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From Cell Biology to Tissue Engineering

Inhibition of the TWEAK/Fn14 pathway attenuates autoimmune arthritis in a SKG mouse model

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Summary. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a proinflammatory cytokine that is involved in pathogenesis of abnormal or disregulated inflammation. To verify how TWEAK/fibroblast growth factor-inducible gene 14 (Fn14) signals affect development of Th17 cells in arthritis, we utilized the SKG mouse, which spontaneously develops Th17-mediated autoimmune arthritis. Fn14-Fc was administered to zymosan A-induced arthritogenic SKG mice, and the effects *in vivo* were examined. Destruction of cartilage and bone damage was assessed by Hematoxylin and Eosin, and safranin O staining of the affected tissues. Phenotypic analysis of cells expressing inflammatory cytokines and angiogenesis-related factors, and the expression of transcription factor STAT3 in the affected joints were determined by immunohistochemistry.

Blockade of Fn14 with Fn14-Fc reduced the clinical and histologic scores of inflammatory arthritis in the mouse model of spontaneously developed chronic autoimmune arthritis. Fn14-Fc suppressed production of inflammatory cytokines and angiogenesis-promoting factors, such as vascular endothelial growth factor and matrix metalloproteinase 3. Moreover, blocking of the TWEAK signal inhibited expression of STAT3 as well as interleukin-17 and -21 produced by Th17 cells. These results implicate TWEAK as a potential molecular target for treatment or prevention of inflammatory arthritis and autoimmune diseases such as rheumatoid arthritis.

Key words: TWEAK, SKG mice, STAT3, IL-17-producing T cells, Angiogenesis

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Introduction

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily, and is a multifunctional cytokine that has been implicated in diverse biological activities including cellular proliferation, stimulation of apoptosis, induction of proinflammatory cytokines, and angiogenesis via its receptor, fibroblast growth factor-inducible-gene 14 (Fn14) (Winkles et al., 2006). Fn14 is a type I transmembrane receptor belonging to the TNF receptor superfamily and is expressed on a wide variety of tissues including the heart, kidneys, placenta, lungs, pancreas, and skeletal muscle (Feng et al., 2000; Wiley and Winkles, 2003).

Rheumatoid arthritis (RA) is the prototypical systemic autoimmune disease characterized by infiltration of inflammatory cells into the joints. RA leads to proliferation of synoviocytes, chronic inflammation, and destruction of articular cartilage and bone (Firestein, 1996, 2003). Angiogenesis is the new formation of capillary outgrowth from pre-existing blood

vessels. It is crucial for RA as it plays an indispensable role in delivering oxygen and nutrients to augmented inflamed tissues that are capable of inducing a hypoxic environment. Angiogenesis also facilitates the migration of inflammatory cells to the synovium in RA (Szekanecz and Koch, 2008).

Several studies have implicated TWEAK/Fn14 signaling directly and indirectly in the processes of RA. TWEAK and Fn14 are abundantly expressed in active inflamed synovial tissue and serum levels of TWEAK are significantly elevated and are reflected in disease activity in patients with RA (Park et al., 2008; van Kuijk et al., 2010). TWEAK is also capable of up-regulating proinflammatory cytokines, such as interleukin (IL)-1 and IL-6, along with prostaglandin E2, adhesion molecules like intercellular adhesion molecule 1 (ICAM-1) and E-selectin, as well as chemokines, such as IL-8, regulated on activation, normal T-expressed and secreted (RANTES), interferon-gamma-inducible protein-10 (IP-10) and monocyte chemoattractant protein 1 (MCP-1) (Chicheportiche et al., 2002; Kamijo et al., 2008).

The SKG mouse, which has a point mutation in the Src homology 2 (SH2) domain of zeta-chain-associated protein kinase 70 (ZAP-70), is genetically prone to develop autoimmune arthritis via altered T cell antigen receptor signal transduction and thymic selection (Sakaguchi et al., 2003; Yoshitomi et al., 2005). Although the effects of TWEAK/Fn14 signaling in RA have been explored, whether a block of Fn14 can affect arthritis in SKG mice has not been elucidated.

Materials and methods

Animals

SKG mice with the BALB/c background were obtained from Professor Shimon Sakaguchi (Department of Experimental Immunology, World Premier International Immunology Frontier Research Center, Osaka University, Osaka, Japan). The mice were maintained in a specific pathogen-free environment under climate-controlled conditions with a 12-h light/dark cycle at the research animal facility of Catholic University of Korea (Seoul, Korea). They were fed standard mouse chow (Ralston Purina, St. Louis, MO, USA) and water *ad libitum*. All animal care and experimental protocols were examined and approved by the Institutional Animal Care and Use Committee (IACUC) at the School of Medicine, Animal Research Ethics Committee of the university and were conducted in accordance with the Laboratory Animals Welfare Act, and the Guide for the Care and Use of Laboratory Animals.

