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A possible role for TSLP in inflammatory arthritis

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Abstract

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that triggers dendritic cell-mediated Th2-type inflammatory responses and is considered as a master switch for allergic inflammation. In this study, we found increased levels of TSLP and, also TNF-α as previously reported, in synovial fluid specimens derived from patients with rheumatoid arthritis (RA) when compared with those from patients with osteoarthritis (OA). In addition, TNF-α up-regulated TSLP expression in RA- and OA-derived synovial fibroblasts, which was inhibited by IFN-γ. Furthermore, anti-TSLP neutralizing antibody ameliorated a TNF-α-dependent experimental arthritis induced by anti-type II collagen antibody in mice. Collectively, these results suggest that TSLP, as a downstream molecule of TNF-α, may be involved in the pathophysiology of inflammatory arthritis. TSLP might thus play a role not only in allergic diseases but also in inflammatory arthritis such as RA.

Keywords: TSLP; Synovial fibroblasts; TNF-α; Rheumatoid arthritis

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine, which binds to the TSLP receptor (TSLPR) consisting of the IL-7 receptor α-chain (IL-7Rα) and a common γ receptor-like chain (TSLPR-γ) [1,2]. TSLP has been shown to be expressed primarily by epithelial cells including keratinocytes, whereas TSLPR is expressed by hematopoietic cells, including monocytes, T cells, B cells, and $CD11c^+$ dendritic cells (DCs) [3–5].

TSLP was originally identified as a factor derived from a thymic stromal cell line that could support the growth of a mouse B cell line [3]. However, recent studies have clearly shown that TSLP potently activates $CD11c^+$ myeloid DCs, leading to the differentiation of CD4 T cells into TNF-α-producing Th2 cells (so-called inflammatory Th2 cells), and plays a key role in the development of allergic inflammation [6]. Transgenic mice expressing TSLP in keratinocytes or in lung epithelial cells have been shown to develop atopic dermatitis- or asthma-like inflammation in the skin or the lung, respectively, while TSLPR null mice failed to develop an inflammatory lung response to inhaled antigen [7–9]. In humans, TSLP has been shown to be expressed by keratinocytes in atopic dermatitis and by bronchial epithelial cells in the asthmatic airways [10,11]. TSLP expression has been also demonstrated to be spontaneously induced in adult mouse keratinocytes in retinoid X receptor (RXR)-α and -β null mice and topical application of vitamin D3 or retinoic acid receptor (RAR) γ-selective

Abbreviations: TSLP, thymic stromal lymphopoietin; RA, rheumatoid arthritis; OA, osteoarthritis; DMARDs, disease modifying anti-rheumatic drugs; NSAIDs, nonsteroidal anti-inflammatory drugs.

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agonist BMS961 induced TSLP in mouse keratinocytes [12,13], thus suggesting that the TSLP expression in mouse keratinocytes is regulated by vitamin D and retinoic acid signaling.

In the current study, we aimed to investigate whether TSLP also plays a role in inflammatory arthritis. We found that (1) TSLP and TNF-α levels are co-elevated in the synovial fluid specimens derived from patients with RA, not with OA, (2) TNF-α stimulates synovial fibroblasts to produce TSLP, and (3) blockade of TSLP activity ameliorates a TNF-α-dependent experimental arthritis. Taken together, these results suggest that TSLP may be involved not only in allergic diseases, but also in inflammatory arthritis such as RA as a downstream effector molecule of TNF-α.

Materials and methods

Reagents. Recombinant human TNF-α, IFN-γ, TGF-β, and IL-6 were purchased from R&D Inc. (Minneapolis, MN).

Synovial fluid samples. Samples of undiluted synovial fluid were obtained from 10 patients with RA and 14 patients with OA, who were diagnosed based on the revised criteria of the American College of Rheumatology for RA [14] or OA [15], by puncture of the knee joint for therapeutic reason and were immediately frozen in liquid nitrogen and stored at −80 °C until use. The profiles of these patients are described in Table 1. The investigation was approved by the Ethics Committee of University of Yamanashi, Faculty of Medicine, and all subjects gave their written informed consent.

