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Substance P ameliorates collagen II-induced arthritis in mice via suppression of the inflammatory response

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ABSTRACT

Current rheumatoid arthritis (RA) therapies such as biologics inhibiting pathogenic cytokines substantially delay RA progression. However, patient responses to these agents are not always complete and long lasting. This study explored whether substance P (SP), an 11 amino acids long endogenous neuropeptide with the novel ability to mobilize mesenchymal stem cells (MSC) and modulate injury-mediated inflammation, can inhibit RA progression. SP efficacy was evaluated by paw swelling, clinical arthritis scoring, radiological analysis, histological analysis of cartilage destruction, and blood levels of tumor necrosis factor-alpha (TNF-α) interleukin (IL)-10, and IL-17 *in vivo*. SP treatment significantly reduced local inflammatory signs, mean arthritis scores, degradation of joint cartilage, and invasion of inflammatory cells into the synovial tissues. Moreover, the SP treatment markedly reduced the size of spleens enlarged by excessive inflammation in CIA, increased IL-10 levels, and decreased TNF-α and IL-17 levels. Mobilization of stem cells and induction of T_{reg} and M2 type macrophages in the circulation were also increased by the SP treatment. These effect of SP might be associated with the suppression of inflammatory responses in RA and, furthermore, blockade of RA progression. Our results propose SP as a potential therapeutic for autoimmune-related inflammatory diseases.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune-related chronic and systemic inflammatory disorder that causes severe cartilage destruction and bone erosion. In RA patients, cytokine balance is usually skewed toward the overexpression of pro-inflammatory markers such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8, IL-12, IL-17, and macrophage colony-stimulating factor (M-CSF) in the synovial tissue and in the serum [1–4]. This causes further infiltration of a variety of inflammatory cells, cellular transformations of synovial fibroblasts and immune cells in the synovial tissue, and finally tissue destruction in the joint. Thus, RA is a progressive deteriorating disease manifested by chronic inflammation.

Several immune-inflammations modulating agents such as methotrexate and steroids or biologics targeting specific pro-inflammatory cytokines have been clinically trialed in RA patients [5,6], showing the substantial improvement in RA. However, in a subset of patients, TNF-α blockade is not effective or long lasting. Non-responders to TNF-α blockers exhibited a high level of IL-17 or a higher IL-17/IL-10 ratio. Therefore, a variety of cytokines and chemokines seem to simultaneously act on both innate and adaptive immune cells during the progression of RA, which may not be resolved with single cytokine inhibition.

Recently, stem cell transplantation has raised interest in their potential use. Bone marrow mesenchymal stem cells (BM-MSCs) modulate the functions of major immune cells such as T lymphocytes, B lymphocytes, and NK cells by skewing their functions to immunosuppressive and regulatory phenotypes. BM-MSCs prevented the occurrence of severe arthritis in the CIA model, accompanied by a decrease in the pro-inflammatory cytokines [7], and induced the differentiation of naïve $CD4^+$ T-cells to $CD4^+$, CD25high $Foxp3^+$ T_{reg} [8,9], and non-classical IL-10-producing regulatory Th17 cells [10]. However, systematic clinical trials in RA patients have not yet been reported. In all likelihood, the cellular heterogeneity of *ex vivo* cultured BM-MSCs will result in inconsistent outcomes. Furthermore, their support of tumor growth is a safety issue that may be one of the hurdles to MSC cell therapy.

Substance P (SP) is endogenous neuropeptide that is involved in neuro-immune modulation in the bone marrow [11] and in re-epithelialization in the cornea [12]. Hong et al. (2009) identified a

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novel role of SP as an injury-inducible messenger that mobilizes endogenous stem cells from the bone marrow to wound sites and accelerates tissue repair [13–17]. SP also had anti-inflammatory function by increasing IL-10 and decreasing TNF-α, prior to MSC mobilization [16]. Considering the novel functions of SP, SP is expected to be used as a therapeutic agent for RA.

In order to assess the efficacy of SP in RA, we used a CIA mouse model that recapitulates the typical immune responses shown in human RA. The therapeutic effects of SP on RA progression were evaluated by analyzing articular erythema, swelling in the paws, cartilage degradation and the secretion of critical cytokines such as TNF-α, IL-10, and IL-17. To examine the effect of SP on immune cell-mediated inflammatory response, T_{reg} and M2 type macrophages were analyzed.