Induction of arthritis and scoring of clinical signs

Zymosan A (Sigma-Aldrich, St. Louis, MO, USA) was suspended in phosphate-buffered saline and incubated for 10 min in boiling water. The zymosan A solution (2 mg/mouse) was injected intraperitoneally into 7- or 8-week-old mice. Clinical scores were monitored weekly; 0=no swelling or redness; 0.1=swelling or redness of digits; 0.5=mild swelling and/or redness of wrists or ankle joint; and 1=severe swelling of larger joints. Scores of affected joints were totaled for each mouse (Sakaguchi et al., 2003).

Treatment with Fn14-Fc

To assess the influence of Fn14-Fc (A&R Therapeutics, Daejeon, Korea) on the severity of symptoms in the arthritis model, SKG mice were treated with 100 μg/mouse Fn14-Fc in saline or control-Fc via intraperitoneal injections three times per week after induction of arthritis with zymosan A.

Histopathological analysis

Joint tissues were fixed in 4% (v/v) para-formaldehyde, decalcified in a histological decalcifying agent (Calci-Clear Rapid; National Diagnostics, Atlanta, GA, USA), embedded in paraffin, and sectioned. The sections were stained with hematoxylin-eosin (H&E) and safranin O. The extent of inflammation and cartilage damage was scored in a manner as previously reported (Rosloniec et al., 2001). Inflammation was scored using the following criteria: 0=no inflammation, 1=slight thickening of the lining or infiltration of some cells into the underlying layer; 2=slight thickening of the lining with infiltration of some cells into the underlying layer, 3=thickening of the lining, with an influx of cells into the underlying layer, and cells evident in the synovial space; and, 4=extensive infiltration of the synovium by inflammatory cells. Cartilage damage was evaluated by staining with safranin-O and toluidine blue, and the extent of such damage was scored using the following criteria: 0=no destruction; 1=minimal erosion (limited to single spots); 2=slight-to-moderate erosion in a limited area; 3=more extensive erosion; and 4=general destruction.

Immunohistochemistry

Tissues were incubated with primary antibodies against TNF-α, IL-1β, IL-17, IL-21, vascular endothelial growth factor (VEGF), matrix metalloproteinase 3 (MMP3) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and STAT3 (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Incubation with a biotinylated secondary antibody was followed by streptavidin-peroxidase complex and incubation continued for a further 1 h. Final colored products were developed using the chromogen diaminobenzidine (Thermo Scientific, Rockford, IL, USA) and the sections were examined under a photo microscope (Olympus, Tokyo, Japan). The slide images stained for TNF-α, IL-1β, IL-17, IL-21, and STAT3 were projected on a screen at higher magnification, and the positive cells were

enumerated visually by four individuals. Mean values were calculated.

Statistical analysis

Statistical analysis was calculated using Prism 5.1 software (GraphPad, San Diego, CA, USA). The experimental values are presented as the means ± SD of at least three experiments. P values were calculated by 2-tailed t-test and two-way analysis of variance (grouped). A p-value of <0.05 was considered to indicate statistical significance.

Results

Suppression of severity of arthritis in Fn14-Fc-treated SKG mice

To clarify the *in vivo* effects of Fn14-Fc on arthritis development, SKG mice were injected with zymosan A and treated with Fn14-Fc. Intraperitoneal injection of Fn14-Fc (100 μg/mouse) reduced the incidence of arthritis compared with injection of control-Fc (Fig. 1). Histologic examination of the ankle joints stained with H&E and safranin O revealed that in a lower degree of inflammation and less severe cartilage destruction in Fn14-Fc-treated mice Fn14-Fc-treated mice 70 days after the first Fn14-Fc injection, when compared to the paws and ankles of control-Fc-treated mice (Fig. 2). These findings suggested that blockade of TWEAK-Fn14 signal suppresses arthritic development in SKG mice.