Cell culture. Human synovial fibroblasts were obtained as previously described [16]. In brief, after enzymatic digestion, human synovial cells were isolated from synovial tissues of the knee joints of three RA patients and three OA patients, diagnosed based on the revised criteria of the American College of Rheumatology for RA [14] or OA [15], at the time of total knee arthroplasty operations. The investigation was approved by the Ethics Committee of University of Yamanashi, Faculty of Medicine, and all subjects gave their written informed consent. All the RA and OA patients were receiving treatment at the time of the study; the majority were on disease modifying anti-rheumatic drugs (DMARDs for RA) while the remainder were being treated with corticosteroid or nonsteroidal anti-inflammatory drugs (NSAIDs). The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco/Invitrogen, Carlsbad, CA) containing 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin G solution, and then were cultured in monolayers. After three to five passages, the subcultured cells were composed of morphologically uniform fibroblastic cells (synovial fibroblasts) that were free of macrophages. Primary human keratinocytes and skin dermal fibroblasts were purchased from Cambrex Cooperation (East Rutherford, NJ) and then were cultured in the medium as recommended by the manufacturer.

Table 1
Patient profiles

Pt	Age	Sex	Treatment
RA1	67	Female	DMARDs + NSAIDs
RA2	79	Male	Predonine + DMARDs
RA3	79	Female	DMARDs + NSAIDs
RA4	83	Female	Predonine
RA5	77	Female	DMARDs + NSAIDs
RA6	62	Female	Predonine
RA7	77	Female	DMARDs + NSAIDs
RA8	61	Male	DMARDs + NSAIDs
RA9	41	Male	Predonine + DMARDs
RA10	85	Female	DMARDs + NSAIDs
OA1	75	Female	NSAIDs
OA2	79	Female	NSAIDs
OA3	70	Female	NSAIDs
OA4	83	Female	NSAIDs
OA5	85	Female	NSAIDs
OA6	82	Female	NSAIDs
OA7	80	Male	NSAIDs
OA8	85	Female	NSAIDs
OA9	78	Female	NSAIDs
OA10	77	Female	NSAIDs
OA11	74	Female	NSAIDs
OA12	78	Female	NSAIDs
OA13	78	Female	NSAIDs
OA14	78	Female	NSAIDs

DMARDs, disease modifying anti-rheumatic drugs.
NSAIDs, nonsteroidal anti-inflammatory drugs.

ELISA. The amounts of TSLP in the culture supernatants or synovial fluid specimens were measured by ELISA using the Human TSLP ELISA Development Kit (R&D, Minneapolis, MN) according to the manufacturer's instructions. The amounts of TNF-α in the synovial fluid specimens were determined using the human TNF-α ELISA kit (R&D, Minneapolis, MN) according to the manufacturer's instructions.

Induction of arthritis. Six-week-old female BALB/c mice were purchased from SLC (Tokyo, Japan) and kept under specific pathogen-free conditions. The arthritogenic anti-collagen type II mAb cocktails obtained from Chondrex (Seattle, WA) contains four mAbs (F10, A2, D8, and D1) in equal amounts. To establish anti-collagen type II antibody-induced arthritis (CAIA) [17], the mice were intraperitoneally injected with 2 mg per mouse of anti-collagen type II mAb cocktail (day 1) and 3 days later (day 4) with 50 μg per mouse of LPS (Chondrex). Rat monoclonal anti-mouse TSLP neutralizing antibody (R&D, Minneapolis, MN) or the isotype control rat IgG2a (R&D, Minneapolis, MN) (15 mg/kg/per mouse) was intraperitoneally administered on day 5. We have previously conducted independent experiments in which both PBS and control rat IgG2a antibody were directly compared and we did not observe any significant differences in the development of CAIA between PBS and the control antibody treatment groups as also previously described by others [18]. Therefore, the effect of anti-TSLP antibody was directly compared with that of the control rat IgG2a antibody in this study. The animal experiments were approved by the Institutional Review Board of University of Yamanashi.

Clinical evaluation of arthritis. Starting on day 1 after mAb injection, the mice were blindly inspected for disease progression. The clinical severity of the disease was scored using a scoring system based on the number of inflamed joints in forepaws, hind paws, and ankles, inflammation being defined by swelling; 0, normal; 1, slight swelling 2, mild swelling 3, moderate swelling, and 4, severe swelling. All paws and ankles were graded, thus resulting in a maximal clinical score of 24 per mouse, and then were expressed as the mean arthritic index on a given day.