2. Materials and methods

2.1. Animals

Five-week old DBA/1J mice were obtained from Nara Biotech (Seoul, Korea). Animals were allowed to acclimatize to the new environment for a period of 1 week before initiation of the experiments. All animal procedures were approved by the institutional animal use committee of Kyung Hee University with the approval number KHUASP (SU)-11-14.

2.2. Induction of CIA

For the induction of CIA, native chicken collagen type II (CII; St. Louis, MO, USA) was dissolved in 50 mM acetic acid at 4 °C overnight and then emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 4 mg/mL *Mycobacterium tuberculosis*. A 100-μL aliquot of the emulsion containing 100 μg CII was injected into the subcutaneous tissue of the tails of 7 weeks old male DBA/1J mice. At 3 weeks after the primary immunization, mice were boosted with 100 μg CII emulsified with an equal volume of incomplete Freund's adjuvant. Paw swelling was assessed by measuring the thickness of the affected hind paws with 0–10 mm calipers (Mitutoyo, Kanagawa, Japan). Clinical arthritis scores were assigned on a scale of 0–4 (0 = no swelling or bone damage, 1 = mild swelling and redness, 2 = moderate swelling and redness, 3 = severe swelling and bone erosion) [18]. The individual mouse arthritic score was obtained by summing the scores recorded for each limb. The severity of arthritis in each mouse was determined independently and blindly by two investigators, and the mean of the two scores was calculated [18].

2.3. Administration of SP

SP (Calbiochem, Darmstadt, Germany) was diluted in PBS (Welgene, Daegu, Korea) immediately before use, and was administered intravenously at dose of 5 nmole/kg twice a week for 2 weeks starting at 4 weeks post immunization. PBS was used as a vehicle. There were 15 animals in each treatment and vehicle groups. For *in vitro* experiments, SP was added to 2×10^7 of PBMCs at the final concentration of 100 nM. The cells were incubated for 4.5 h or 24 h.

2.4. Radiological analysis

Inflammatory swelling of the soft tissues and cartilage destruction were evaluated by taking radiographs at 60 kV with a 600-s exposure time (LX-60, FAXITRON, Lincolnshire, IL, USA). The degree of bone erosion in the joint was scored as the previous [19].

2.5. Histological analysis

Whole knee was fixed in 3.7% paraformaldehyde for 2 days. After decalcification in 0.2 M EDTA for 4 weeks, samples were processed with a TP1020 tissue processor (Leica Biosystems, Wetzlar, Germany). Histological damage was scored as follows: 0 = no damage; 1 = edema; 2 = inflammatory cell presence; and 3 = bone resorption [19].

2.6. Measurement of cytokines

Serum levels of IL-10, TNF-α, and IL-17 were examined by using Quantikine ELISA kits (R&D Systems) according to the manufacturer's protocol.

2.7. FACS analysis

For analysis of T_{reg} in PBMCs, 2×10^7 PBMCs were incubated with allophycocyanin-conjugated anti-foxp3 antibody and fluorescein isothiocyanate (FITC)-conjugated CD4 antibody (Miltenyi, Bergisch Gladbach, Germany). In order to check the percentage of M2 type macrophages, 2×10^7 PBMCs were treated with FITC-conjugated anti-CD11b antibody (Miltenyi, Bergisch Gladbach, Germany) and Cy5.5-conjugated anti-CD206 antibody (Biolegend, San Diego, CA, USA). To examine MSCs, 2×10^7 of PBMCs were incubated with FITC-conjugated anti-CD 29 antibody, allophycocyanin-conjugated anti-CD105 antibody, and PE-conjugated anti-CD45 antibody (Miltenyi, Bergisch Gladbach, Germany). The fractions of T_{reg}, M2 type macrophage, and MSCs were analyzed by FACS Calibur Flow cytometer utilizing the CELLQuest software (Becton Dickinson, San Jose, CA, USA).

2.8. Statistical analysis

Data are presented as the mean ± standard deviation of the mean (SD) determined from 3 independent experiments. Between-group differences were determined using *t*-test, the Kruskal–Wallis test followed by the Mann–Whitney *U* test. p values < 0.05 were interpreted as statistically significant.

