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ORIGINAL ARTICLE

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Thymic stromal lymphopoietin secretion of synovial fibroblasts is positively and negatively regulated by Toll-like receptors/nuclear factor-κB pathway and interferon-γ/dexamethasone

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Abstract Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-7-like cytokine produced by epithelial cells and triggers dendritic cell-mediated Th2 type allergic inflammatory responses. This study investigated whether Toll-like receptor (TLR) ligands, lipopolysaccharide (LPS) and poly-IC affect TSLP production in synovial fibroblasts. Enzyme-linked immunosorbent assay showed that LPS and poly-IC upregulated TSLP production in synovial fibroblasts obtained from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). In addition, we found that nuclear factor (NF)-κB inhibitor IMD-0354, dexamethasone, and interferon (IFN)-γ inhibited the LPS- and poly-IC-induced TSLP production in RA and OA synovial fibroblasts. Thus, LPS and poly-IC can upregulate TSLP via a NF-κB pathway in synovial fibroblasts, which is downregulated by dexamethasone and interferon (IFN)-γ. The current findings suggest that TSLP may be involved in the pathophysiology of inflammatory arthritis as well as allergic disease.

Key words Nuclear factor-κB · Rheumatoid arthritis · Synovial fibroblasts · Thymic stromal lymphopoietin (TSLP) · Toll-like receptor ligands

Introduction

Thymic stromal lymphopoietin (TSLP) is an interleukin (IL)-7-like cytokine, which binds to the TSLP receptor (TSLPR) consisting of the IL-7 receptor α-chain (IL-7Rα) and a commonγ receptor-like chain (TSLPR-γ).[1,2] Thymic stromal lymphopoietin 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]

Thymic stromal lymphopoietin 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 were 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 was shown to be expressed by keratinocytes in atopic dermatitis and by bronchial epithelial cells in the asthmatic airways.[10,11]

Most recently, we have suggested that TSLP may be also involved in inflammatory arthritis as an effector molecule downstream of tumor necrosis factor (TNF)-α.[12] This study demonstrated that (1) synovial fluid specimens from patients with rheumatoid arthritis (RA) showed increased levels of TSLP compared with those from patients with osteoarthritis (OA), (2) TNF-α upregulated TSLP production in RA- and OA-derived synovial fibroblasts, (3) anti-TSLP neutralizing antibody ameliorated a TNF-α-dependent experimental arthritis induced by anti-type II collagen antibody (CAIA) in mice: an arthritis model which is different from a classical collagen-induced arthritis (CIA) model in that the CAIA model can focus on effector inflammatory phase of RA.[13] In the current study, we extend this study by investigating whether Toll-like receptor (TLR) ligands, lipopolysaccharide (LPS) (TLR4 ligand) and poly-IC (TLR3 ligand), affect TSLP production in synovial fibroblasts because synovial fibroblasts express several TLRs including TLR3 and TLR4[14,15] and bacterial substances or endogenous molecules that can activate TLRs are suggested to be present and play some roles in RA joints.[16] We also investi-

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gated the factors that downregulate TSLP expression in synovial fibroblasts.

Materials and methods

Reagents

Lipopolysaccharide, poly-IC, IMD-0354 (a selective inhibitor of IκB kinase), and dexamethasone were purchased from Sigma Aldrich (St. Louis, MO, USA). Recombinant human IFN-γ, human transforming growth factor (TGF)-β, human TNF-α, and human IL-6 were purchased from R&D (Minneapolis, MN, USA).

Patient profile

Synovial tissue samples of the knee joints were obtained from two RA patients (two males, age 79 and 65 years) and two OA patients (two females, age 84 and 85 years) diagnosed based on the revised criteria of the American College of Rheumatology for RA[17] or OA.[18] All RA and OA patients were receiving treatment at the time of the study; two RA patients were on disease-modifying anti-rheumatic drugs (DMARDs) and prednisolone, while the two OA patients were being treated with nonsteroidal anti-inflammatory drugs (NSAIDs).

Cell culture

Human synovial fibroblasts were obtained as previously described.[19] In brief, after enzymatic digestion, human synovial cells were isolated from synovial tissues of the knee joints of RA and OA patients (see the Patient profile section) at the time of total knee arthroplasty operations. The investigation was approved by the Ethics Committee of the University of Yamanashi, Faculty of Medicine, and all subjects gave their written informed consent. The cells were suspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS), 100 μg/ml streptomycin, and 100 units/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.

Enzyme-linked immunosorbent assay (ELISA)

The amounts of TSLP in the culture supernatants were measured by ELISA using the Human TSLP sandwich ELISA Development Kit (R&D) according to the manufacturer's instructions. The minimum detection level of ELISA was 31.25 pg/ml.