Histology. The hind paws (torsocrural joint) were removed postmortem on day 10 after mAb injection, fixed, and decalcified. The decalcified paws were embedded in paraffin, sectioned, and stained with hematoxylin and eosin or with anti-mouse TSLP antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) following de-paraffinization through the use of peroxidase-based VECTASTAIN ABC kits with DAB substrate (Vector Laboratories, Burlingame, CA).

Data analysis. The data are summarized as means ± SD. The unpaired Student's *t*-test was used for the statistical analysis of the results except for Fig. 1. Mann and Whitney U test was used for the statistical analysis of Fig. 1. A value of $p < 0.05$ was considered to be significant.

Results and discussion

Increased TSLP levels in RA, not OA, synovial fluid

To investigate the roles of TSLP in rheumatoid arthritis, we measured TSLP levels in synovial fluid specimens

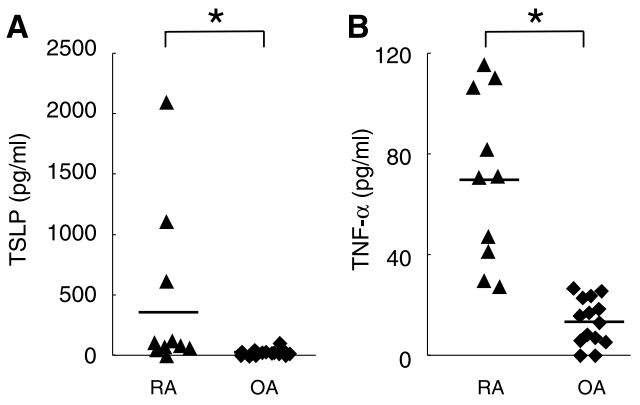

Fig. 1. TSLP and TNF-α are co-elevated in RA synovial fluid, but not in OA synovial fluid. The TSLP (A) and TNF-α (B) levels in the synovial fluid specimens of the knee joints from RA and OA patients (RA 1–10, OA 1–14) were measured by ELISA. Due to the scale, it appears that only three RA patients show elevated synovial fluid TSLP levels, but, actually, 6 out of 10 RA patients showed detectable levels of TSLP in the synovial fluid specimens. The patients' profiles are shown in Table 1. $^{*}p < 0.05$ compared with the corresponding control.

obtained from patients with RA and OA by ELISA. We examined the TSLP expression in 24 synovial fluid samples obtained from 10 RA and 14 OA patients. These patients showed different clinical profiles regarding age, sex, and their treatment (Table 1). The synovial fluid levels of TSLP were elevated in 6 out of the 10 patients with RA, whereas only 1 out of 14 patients showed detectable TSLP levels in the synovial fluid specimens from the patients with OA (Fig. 1A). There was considerable variability in the concentration of TSLP in the RA synovial fluid specimens (RA; 391.5 ± 667.5 pg/ml, $n = 10$) possibly due to the different clinical profiles in their disease severity and/or treatment. We found that the synovial fluid levels of TNF-α were also elevated in RA patients in comparison to those in OA patients (Fig. 1B) as previously described [19]. Because OA has traditionally been regarded as non-inflammatory arthritis, these results may suggest a potential role of TSLP in inflammatory arthritis and also a possible pathological link between TSLP and TNF-α in RA synovial joints, though no correlation was observed statistically