3. Results

3.1. SP injection inhibits CIA progression

DBA/1J mice received CII with adjuvant at day 0 and a secondary boost at 3 weeks. Daily injections of SP or PBS were administered intravenously on the first 2 days of weeks 4 and 5 (Fig. 1A). Photographs of the paw taken at 0, 5, and 6 weeks after the induction of RA are shown in Fig. 1B. The CIA mice treated with the vehicle showed severe inflammation characterized by marked swelling and erythema of the paws, whereas SP-injected CIA mice displayed much milder local inflammatory signs with a 25% reduction in paw thickness compared to the vehicle-treated group (Fig. 1C). An evaluation was conducted based on the clinical arthritis scores rated on a scale of 0–4 as described previously [18]. The vehicle-treated mice developed severe clinical signs of arthritis (10.08/12 at 6 weeks). These signs were much less severe in the SP-injected CIA mice (5.58/12 at 6 weeks) (Fig. 1D).

Radiograph analysis revealed that the vehicle-injected mice had severely damaged cartilage and swelling of the paw, whereas the SP-injected mice displayed a more preserved cartilage structure without edema (Fig. 2A and B, white dot oval and arrow). The severity of the bone and cartilage damage was scored based on the radiological data. While the vehicle-injected mice showed a mean radiographic score of 3.15 ± 0.41, the SP-injected mice got