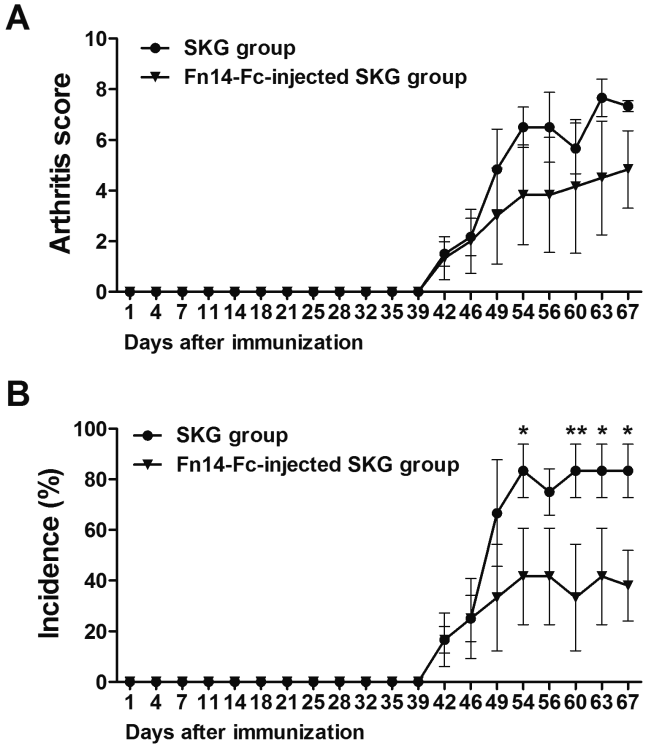

Fig. 1. Suppression of arthritis development of SKG mice by Fn14-Fc treatment. SKG mice were each treated with 100 μg Fn14-Fc in saline or with control-Fc by intraperitoneal injection three times a week beginning 7 days after zymosan A injection and continuing for 10 weeks (n=5/group). Panel A displays clinical scores and panel B displays incidence of arthritis. *P<0.05, **P<0.01.

Anti-inflammatory effects of Fn14-Fc

The proinflammatory cytokines TNF-α and IL-1β are considered to be key proinflammatory factor players in RA, and have been implicated in the pathogenesis of RA (Choy, 2012). To verify the effects of Fn14-Fc on regulation of inflammatory cytokines, immunohistochemical analysis of inflammatory cytokines was done in the joint tissue of mice treated with Fn14-Fc or control-Fc. Fewer TNF-α- and IL-1β-positive cells were observed in the joints of Fn14-Fc-treated mice compared to control-Fc-treated mice (Fig. 3).

VEGF is important in angiogenesis and lymphangiogenesis. It acts as a highly specific mitogen for endothelial cells and promotes cell migration and inhibits apoptosis (Neufeld et al., 1999). MMP3 also induces angiogenesis by enhancing extracellular matrix degradation (Jin et al., 2006). In comparison with control-Fc-treated group, the joint tissue from mice treated with Fn14-Fc showed decreased numbers of VEGF+ cells and MMP3+ cells (Fig. 4). These findings suggest that blockade of Fn14 signal could modulate the expression of inflammatory cytokines in the pathogenesis of RA.

Inhibition of STAT3-mediated IL-17 and IL-21 expression by blockade of Fn14

Activated STAT3 in the inflamed synovium has important roles in inflammatory arthritis (de Hooge et al., 2004; Sawa et al., 2006; Mori et al., 2011). STAT3 is a transcription factor promoting differentiation of IL-17-producing T helper cells (Th17 cells) (Yang et al., 2007). Th17 cells produce IL-17A, IL-17F, and IL-21 and are the major effector cells in the pathogenesis of numerous autoimmune disorders including RA (Bettelli et al., 2006; Liang et al., 2006; Tesmer et al., 2008; Miossec et al., 2009). To investigate whether blocking Fn14 could suppress the expression of STAT3, immunohistochemical analysis of STAT3 was done in the joint tissue of mice treated with Fn14-Fc or control-Fc. Treatment of Fn14-Fc markedly decreased the expression of STAT3 in arthritic SKG mice compared to that in control-Fc-treated SKG mice (Fig. 5). Expression of IL-17 and IL-21 was also evaluated in the Fn14 blocked arthritic SKG mice, as IL-17 and IL-21 are the major cytokines produced from activation of Th17 cells. As expected, mice treated with Fn14-Fc showed a decreased expression of IL-17 and IL-21 (Fig. 6). These findings verify that blockade of Fn14 signal could modulate the