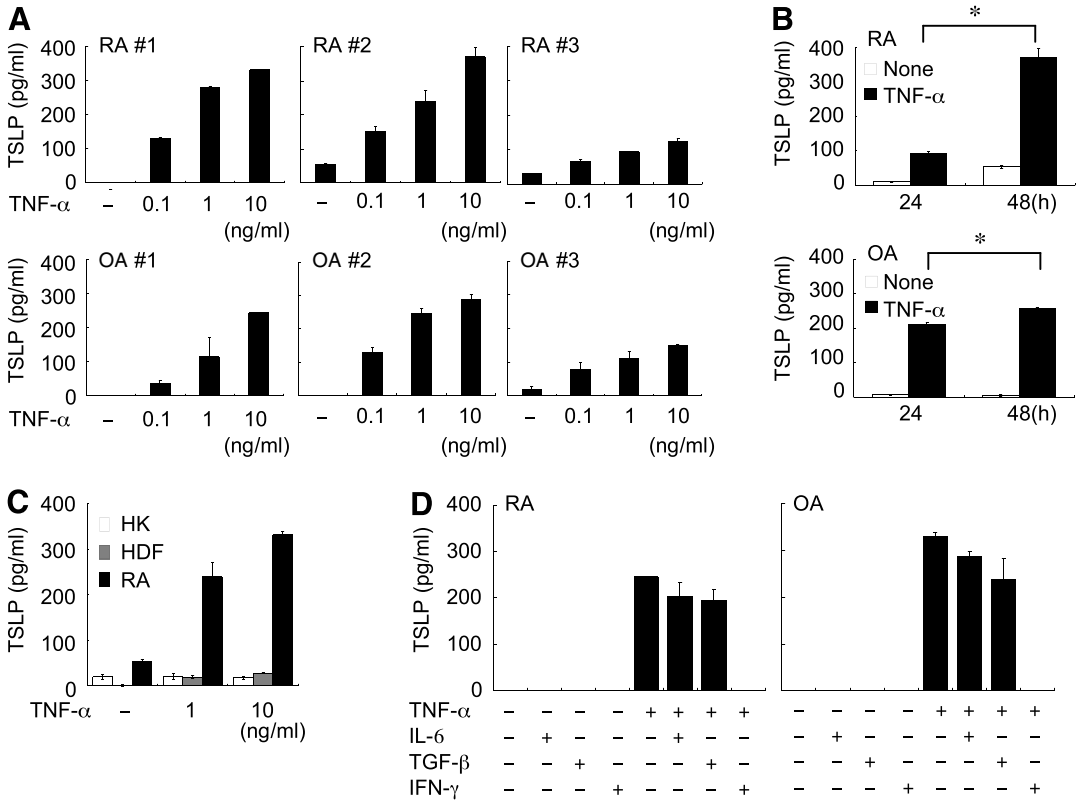

Fig. 2. TNF-α induces TSLP production in RA and OA synovial fibroblasts. (A) RA and OA synovial fibroblasts (2.5×10^5 cells/well) derived from three RA and three OA patients (RA #1–3, OA #1–3) were cultured in the presence or absence of the indicated doses of TNF-α for 24 h. The culture supernatants were then collected and the TSLP concentrations were measured by ELISA. (B) Representative RA and OA synovial fibroblasts (2.5×10^5 cells/well) were cultured in the presence or absence of 10 ng/ml TNF-α for 24 and 48 h. The culture supernatants were then collected and the TSLP concentrations were measured by ELISA. (C) Human keratinocytes (HK) and human dermal fibroblasts (HDF) derived from normal adult skin (2.5×10^5 cells/well) were cultured in the presence or absence of the indicated doses of TNF-α for 24 h. The culture supernatants were then collected and the TSLP concentrations were measured by ELISA. The TSLP levels were compared with representative RA synovial fibroblasts (2.5×10^5 cells/well). (D) Representative RA and OA synovial fibroblasts (2.5×10^5 cells/well) were cultured in the presence or absence of 10 ng/ml TNF-α and/or 10 ng/ml IFN-γ, TGF-β, IL-6 for 24 h. The culture supernatants were then collected and the TSLP concentrations were measured by ELISA. Representative results of four independent experiments using synovial fibroblasts derived from four RA or OA patients are shown (B–D). Values represent means ± SD. $^{*}p < 0.05$ compared with the corresponding control.

between the two cytokine levels in the small number of synovial fluid samples.

TNF-α up-regulates TSLP in synovial fibroblasts

Based on the clinical findings described above, we investigated whether RA synovial fibroblasts expressed TSLP upon stimulation with TNF-α. A total of six synovial fibroblasts were isolated from three patients with RA and three patients with OA at the time of total knee arthroplasty operations and were then stimulated with TNF-α (Fig. 2). TNF-α significantly up-regulated TSLP expression in RA and OA synovial fibroblasts in a dose- and time-dependent manner (Fig. 2A and B). In some synovial fibroblasts, TSLP was found to be constitutively produced for unknown reasons (Fig. 2A). Dermal fibroblasts obtained from human adult skin showed only marginal elevation of TSLP upon stimulation with TNF-α (Fig. 2C). TNF-α did not up-regulate TSLP in human primary keratinocytes (Fig. 2C). In addition, the TNF-α-induced TSLP production in RA and OA synovial fibroblasts was significantly inhibited by IFN-γ, but not by IL-6 and TGF-β (Fig. 2D). These results indicated that TNF-α up-regulated TSLP production in RA and OA synovial fibroblasts in vitro.