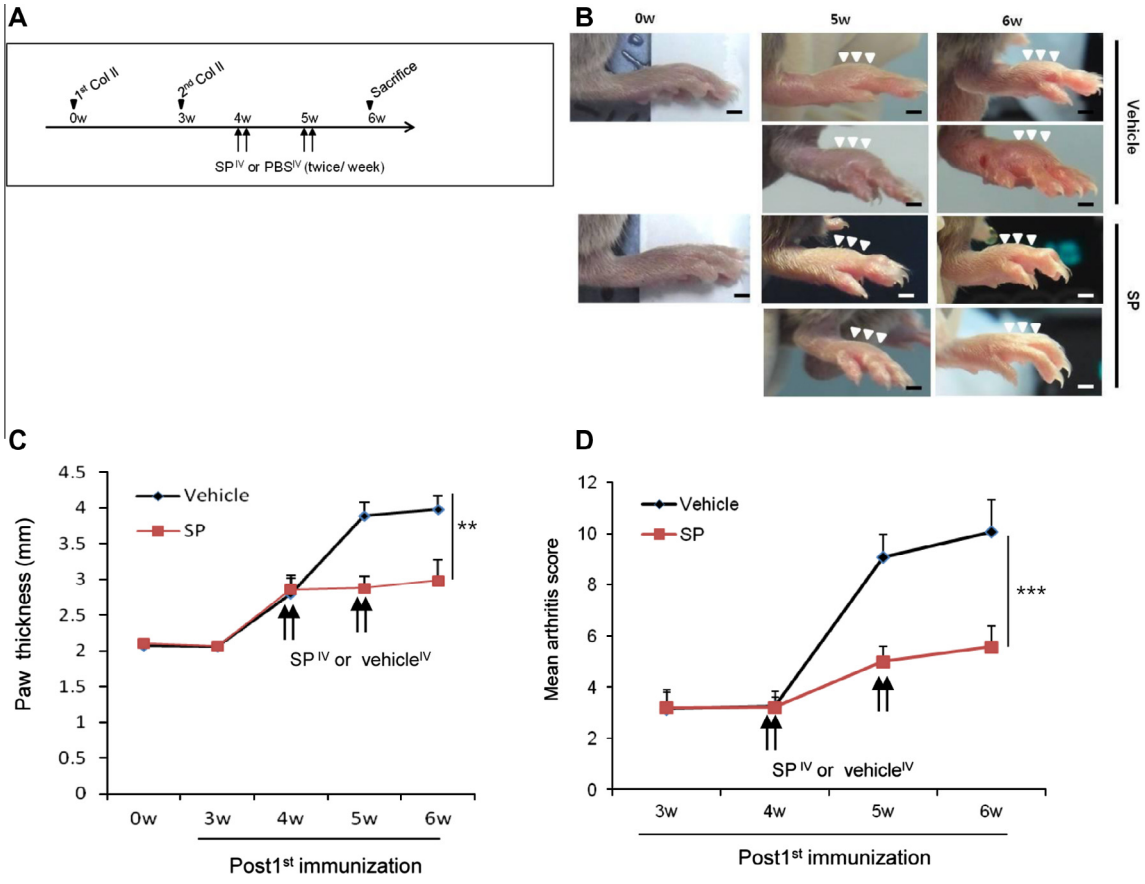

Fig. 1. SP reduces local inflammation in the paws of CIA mice. (A) Experimental scheme for RA induction and SP treatment. (B) Photographs of paw swelling in DBA/1J mice at 0, 5, and 6 weeks after the primary induction of RA. (C) Time course of paw thickness. (D) Time course of arthritis score. Values of $p < 0.05$ were interpreted as statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

the mean radiographic score of 1.4 ± 0.54 (Fig. 2C). The capacity of SP to attenuate RA progression was also confirmed by the histological analysis (Fig. 2D and E). The vehicle-treated CIA mice showed severe erosion of the superficial cartilage with synovial tissue characterized by numerous inflammatory cells and fibrous tissue. By contrast, the SP-injected group showed no sign of cartilage erosion and had normal-looking synovial tissue with only few infiltrated immune cells. Quantification of the thickness of the cartilage showed that the SP-injected mice had a 2-fold greater cartilage thickness than that of the vehicle-injected mice (Fig. 2F). Scoring for histology revealed mean scores were 5.8 ± 0.44 in the vehicle-treated mice and 2.6 ± 0.89 in the SP-treated mice (Fig. 2G).

Taken together, these data suggest that SP can prevent cartilage destruction, which may be occurred possibly by suppressing inflammatory response at the wound sites.

3.2. The therapeutic effect of SP may be due to immune suppression via the regulation of IL-10, TNF-α, and IL-17

We investigated whether the therapeutic effect of SP is related to the regulation of systemic immune responses. The spleen promptly becomes enlarged in response to inflammatory signals, and thus its size is generally used to estimate the extent of systemic inflammation. As predicted, the vehicle-treated mice had strikingly enlarged spleens but the SP-treated mice had reduced size to near-normal levels. Furthermore, the spleen of the vehicle-treated CIA mice showed an enlarged white pulp consisting of germinal centers and peripheral T-cell compartments, indicating elevated numbers of immune cells. By comparison, the SP-injected mice showed reduced white pulp areas (Fig. 3A and B). These data indicate that SP may block CIA-induced inflammation systemically as well as locally.

Ivanov II et al. reported that IL-10 reduces the secretion of IL-17 and TNF-α, a major target cytokine in RA, to induce RA recovery [20]. SP increased the levels of IL-10 to suppress inflammatory responses in SCI [16]. Thus, we presumed that SP may induce the secretion of IL-10 under RA, which can negatively affect the IL-17 or TNF-α level. In order to check this, serum IL-10, TNF-α, and IL-17 were measured by ELISA. IL-10 was increased in the SP-injected CIA mice by 60% compared to the vehicle-injected CIA mice (Fig. 3C). Moreover, SP-injected mice showed reduced levels of TNF-α by 59% (Fig. 3D) and IL-17 by 27% (Fig. 3E). This result indicates that SP may increase IL-10 levels and reduce TNF-α and IL-17 levels, which might eventually contribute to the reduction of the paw swelling and the prevention of the cartilage damage in the CIA mouse model.

3.3. SP-mediated immune suppression is associated with increased T_{reg}, M2, and MSCs in the circulation

In RA, Foxp3^{+} T_{reg} cells are defective in their ability to suppress pro-inflammatory cytokine production [21–24]. IL-10 injection is known to promote the ability of Foxp3^{+} T_{reg} cells to secrete IL-10 [25,26]. Jiang et al. reported that SP induced M2 macrophages and IL-10 in spinal cord injury, leading to accelerated tissue repair [16]. Therefore, it was expected that SP is capable of increasing the level of circulating T_{reg} and M2 macrophages in RA. Fig. 3F and G illustrate that SP could increase the levels of T_{reg} and M2 macro-