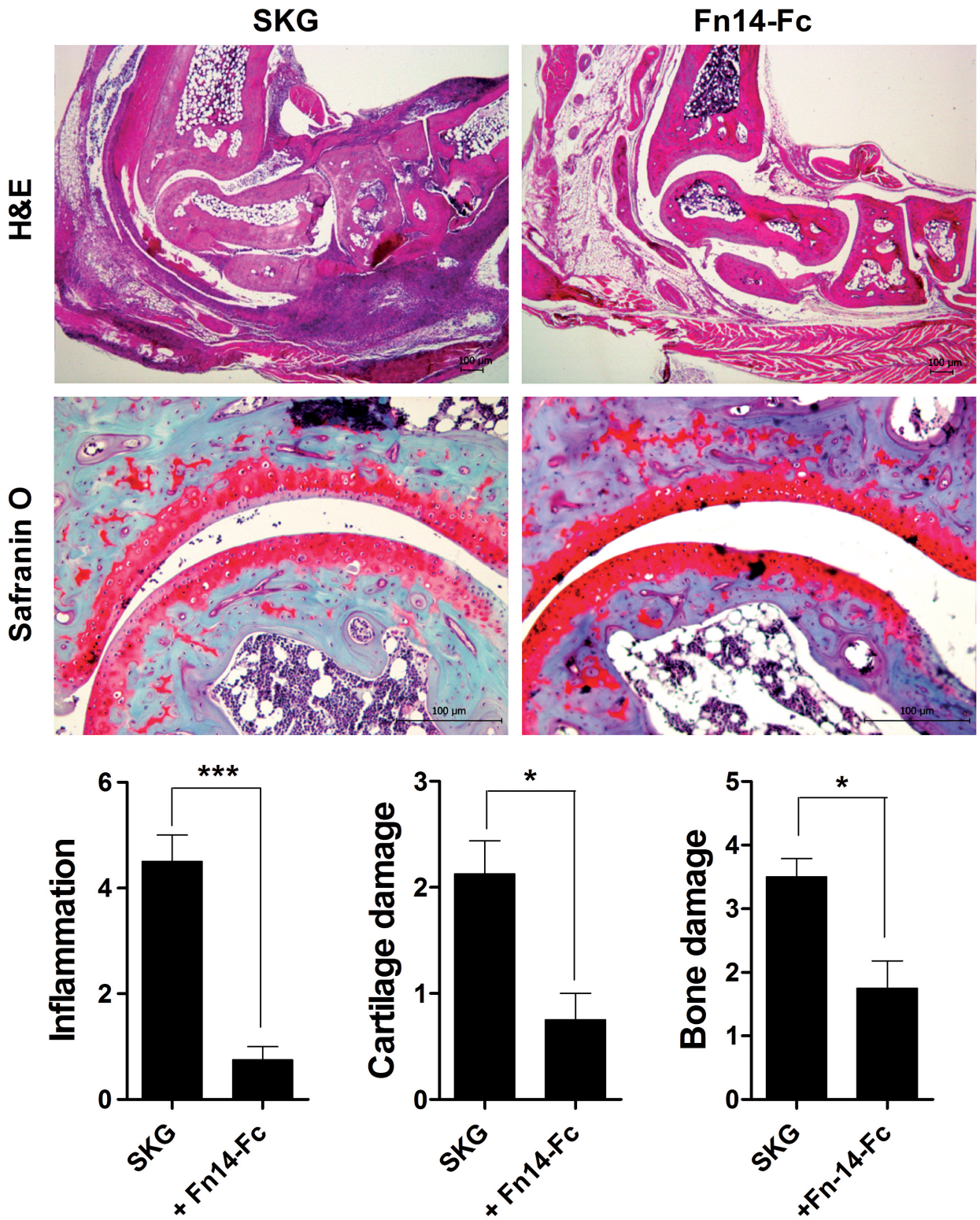

Fig. 2. Lower degree of inflammation and cartilage destruction in Fn-14-treated SKG mice. Representative histological features of the joints from Fn14-Fc- or control-Fc-treated mice (four joints/group). Specimens were stained with H&E and safranin O. Histologic scores for inflammation, cartilage damage and bone damage in SKG mice treated with Fn14-Fc or control-Fc are shown. Original magnification: × 40 for H&E and × 400 for safranin O; *P<0.05, ***P<0.001.