Anti-TSLP neutralizing antibody ameliorates the arthritis induced by anti-collagen type II antibody in mice

Because TNF-α is a critical cytokine in the pathophysiology of RA [19], we investigated whether TSLP is involved in the development of RA-like TNF-α. For this purpose, we used a mouse model of arthritis which was developed by the injection of monoclonal antibodies (mAbs) against type II collagen followed by the subsequent injection of bacterial LPS to reduce the threshold of the arthritogenic dose of mAbs and the required number of mAb clones (collagen type II antibody-induced arthritis; CAIA) [17]. The development of this arthritis model, in particular in the early phase, is independent of T cells and B cells [20,21], and it is dependent on TNF-α [18], with neutrophils and macrophages being the major mediator of this inflammation [17]. Therefore, the CAIA model provides an opportunity to study the inflammation phase without involving the priming phase of the immune responses. Wild-type mice

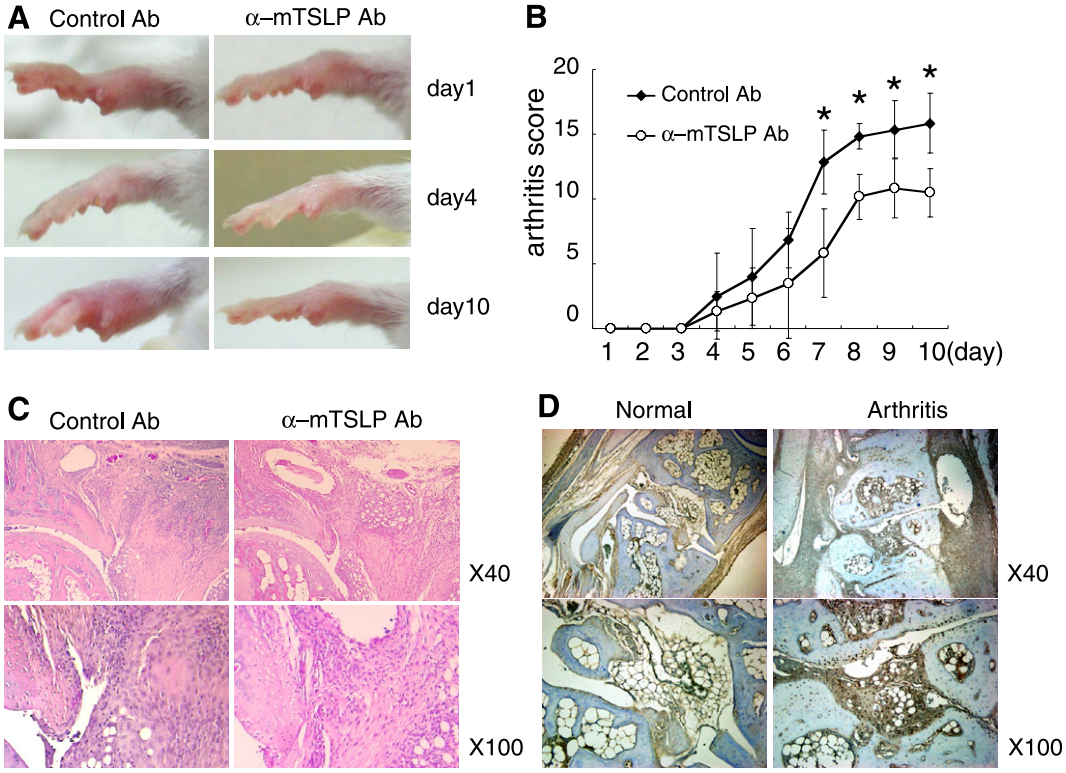

Fig. 3. Anti-TSLP neutralizing antibody ameliorates the arthritis induced by anti-collagen type II antibody in mice. The mice were intraperitoneally injected with 2 mg per mouse of anti-collagen type II mAb cocktail (day 1) and three days later (day 4) with 50 μg per mouse of LPS. Rat anti-mouse TSLP neutralizing antibody or the isotype control rat IgG2a (15 mg/kg per mouse) was intraperitoneally administered on day 5. (A) Representative photographs showing the fore paws of the mice treated or untreated with anti-TSLP antibody on day 1, day 4, and day 10. (B) Clinical scoring measured during the course of study. The values represent means ± SD of six mice per group. $^{*}p < 0.05$ in comparison with the corresponding control. Similar results were obtained from three independent experiments. (C) A histological examination of the hind paws (torsocrural joint) of the mice treated or untreated with anti-TSLP antibody on day 10. Representative photographs of hematoxylin and eosin (HE) staining are shown. (D) An immunohistochemical examination of the hind paws (torsocrural joint) of the anti-collagen antibody-induced arthritis. Tissue sections obtained from paw joints of normal untreated mice (left panels) and mice on day 10 after arthritis induction (right panels) were stained with anti-mouse TSLP antibody. Representative photographs are shown. Positive staining is indicated as brown.

receiving control rat IgG antibody developed arthritis after the injection of anti-type II collagen mAbs plus LPS, beginning on day 5 after the administration of the mAbs, with subsequent increasing severity of inflammation until day 10 (Fig. 3A and B). The mice receiving rat anti-mouse TSLP antibody developed limited clinical manifestations of arthritis based on a clinical assessment (Fig. 3A and B). A histological examination of the hind paws on day 10 showed a significantly reduced cellular infiltration and synovial hyperplasia in the mice receiving anti-mouse TSLP antibody (Fig. 3C). In addition, we immunohistochemically observed that TSLP expression was to be up-regulated in the joint tissues after the induction of arthritis on day 10 (Fig. 3D). It appeared that TSLP was mainly expressed in the inflamed synovium (Fig. 3D) and this result was consistent with our in vitro findings. These results suggest that TSLP is involved in the development of the arthritis induced by anti-collagen type II antibody in mice.