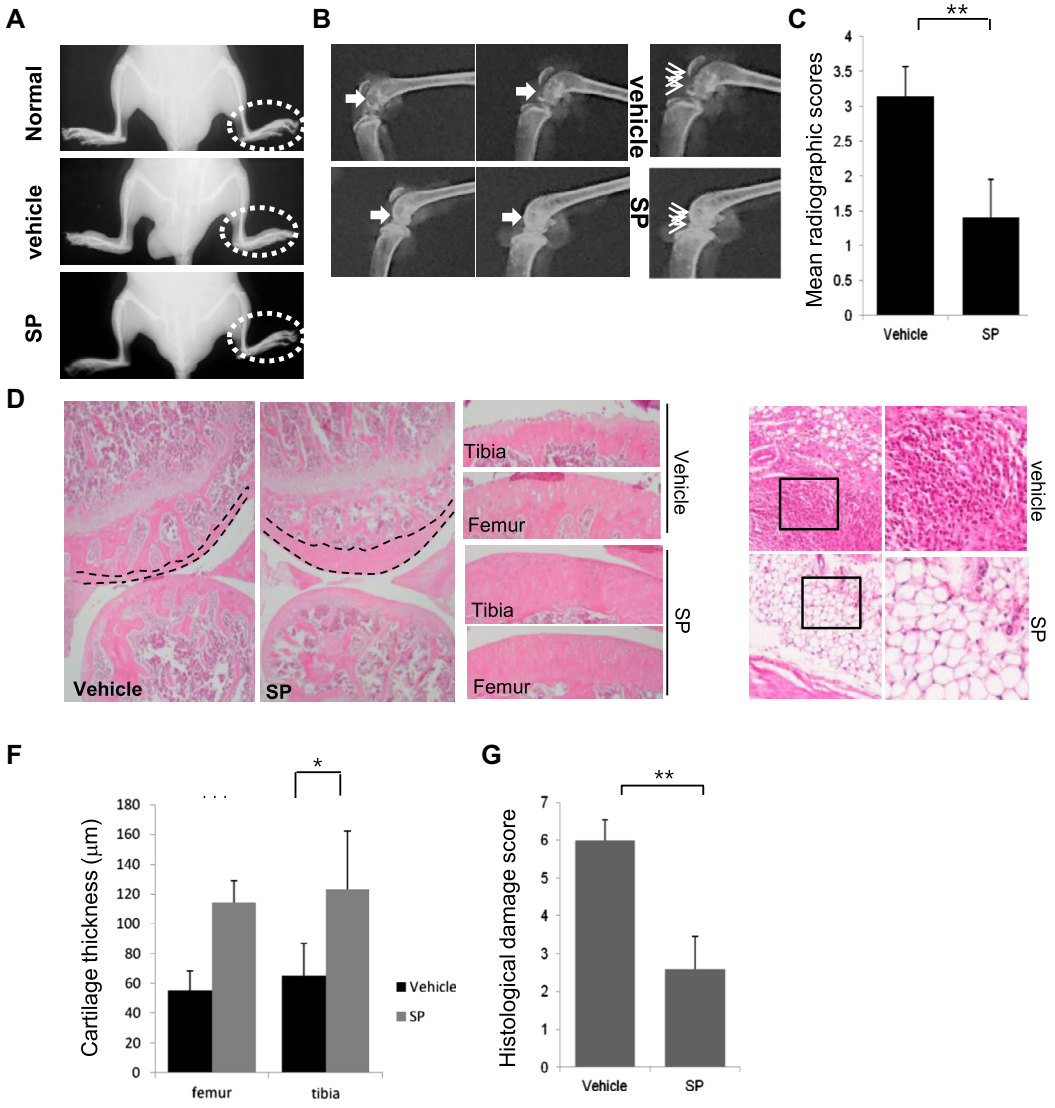

Fig. 2. SP prevents cartilage destruction in the CIA mouse. Radiograph images of paws (A) and joints (B) of CIA mice. (C) Radiographic scores of damage. H&E staining for cartilage (D) and synovial tissue (E) 40×. (F) The thickness of cartilage. The average of measurements for 5 images in each sample was determined for each animal. (G) Quantitative score of severity for inflammation and damage. Values of $p < 0.05$ were interpreted as statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

phages in the circulation within 4.5 h, whereas the total $CD4^{+}$ or $CD11b^{+}$ cellular pools of PBMC were not affected by SP (Supplementary Fig. 1), suggesting that SP can specifically trigger the generation of M2 macrophages and T_{reg}.