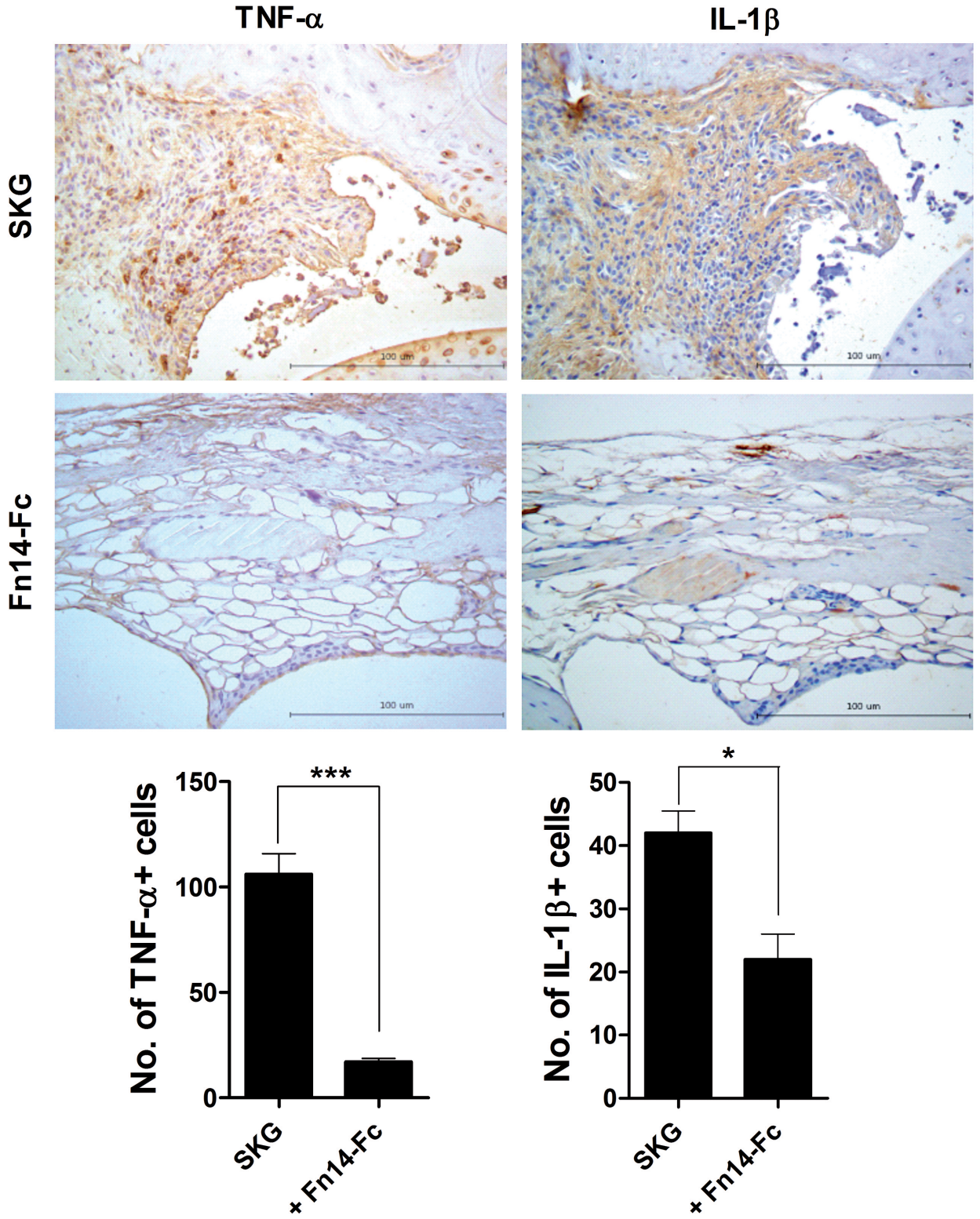

Fig. 3. Effects of Fn14-Fc on inflammatory cytokines (TNF-α and IL-1β) in the joints of Fn14-Fc-treated SKG mice (four joints/group). Representative histological features of the joints from Fn14-Fc- or control-Fc-treated mice stained with anti-TNF-α- and IL-1β. Cells that stained positively for each antibody are brown colored. Histologic scores for TNF-α and IL-1β in SKG mice treated with Fn14-Fc or control-Fc are shown. *P<0.05, ***P<0.001. × 400