We found that not only RA- but also OA-derived synovial fibroblasts up-regulated TSLP production upon TNF-α stimulation (Fig. 2A), suggesting that the TNF-α-induced TSLP production in synovial fibroblasts is not specific to RA and it largely depends on TNF-α. Since it is well accepted that TNF-α levels are elevated in RA synovial fluid [19], the TNF-α dependence of TSLP production could thus explain why TSLP elevation was observed in RA, not OA, synovial fluid specimens (Fig. 1).

The TNF-α-induced TSLP production in synovial fibroblasts was inhibited by IFN-γ (Fig. 2). IFN-γ inhibits arthritis and osteoclast genesis [22] and IFN-γ is not highly expressed in the RA joints [23], suggesting that IFN-γ may be anti-arthritic. The inhibition of TSLP production by such anti-arthritic IFN-γ also suggests that TSLP might be involved in the pathophysiology of RA.

We showed that anti-TSLP neutralizing antibody diminished the clinical manifestations and histology observed in the arthritis induced by anti-collagen type II antibody (CAIA) (Fig. 3). This arthritis model represents the effector inflammatory phase of arthritis, depending on TNF-α [18]. Thus, the endogenous TSLP signaling pathway is likely to be important for the proinflammatory cytokine-dependent, T cell-independent, inflammatory phase of the arthritis. The roles of TSLP in the priming phase of arthritis or in other arthritis models remain uncertain and should be investigated in future studies.

Roles of TSLP in the pathophysiology of RA remain unclear and should be investigated in future studies. Recent studies have shown that TSLP potently activates $CD11c^{+}$ myeloid DCs, leading to the differentiation of CD4 T cells into TNF-α-producing Th2 cells and, as a result, plays a key role in the development of allergic inflammation (6). Dendritic cells was shown to be abundant both in the synovial tissue and synovial fluid of RA and attention has been therefore directed at the possible roles of synovial dendritic cells in the initiation and perpetuation of RA [24]. It is thus possible that TSLP, induced by TNF-α in the synovial fibroblasts, may regulate the activation of the synovial dendritic cells in RA. We are currently investigating this point.

In summary, the current results suggest that TSLP may play a role in inflammatory arthritis such as RA. Because TSLP is considered to be a critical cytokine for the development of allergic inflammation and also regulatory mechanisms of TSLP expression are not fully understood, these results reveal a novel function and regulation of TSLP in the synovial joints. These findings may also support the notion that, although RA is generally considered to be a Th1-type immune disorder, the application of a simple Th1/Th2 paradigm to RA can mislead the effort to understand the pathogenic mechanisms.

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