Previous studies have consistently shown that administration of SP can mobilize MSCs to the circulation to be engaged in tissue repair [13,27,28]. In mice, SP-mobilized MSCs were observed in the circulation at day 1 post SP injection [13]. Thus, we analyzed the CD105+, CD29+ CD45− MSCs level at day 1 after the final injection of SP. RA itself caused the MSC level of PBMCs to drop below the normal level, which was significantly recovered by SP injection. SP-mobilized MSCs are anticipated to contribute to immune regulation and tissue repair (Fig. 3H). Together, these results provide evidence for the clinical role of SP is in regulating immune response and sequentially mobilizing stem cells, which eventually leads to accelerated tissue repair.

3.4. SP directly react immune cells to induces immune suppression

In an attempt to examine the mechanism of SP responsible for immune suppression in RA, we investigated the change of T_{reg}, M2 type macrophages and cytokines by treating PBMCs with SP *in vitro*. We measured the fractions of T_{reg} and M2 type macrophages in PBMCs that were treated with SP for 4.5 h to explore the early effect of SP. The fraction of T_{reg} and M2 type macrophages in the SP-treated cells was 2-fold higher than that in the vehicle-treated cells (Fig 4A and B). Moreover, the SP treatment increased IL-10 (Fig. 4C) and decreased TNF-α (Fig. 4) in the supernatant of PBMCs within 4.5 h as well as 24 h. These data ascertained that SP can directly enrich T_{reg} or M2 macrophages of PBMCs in combination with increasing the IL-10 and reducing the TNF-α levels, which might be relevant to systemic suppression of inflammation in diseases like RA.

4. Discussion

Biologic therapies targeting inflammatory cytokines and their pathways have greatly reduced the severity of RA. However, approximately 30–40% of RA patients do not respond to the current biologic therapies such as TNF-α blockers, and even in cases where an initial response is present, it is not always maintained. Recently, the pathological role of Th17 cells and their cytokines in autoim-

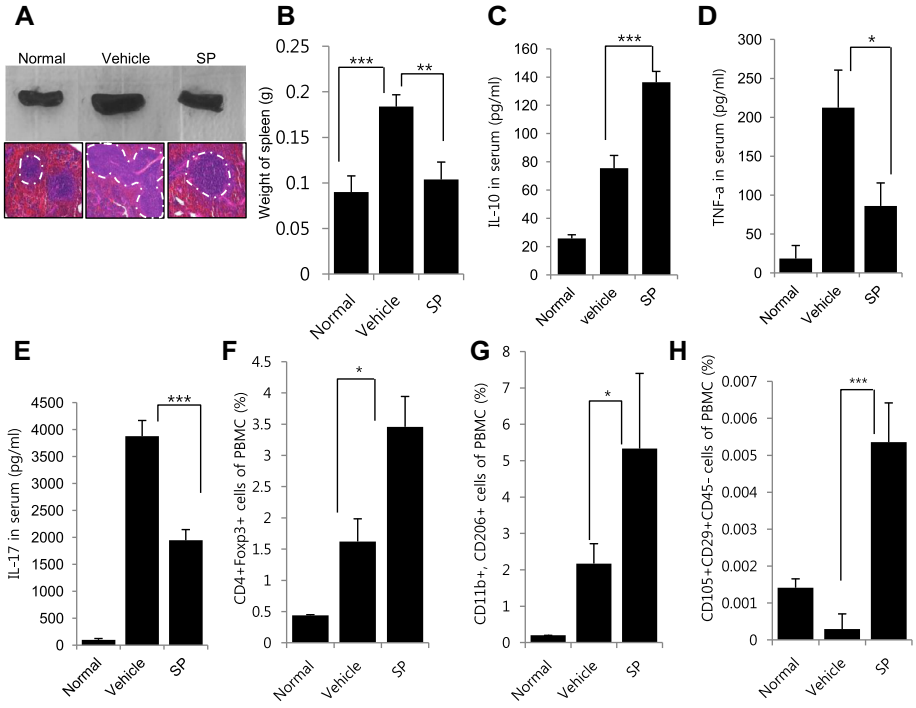

Fig. 3. SP systemically suppresses the inflammatory response *in vivo*. (A) Comparison of spleen sizes and area of germinal center at 6 weeks after the primary induction of RA. (B) Spleen weight. Serum level of IL-10 (C) TNF-α (D) and IL-17 (E). The percentage of T_{reg} (F) and M2 type macrophages (G) of PBMCs. (H) The percentage $CD45^{-}CD29^{+}CD105^{+}$ MSC of PBMCs. Values of $p < 0.05$ were interpreted as statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

