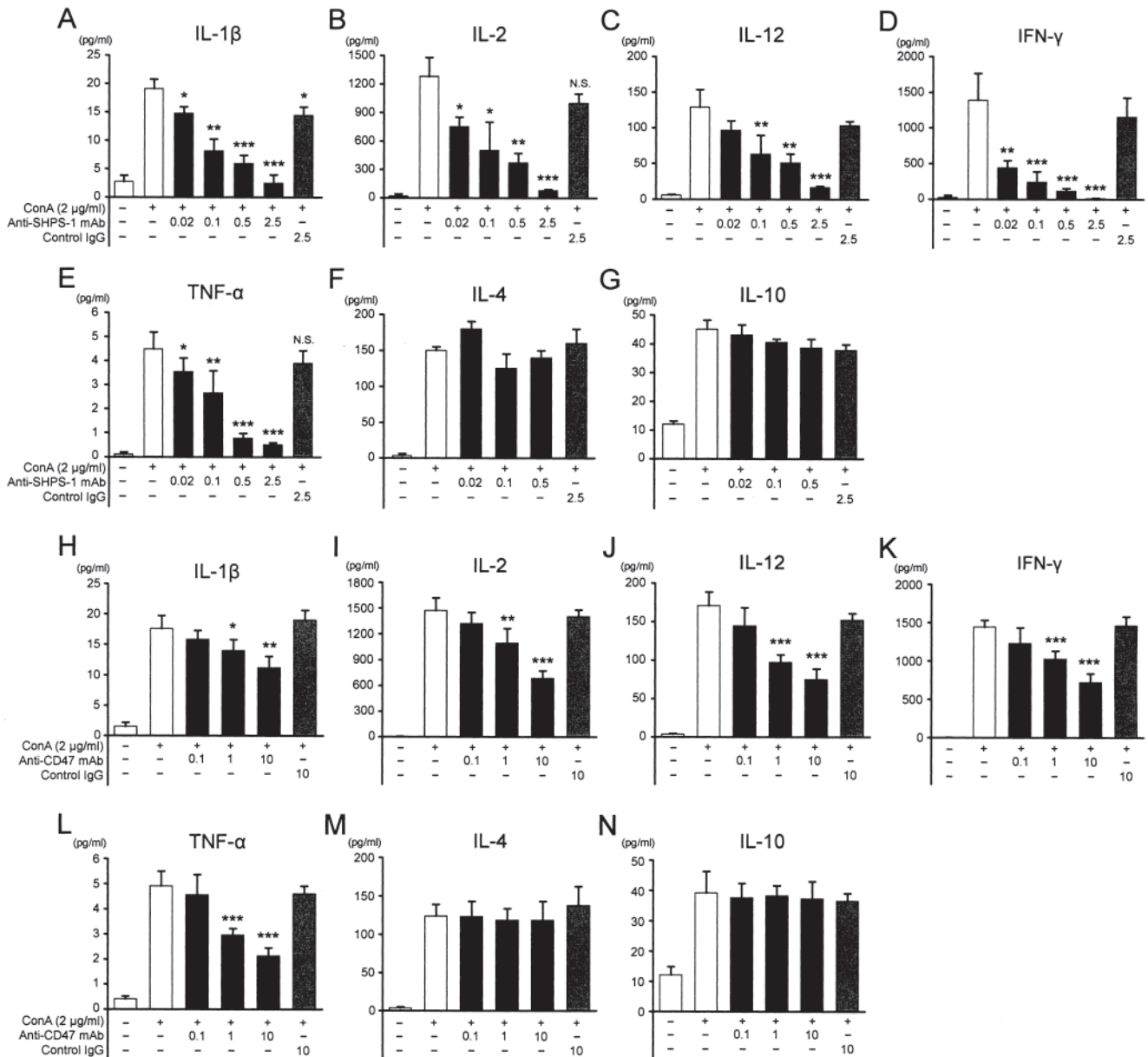
Figure 5. (A-G) Murine spleen cells were stimulated with ConA (2 μg/ml) in media containing graded doses of anti-SHPS-1 mAb or control IgG (2.5 μg/ml). 24 h later, supernatants were collected, and concentrations of IL-1ß (A), IL-2 (B), IL-12 (C), IFN-γ (D), TNF-α (E), IL-4 (F), and IL-10 (G) were determined by ELISA. (H-N) Experiments were repeated with anti-CD47 mAb, and concentrations of IL-1ß (H), IL-2 (I), IL-12 (J), IFN-γ (K), TNF-α (L), IL-4 (M), and IL-10 (N) were determined. Experiments were repeated 3 or 4 times. Data are mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control IgG.