Western blot

Western blot analysis was performed as previously described[20] with specific antibodies for IκB-α (Cell Signaling Tech., Beverly, MA, USA) andβ-actin (Santa Cruz Biotech, Santa Cruz, CA, USA).

Cell viability assay

Cells (2.5×10^3 cells/well) were cultured in DMEM containing 10% FCS with or without the indicated doses of IMD-0354 or dexamethasone for 24 h in a flat-bottomed 96-well microtiter plate. Cell viability was then determined by measuring the metabolic activity using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)[^{2}H]tetrazolium monosodium salt (WST) using Tetra Color ONE kit (Seikagaku, Tokyo, Japan) according to the manufacturer's instructions. In our preliminary studies, the metabolic activity measured in this assay was proportional to the cell number which was directly counted by a trypan blue exclusion assay.

Data analysis

The data are summarized as the mean ± SD. The unpaired Student's *t*-test was used for the statistical analysis of the results. $P < 0.05$ was considered to be significant.

Results

Lipopolysaccharide and poly-IC upregulate TSLP production in RA and OA synovial fibroblasts

We first examined TSLP production in synovial fibroblasts obtained from patients with RA and OA by ELISA. As shown in Fig. 1, RA and OA synovial fibroblasts constitutively produced some amounts of TSLP. Synovial fibroblasts were then stimulated with TLR ligands, LPS, and poly-IC, and also with several cytokines such as IL-6, IFN-γ, and TGF-β. Among the stimuli that we examined, LPS and poly-IC significantly upregulated TSLP production in 2 RA and 2 OA synovial fibroblasts in a dose-dependent manner (Fig. 1). These results indicated that LPS and poly-IC upregulated TSLP production in RA and OA synovial fibroblasts.

Activation of NF-κB contributes to LPS- and poly-IC-induced TSLP production in synovial fibroblasts

Because TNF-α, LPS, and poly-IC share a NF-κB pathway as a common element for these signaling intermediates, we hypothesized that LPS- and poly-IC induced TSLP production in synovial fibroblasts via a NF-kB pathway. To test this hypothesis, we examined the effects of IMD-0354, a selective inhibitor of IκB kinase, and dexamethasone, a steroid hormone that inhibits NF-κB activation,[21] on the TSLP production.

We first assessed the effective doses of IMD-0354 and dexamethasone. As shown in Fig. 2A, 10 μM IMD-0354 significantly inhibited TNF-α-induced degradation of IκB-

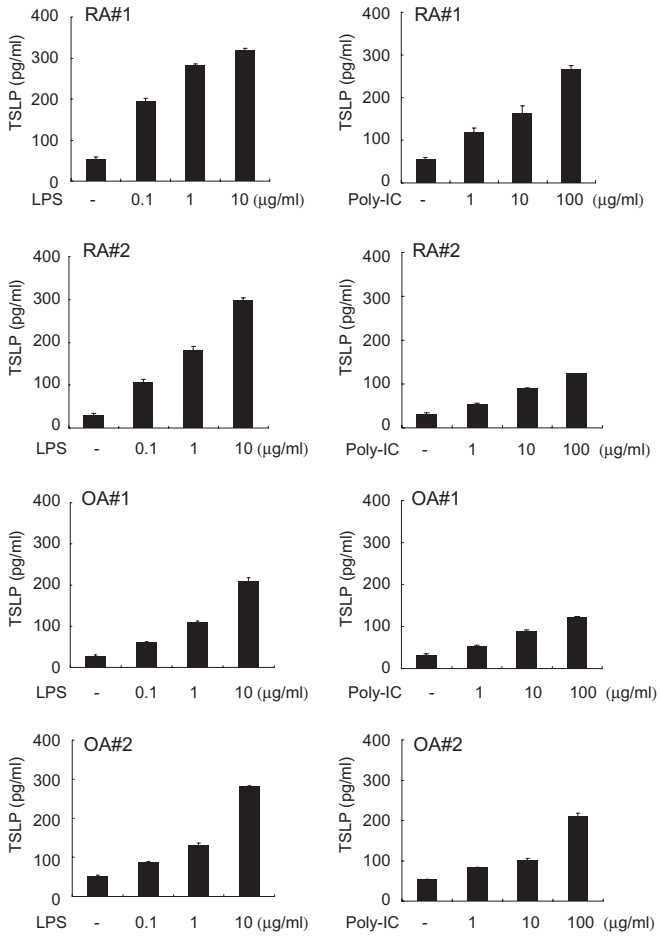

Fig. 1. Lipopolysaccharide (*LPS*) and poly-IC induce thymic stromal lymphopoietin (*TSLP*) expression in rheumatoid arthritis (*RA*) and osteoarthritis (*OA*) synovial fibroblasts. RA and OA synovial fibroblasts (2.5×10^5 cells/well) derived from 2 RA and 2 OA patients (RA #1, 2, OA #1, 2) were cultured in the presence or absence of the indicated doses of LPS or poly-IC for 24 h. The culture supernatants were then collected and the TSLP concentrations were measured by enzyme-linked immunosorbent assay (ELISA)