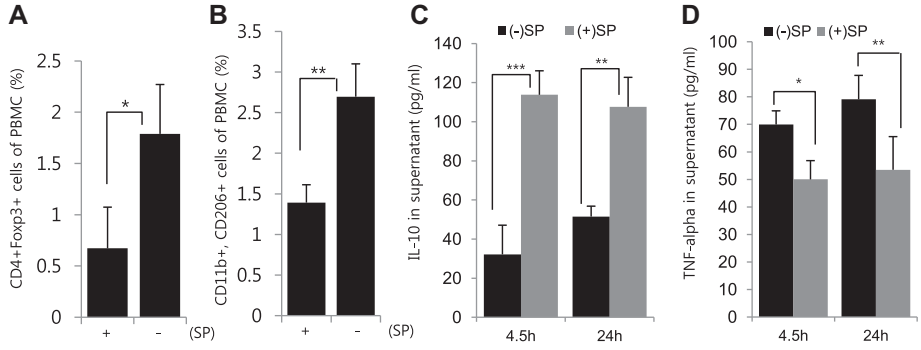

Fig. 4. The direct effect of SP on M2 macrophage/T_{reg}, IL-10 and TNF-α *in vitro*. PBMCs were treated with SP for 4.5 h or 24 h *in vitro*. (A and B) The percentage of T_{reg}/M2 type macrophages of PBMCs. (C and D). The level of IL-10 and TNF-α in supernatant. Values of $p < 0.05$ were interpreted as statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

mune disease development has been evaluated in non-responders to TNF-α blockers. Among these patients, pro-inflammatory cytokines such as IL-12 and IL-17 were elevated in the blood and joints.

Recombinant IL-10 was proposed as an anti-inflammatory therapy in RA. The frequency and severity of CIA was suppressed when the level of IL-10 was increased systemically [5,29–32]. Moreover, disease acceleration is also observed in cases of CIA if anti-IL-10 treatment is administered or IL-10-deficient mice are used [33,34]. Treatment with recombinant IL-10 is now being investigated in patients with RA, and a phase I study of the IL-10 treatment was already completed. However, its clinical result was very marginal and unpredictable [35–36].

In this study, we found that injection of SP after the initiation of the CIA phenotype can successfully abrogate the progression of RA. This effect was confirmed by reductions in the arthritis score, radiographic score, and histological cartilage damage score. As part of its mechanism of action on the RA pathology, it was shown that SP skewed the systemic cytokine profiles toward anti-inflammation by reducing the levels of TNF-α and IL-17 and increasing that of IL-10. Moreover, SP could increase the percentage of T_{reg} and M2 type macrophages in the circulation within 4.5 h after SP administration. These results demonstrate the anti-inflammatory role of SP, which might block the progression of RA by suppressing inflammation systemically.

Additionally, *in vitro* experiments showed that SP promoted production of T_{reg} and M2 type macrophages in PBMCs, in which the level of IL-10 was increased and that of TNF-α was reduced, compared to that of the vehicle-treated cells. Therefore, the change in the cytokine profiles induced by SP could be considered as a consequence of the direct regulation of blood immune cells by SP, which was independent of the function of SP in mobilization of MSCs to the wound sites.

Since the SP treatment in this study started at 4 weeks after the primary immunization, when the primary signs of RA had emerged, it remains unclear whether SP can work at the onset of autoimmune disease. However, its efficacy in the alleviation of

RA severity was clearly shown. In this regard, the SP-mediated blockade of RA progression would be clinically effective and should be considered as a potential RA therapeutic. Further studies of the effects of SP on innate immune cells as well as adaptive immune cells in the joints will be undertaken to precisely examine the mechanism of action of SP in RA.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.090.

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