0.079, and 1.18, respectively. Anti-CD47 mAb showed similar effects on cytokine release (Figure 5H-5N). This antibody reduced the concentration of IL-2, IL-12, IFN-γ, and TNF-α in the media in a dose-dependent manner. However, its inhibitory effect was much lower than that of anti-SHPS-1 mAb, and the suppression was no more than 60% even with 10 μg/ml of the antibody.

DISCUSSION
The results of our study demonstrate that the administration of an anti-SHPS-1 mAb successfully reduces the severity of arthritis in CIA mice. CIA is an animal model often used to study the pathology of RA, in which both humoral and cell-mediated immunity is necessary for the development of arthritis[31,32]. The treatment with the anti-SHPS-1 mAb virtually did not suppress the humoral immunity, since it did not alter the concentration of anti-CII antibodies in the sera of mice (Figure 6). Thus, the therapeutic effect of the anti-SHPS-1 mAb could be ascribed entirely to the suppression of the cell-mediated immune response. In *in vitro* experi-
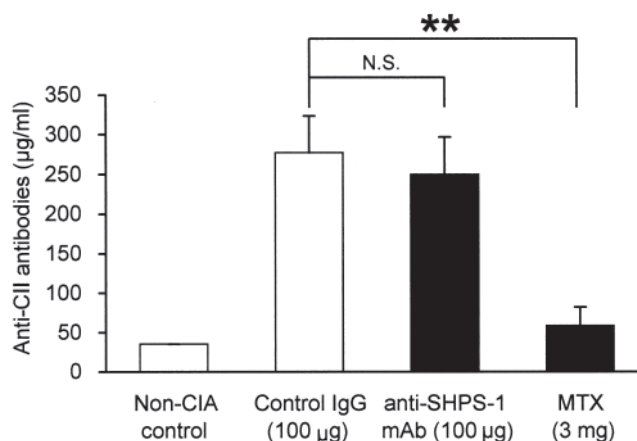
Figure 6. Concentration of anti-CII antibodies in the sera. CIA mice were treated for 11 days with control IgG (100 μg), anti-SHPS-1 mAb (100 μg), or MTX (3 mg), and the sera were obtained. Sera were also obtained from mice in which CIA was not induced, and the concentrations of anti-CII antibodies were determined by ELISA. Data are mean $\pm$ SD of 5–8 mice. **p < 0.01 compared with control IgG. NS: nonsignificant.

ments using murine spleen cells, addition of the anti-SHPS-1 mAb inhibited the release of IL-1ß, IL-2, IL-12, IFN-γ, and TNF-α into the media upon stimulation by ConA, while the release of IL-4 or IL-10 was almost unaffected. The finding that the anti-SHPS-1 mAb suppressed the release of cytokines primarily from Th1 cells but not those from Th2 cells further supports the idea that the anti-SHPS-1 mAb affects cellular immunity rather than humoral immunity. In human RA, TNF-α is profoundly involved in the progression of the disease as shown by the efficacy of anti-TNF-α therapy[33-35]. Also, IL-2 and IFN-γ are known to be involved in the catabolism in affected joints[36]. Since the pathology of arthritis in the CIA mouse closely resembles that of human RA[37], the reduction in the release of those cytokines could reasonably explain the therapeutic effects of the anti-SHPS-1 mAb observed in this work.

For such change in cytokine release, ligation of SHPS-1 by anti-SHPS-1 mAb may play a significant role, in addition to the inhibitory role of the antibody upon SHPS-1/CD47 interaction. We previously showed that SHPS-1 ligation by the antibody inhibits the migration and maturation of epidermal Langerhans cells, which suggests that DC function could be regulated by SHPS-1 engagement[23,24]. Ligation of SHPS-1 has been shown to inhibit TNF-α production by lipopolysaccharide-stimulated monocytes[38]. Thus, the observed reduction in TNF-α release by anti-SHPS-1 mAb could be ascribed, at least in part, to the suppression of TNF-α production by macrophages or DC by SHPS-1 ligation. Again, since the antibody inhibits IL-12 production by DC[16], the observed suppression of IL-12 release could be partly caused by SHPS-1 ligation. Because IL-12 is an essential cytokine for Th1 development, reduced IL-12 production favors the development of Th2 cells rather than Th1 cells. This is compatible with the finding that the production

of all Th1 cytokines, but not those of Th2, was suppressed by anti-SHPS-1 mAb. The difference between anti-SHPS-1 and anti-CD47 mAb in the effects on cytokine release may be reasonable if these direct actions are assumed with the former antibody.