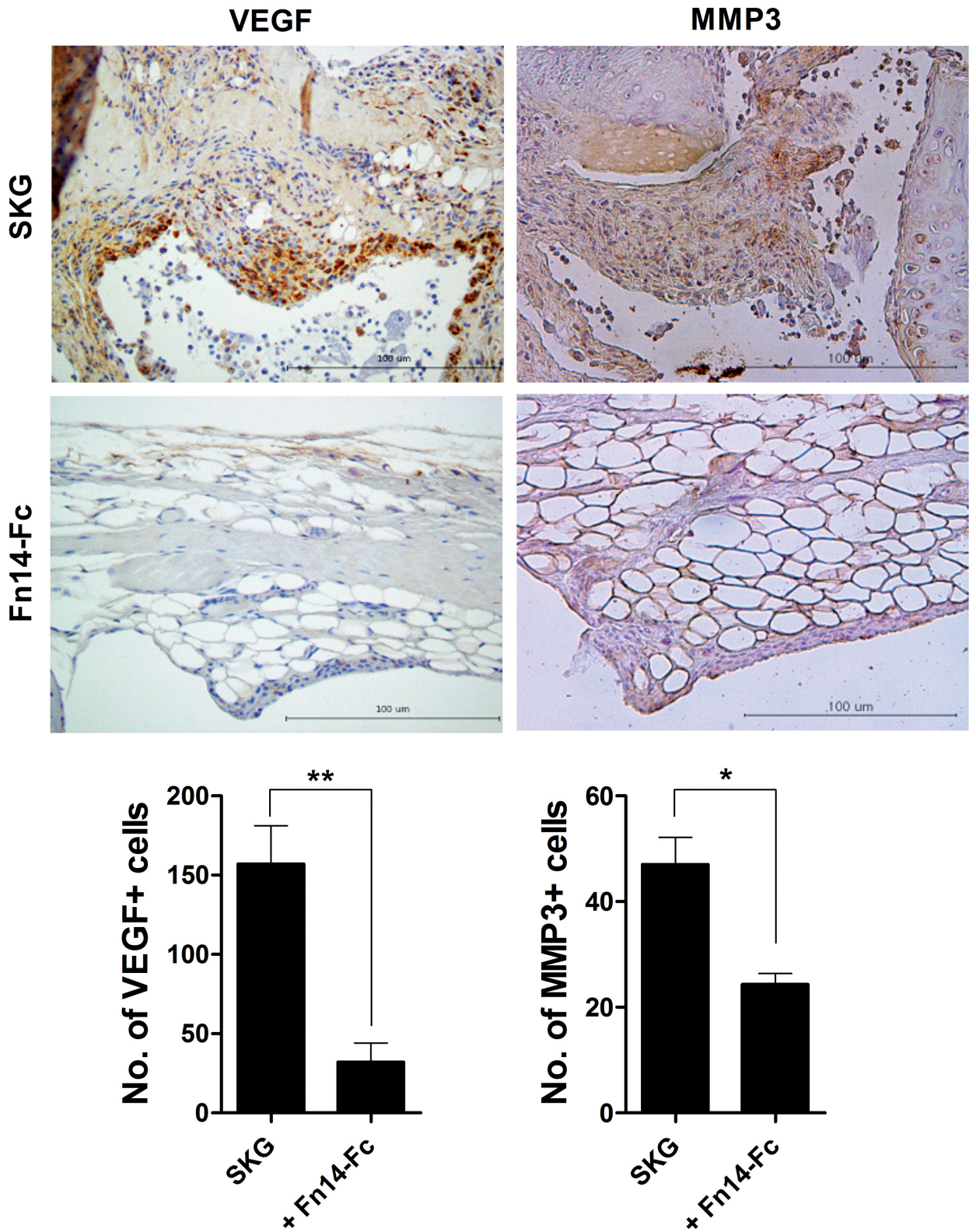

Fig. 4. Suppressive effects of Fn14-Fc on angiogenesis related factors VEGF and MMP3 in the joints of Fn14-Fc-treated SKG mice (four joints/group). Representative histological features of the joints from Fn14-Fc- or control-Fc-treated mice stained with anti-VEGF- and MMP3. The cells positively stained with each antibody are brown colored. Histologic scores for VEGF and MMP3 in SKG mice treated with Fn14-Fc or control-Fc are shown. *P<0.05, **P<0.01. × 400

effects of STAT3-mediated Th17 cells. These results indicate that inhibition of Fn14 signal suppresses Th17 cell differentiation via control of STAT3.

Discussion

We sought to verify the role of TWEAK-Fn14 signaling in arthritic mice caused by altered signal transduction in T cells. Treatment with Fn14-Fc reduced the clinical and histologic scores in SKG mice, a model of spontaneously generated chronic autoimmune arthritis. Our data demonstrate that blockade of TWEAK-Fn14 signal can alleviate arthritis parameters by suppressing inflammatory cytokines and angiogenesis related factors, as well as modulating STAT3-mediated Th17 cell differentiation.

Upon antigen stimulation, naïve CD4+ T cells undergo rapid growth and proliferation, and can differentiate into various subtypes of effector cells depending on the cytokine milieu during activation. Each of these subsets has distinct functions and cytokine profiles in the adaptive immune system. Among the various effector T cell subsets, Th17 cells can be distinguished from Th1 or Th2 cells based on their expression of IL-17A, IL-17F, and IL-21 and play a major role in the pathogenesis of numerous autoimmune disorders, such as autoimmune arthritis, while regulatory T cells play an opposing role, controlling excessive inflammatory response (Bettelli et al., 2006; Miossec et al., 2009). IL-6 is an essential cytokine that generates Th17 cells through activation of STAT3, which is the major transcription factor in the differentiation of Th17 cells (Yang et al., 2007).

Sakaguchi and colleagues first generated SKG mice, which develop self-reactive T cells as a consequence of a point mutation in the SH2 domain of ZAP-70, a highly T cell-restricted molecule. This genetic defect impairs positive and negative selection in the thymus, causing the production of self-reactive arthritogenic T cells, which mediate autoimmune arthritis (Sakaguchi et al., 2003; Hata et al., 2004; Tanaka et al., 2010). *In vitro* self-reactivity of CD4+ T cells from SKG mice could stimulate antigen presenting cells to form cytokine milieu including IL-6, TNF-α, and IL-17; this cytokine milieu facilitates preferential differentiation of self-reactive T cells into Th17 cells (Hirota et al., 2007a). Furthermore, it was shown in this model that the expression of CCL20 is required for recruitment of CCR6-expressing Th17 cells to the inflamed synovial tissues, leading to an autoimmune reaction in the joints (Hirota et al., 2007b).

Several studies have reported direct and indirect evidence of the capability of TWEAK to promote RA-related pathogenesis. TWEAK and Fn14 are abundantly expressed in synovial tissues from RA patients, and synovial fluid and serum level of TWEAK are elevated and correlate well with the disease index in patients with RA (Park et al., 2008). Induction of proinflammatory cytokines by TWEAK has also been observed in cultured fibroblast-like synoviocytes (FLSs) from patients with RA (Chicheportiche et al., 2002; Kamijo et al., 2008). We demonstrated that TWEAK promotes osteoclastogenesis through induction of RANKL expression in FLSs (Park et al., 2013). We have also reported that TWEAK enhances the expression of IL-17 by itself and synergistically by cooperation with other inflammatory cytokines, such as IL-23 or IL-21, and blockade of Fn14 suppresses Th17 cell differentiation *in vitro* (Park et al., 2012).