On the other hand, the supposed suppression of cellular immunity by anti-SHPS-1 mAb may be caused primarily by the inhibition of interaction between SHPS-1 and CD47. T cells express CD47 at a high density[39]. Since SHPS-1/CD47 interaction positively regulates T cell responses[21], it is possible that the anti-SHPS-1 mAb suppressed T cell activation by blocking that interaction. Anti-SHPS-1 mAb may inhibit proliferation of T cells via the suppression of TNF-α production by antigen-presenting cells[22]. Other studies have shown that SHPS-1/CD47 interaction may downregulate DC-T cell interaction, by reducing IL-12 production by DC and IL-12 receptor expression on T lymphocytes[16,17,39]. Reduced T cell activation by these mechanisms could be involved in the amelioration of arthritis by anti-SHPS-1 mAb.

Meanwhile, a mechanism for the reduction of bone erosion by the antibody was suggested by an *in vitro* experiment. Our current investigation and that of others consistently indicate that anti-SHPS-1 mAb and anti-CD47 mAb both inhibited induction of osteoclasts from macrophages[40]. Macrophages express SHPS-1 and CD47 abundantly, and utilize them for cell fusion, which is an essential step for osteoclast formation[7,11,20]. Therefore, it is likely that the antibodies for these molecules reduced the formation of osteoclasts through the inhibition of multinucleation. In addition to this, anti-SHPS-1 mAb might have reduced osteoclast formation through the change in released cytokines discussed above: among the cytokines whose release was suppressed by the antibody, IL-1ß and TNF-α are known to play essential roles in the formation of osteoclasts[3,4]. In our study, suppression of osteoclast formation was more obvious with anti-SHPS-1 mAb than with anti-CD47 mAb (Figure 4). This difference, again, could be ascribed to the lack of SHPS-1 ligation with the latter antibody.

Our results show that the use of anti-SHPS-1 antibody could be a promising strategy to treat patients with RA. Although our current results are based on an animal model of RA, the treatment with the antibody seems attractive because the antibody could regulate T cell immunity and osteoclast formation together, both of which are essential in treating RA[3,4,41]. Further studies are awaited to determine the feasibility of the antibody treatment.

REFERENCES

1. Koopman WJ. Arthritis and allied conditions: A textbook of rheumatology. 15th ed. Philadelphia: Williams and Wilkins; 1997.
2. Tak PP, Smeets TJ, Daha MR, et al. Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. Arthritis Rheum 1997;40:217-25.

3. Teitelbaum SL. Osteoclasts; culprits in inflammatory osteolysis. Arthritis Res Ther 2006;8:201.

4. Goldring SR. Pathogenesis of bone and cartilage destruction in rheumatoid arthritis. Rheumatology Oxford 2003;42 Suppl:ii11-6.

5. Kharitonenkov A, Chen Z, Sures I, Wang H, Schilling J, Ullrich A. A family of proteins that inhibit signalling through tyrosine kinase receptors. Nature 1997;386:181-6.

6. Ohnishi H, Kubota M, Ohtake A, Sato K, Sano S. Activation of protein-tyrosine phosphatase SH-PTP2 by a tyrosine-based activation motif of a novel brain molecule. J Biol Chem 1996;271:25569-74.

7. Saginario C, Sterling H, Beckers C, et al. MFR, a putative receptor mediating the fusion of macrophages. Mol Cell Biol 1998;18:6213-23.

8. Comu S, Weng W, Olinsky S, et al. The murine P84 neural adhesion molecule is SHPS-1, a member of the phosphatase-binding protein family. J Neurosci 1997;17:8702-10.

9. Fujioka Y, Matozaki T, Noguchi T, et al. A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. Mol Cell Biol 1996;16:6887-99.

10. Adams S, van der Laan LJ, Vernon-Wilson E, et al. Signal-regulatory protein is selectively expressed by myeloid and neuronal cells. J Immunol 1998;161:1853-9.

11. Han X, Sterling H, Chen Y, et al. CD47, a ligand for the macrophage fusion receptor, participates in macrophage multinucleation. J Biol Chem 2000;275:37984-92.

12. Jiang P, Lagenaur CF, Narayanan V. Integrin-associated protein is a ligand for the P84 neural adhesion molecule. J Biol Chem 1999;274:559-62.

13. Seiffert M, Cant C, Chen Z, et al. Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. Blood 1999;94:3633-43.

14. Vernon-Wilson EF, Kee WJ, Willis AC, Barclay AN, Simmons DL, Brown MH. CD47 is a ligand for rat macrophage membrane signal regulatory protein SIRP (OX41) and human SIRP alpha 1. Eur J Immunol 2000;30:2130-7.