Several anti-TWEAK drugs are currently being developed for the treatment of autoimmune diseases, such as systemic lupus erythematosus-related nephritis and cancers. BIIB023, a monoclonal antibody against TWEAK, has been evaluated for the treatment of

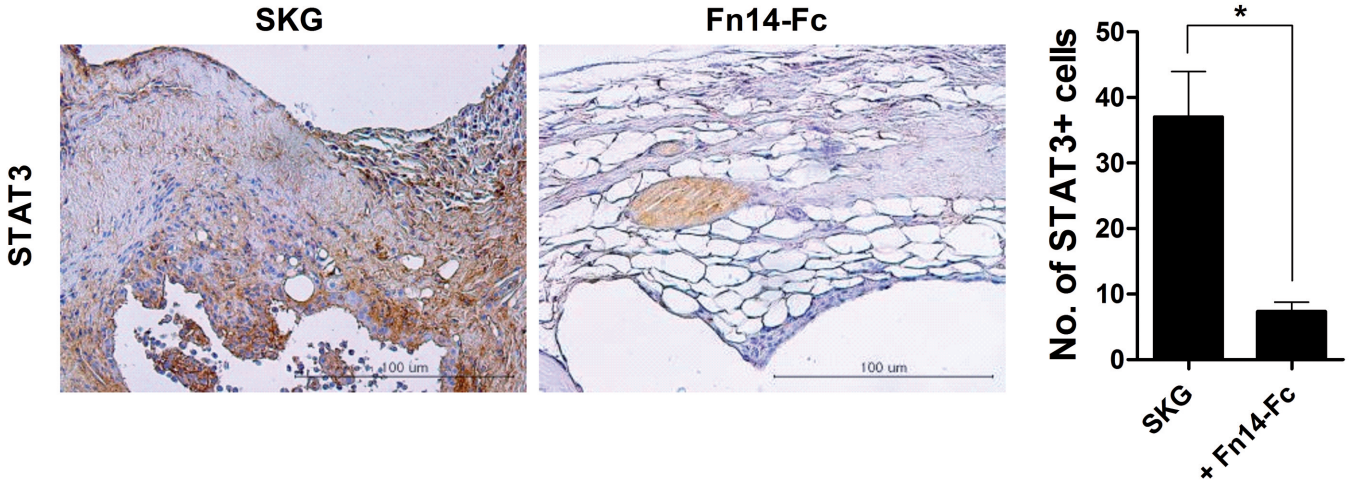

Fig. 5. Suppression of STAT3 expression in the joints of Fn14-Fc-treated SKG mice (four joints/group). Representative histological features of the joints from Fn14-Fc- or control-Fc-treated mice stained with anti-STAT3. The cells stained positively are brown colored. Histologic scores for STAT3 staining in SKG mice treated with Fn14-Fc or control-Fc are shown. *P<0.05. × 400

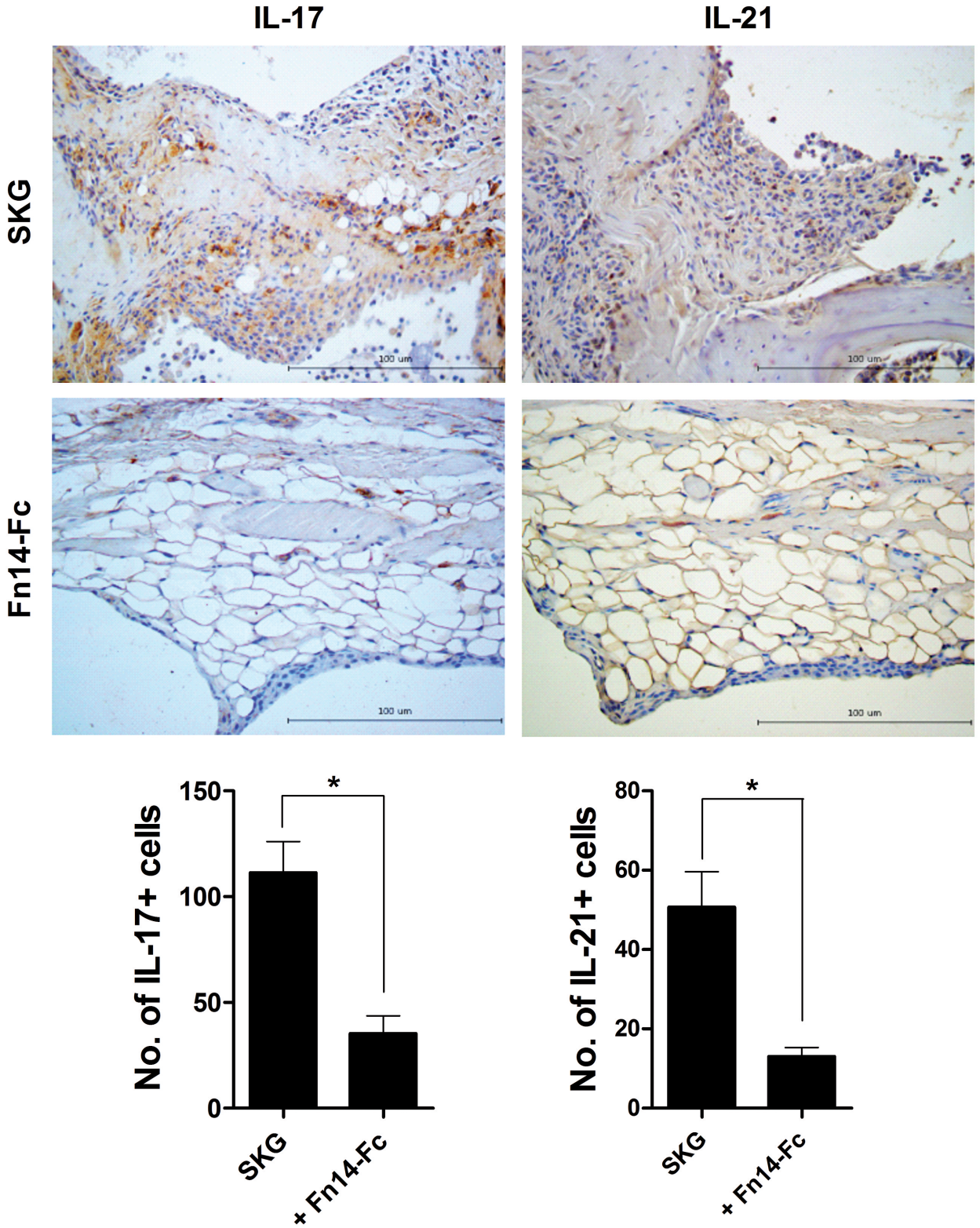

Fig. 6. Effects of Fn14-Fc on Th17 related inflammatory cytokines IL-17 and IL-21 in the joints of Fn14-Fc-treated SKG mice (four joints/group). Representative histological features of the joints from Fn14-Fc- or control-Fc-treated mice stained with anti-IL-17 and anti-IL-21. The cells staining positively for each antibody are brown colored. Histologic scores for IL-17 and IL-21 in SKG mice treated with Fn14-Fc or control-Fc are shown. *P<0.05. × 400

rheumatoid arthritis (Phase 1) and lupus nephritis (Phase 2) with good tolerance and safety (Wisniacki et al., 2013). RO5458640, a TWEAK-specific antibody, and PDL192, an anti-TWEAK receptor monoclonal IgG1 antibody, have been assessed for the treatment of solid tumors in phase 1. These results suggest that improvement of TWEAK targeting could be a good therapeutic strategy to treat patients with RA.

The cytoplasmic region of TWEAK can bind TRAF family members 1, 2, 3, and 5, but not TRAF 4 and 6. This binding mediates the nuclear factor-kappa B (NF-κB) transcription factor signaling pathway, with the NF-κB pathway playing important roles in the pathogenesis of inflammatory disease (Wiley et al., 2001; Brown et al., 2003; Saitoh et al., 2003). It has been speculated that TWEAK acts as a NF-κB pathway activator, playing a role in autoimmune disease such as RA; however, the intrinsic function and the roles of TWEAK in implicating Th17 cells was not clearly shown. To verify how TWEAK/Fn14 signal affects the Th17 cells, we utilized SKG mice that spontaneously develop Th17-mediated autoimmune arthritis.

Treatment with anti-TWEAK Ab inhibits arthritogenic mediators including MMP-9, IP-10, MCP-1, and TNF-α, resulting in a reduction in joint inflammation (Kamata et al., 2006; Perper et al., 2006). Presently we confirmed that blockade of TWEAK/Fn14 signal reduced the symptoms of arthritis, including cartilage damage and inflammatory cytokines, such as TNF-α and IL-1β, in arthritogenic SKG mice. Of note, blockade of Fn14 with Fn14-Fc also inhibited the expression of STAT3 as well as IL-17 and IL-21, which are produced by STAT3-mediated Th17 cells. Persistent activation of STAT3 also stabilizes hypoxia-induced factor 1α via a direct interaction and enhances VEGF expression under hypoxia conditions (Jung et al., 2005). Based on our findings, blocking of TWEAK/Fn14 signal is expected to inhibit angiogenesis related factor VEGF via targeting of STAT3 and its downstream pathway. Further molecular analyses are required to fully explore the action of TWEAK/Fn14 signal in STAT3-mediated immune responses.

In conclusion, we demonstrated that blockade of Fn14 with Fn14-Fc improved the clinical score of inflammatory arthritis in SKG mice. Fn14-Fc suppressed the production of inflammatory cytokines and angiogenesis promoting factors, such as VEGF and MMP3. Moreover, blocking of the TWEAK signal inhibited expression of STAT3 as well as IL-17 and IL-21, which are produced by Th17 cells. These results suggest that TWEAK is a potential molecular target for treatment or prevention of inflammatory arthritis and autoimmune diseases such as RA.

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