15. Babic I, Schallhorn A, Lindberg FP, Jirik FR. SHPS-1 induces aggregation of Ba/F3 pro-B cells via an interaction with CD47. J Immunol 2000;164:3652-8.

16. Latour S, Tanaka H, Demeure C, et al. Bidirectional negative regulation of human T and dendritic cells by CD47 and its cognate receptor signal-regulator protein-alpha: down-regulation of IL-12 responsiveness and inhibition of dendritic cell activation. J Immunol 2001;167:2547-54.

17. Lienard H, Bruhns P, Malbec O, Fridman WH, Daeron M. Signal regulatory proteins negatively regulate immunoreceptor-dependent cell activation. J Biol Chem 1999;274:32493-9.

18. Oldenborg PA, Zhelezenyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. Science 2000;288:2051-4.

19. Oshima K, Ruhul Amin AR, Suzuki A, Hamaguchi M, Matsuda S. SHPS-1, a multifunctional transmembrane glycoprotein. FEBS Lett 2002;519:1-7.

20. Vignery A. Osteoclasts and giant cells: macrophage-macrophage fusion mechanism. Int J Exp Pathol 2000;81:291-304.

21. Seiffert M, Brossart P, Cant C, et al. Signal-regulatory protein alpha (SIRP alpha) but not SIRP beta is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34(+)CD38(-) hematopoietic cells. Blood 2001;97:2741-9.

22. Patel V, Smith RE, Serra A, Brooke G, Howard CJ, Rigley KP. MyD-1 (SIRP alpha) regulates T cell function in the absence of exogenous danger signals, via a TNF alpha-dependent pathway. Eur J Immunol 2002;32:1865-72.

23. Fukunaga A, Nagai H, Noguchi T, et al. Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 regulates the migration of Langerhans cells from the epidermis to draining lymph nodes. J Immunol 2004;172:4091-9.

24. Fukunaga A, Nagai H, Yu X, et al. Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 regulates the induction of Langerhans cell maturation. Eur J Immunol 2006;36:3216-26.

25. Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. J Exp Med 1977;146:857-68.

26. Campbell IK, Bendele A, Smith DA, Hamilton JA. Granulocyte-macrophage colony stimulating factor exacerbates collagen-induced arthritis in mice. Ann Rheum Dis 1997;56:364-8.

27. Chuang W, Lagenaur CF. Central nervous system antigen P84 can serve as a substrate for neurite outgrowth. Devel Biol 1990;137:219-32.

28. Fishman P, Bar-Yehuda S, Madi L, et al. The PI3K-NF-kappa-B signal transduction pathway is involved in mediating the anti-inflammatory effect of IB-MECA in adjuvant-induced arthritis. Arthritis Res Ther 2006;8:R33.

29. Campbell IK, Rich MJ, Bischof RJ, Dunn AR, Grail D, Hamilton JA. Protection from collagen-induced arthritis in granulocyte-macrophage colony-stimulating factor-deficient mice. J Immunol 1998;161:3639-44.

30. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunisation against heterologous type II collagen induces arthritis in mice. Nature 1980;283:666-8.

31. Seki N, Sudo Y, Yoshioka T, et al. Type II collagen-induced murine arthritis. I. Induction and perpetuation of arthritis require synergy between humoral and cell-mediated immunity. J Immunol 1988;140:1477-84.

32. Holmdahl R, Klareskog L, Rubin K, et al. Role of T lymphocytes in murine collagen-induced arthritis. Agents Actions 1986;19:295-305.

33. Moreland LW, Schiff MH, Baumgartner SW, et al. Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. Ann Intern Med 1999;130:478-86.

34. Lipsky PE, van der Heijde DM, St. Clair EW, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 2000;343:1594-602.

35. Bathon JM, Martin RW, Fleischmann RM, et al. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. N Engl J Med 2000;343:1586-93.

36. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145-73.

37. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. Nature Protocols 2007;2:1269-75.

38. Smith RE, Patel V, Seatter SD, et al. A novel MyD-1 (SIRP alpha) signaling pathway that inhibits LPS-induced TNF alpha production by monocytes. Blood 2003;102:2532-40

39. Ticchioni M, Deckert M, Mary F, Bernard G, Brown EJ, Bernard A. Integrin-associated protein (CD47) is a comitogenic molecule on CD3-activated human T cells. J Immunol 1997;158:677-84.

40. Lundberg P, Koskinen C, Baldock PA, et al. Osteoclast formation is strongly reduced both in vivo and in vitro in the absence of CD47/SIRP alpha-interaction. Biochem Biophys Res Commun 2007;352:444-8.

41. Kremer JM, Westhovens R, Leon M, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. N Engl J Med 2003;349:1907-15.