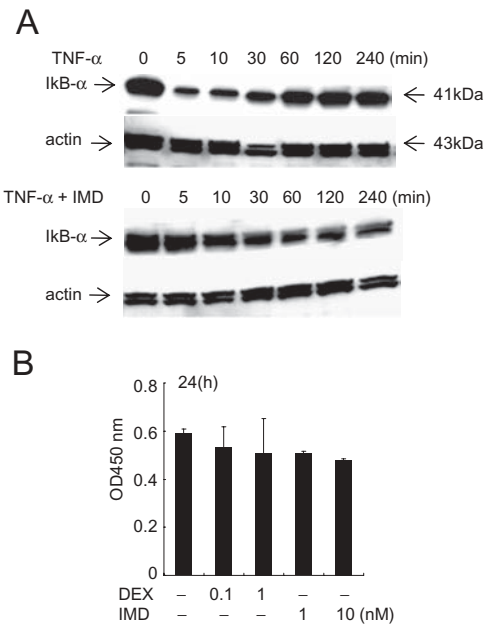

Fig. 2A,B. IMD-0354 inhibits tumor necrosis factor-α (*TNF*-α)-induced degradation of IκB-α in synovial fibroblasts without affecting the cellular viability. **A** RA synovial fibroblasts (2.5×10^5 cells/well) were cultured with 10 ng/ml TNF-α in the presence or absence of 10 μM IMD-0354 for the indicated times. The cell lysates were then subjected to a Western blot analysis with specific antibodies for IκB-α and β-actin. Note that TNF-α-induced reduction of IκB-α expression during 5–30 min was significantly inhibited by IMD-0354. **B** RA synovial fibroblasts (5×10^3 cells/well) were cultured with the indicated doses of IMD-0354 or dexamethasone for 24 h, and then cell viability was measured by WST assay. Values represent the mean ± SD. $^*P < 0.05$ compared with the corresponding control

α in RA synovial fibroblasts. We confirmed that 10 μM IMD-0354 did not affect the viability of RA synovial fibroblasts (Fig. 2B). Dexamethasone at 1 nM also inhibited TNF-α-induced degradation of IκB-α in RA synovial fibroblasts without affecting the cellular viability (Fig. 2B and data not shown). These results indicated that 10 μM IMD-0354 and 1 nM dexamethasone are effective doses for the inhibition of NF-κB activation without affecting cellular viability.

We then examined the effects of IMD-0354 and dexamethasone on LPS- and poly-IC-induced TSLP production in synovial fibroblasts (Fig. 3A). The LPS- and poly-IC-induced TSLP production in RA and OA synovial fibroblasts was completely inhibited by 1 and 10 μM IMD-0354 and also by 0.1 and 1 nM dexamethasone. Although the basal levels of TSLP were also suppressed by IMD-0354 and dexamethasone, the inhibitory effects of these reagents on the LPS- or poly-IC-induced TSLP expression were much stronger than those on the basal TSLP production (Fig. 3A). We confirmed that LPS and poly-IC indeed induced activation of NF-κB pathway as demonstrated by degradation of IκB-α in RA synovial fibroblasts, which was inhibited by 10 μM IMD-0354 and 1 nM dexamethasone (Fig. 3B and data not shown). These results indicated that LPS- and poly-IC-induced TSLP production in synovial fibroblasts via a NF-κB pathway.

Interferon-γ inhibits LPS- and poly-IC-induced TSLP production in synovial fibroblasts

We also investigated whether the LPS- and poly-IC-induced TSLP production in RA and OA synovial fibroblasts can be regulated by several cytokines such as IFN-γ, TGF-β, and IL-6. Among the cytokines that we examined, IFN-γ significantly inhibited LPS and poly-IC-induced TSLP production in RA and OA synovial fibroblasts in a dose-dependent manner (Fig. 4). In some RA synovial fibroblasts, the basal levels of TSLP production were also inhibited by IFN-γ (Fig. 4).

Discussion

Thymic stromal lymphopoietin has been suggested to play an important role in the development of allergic inflammation.[1,2] Thymic stromal lymphopoietin may be also involved

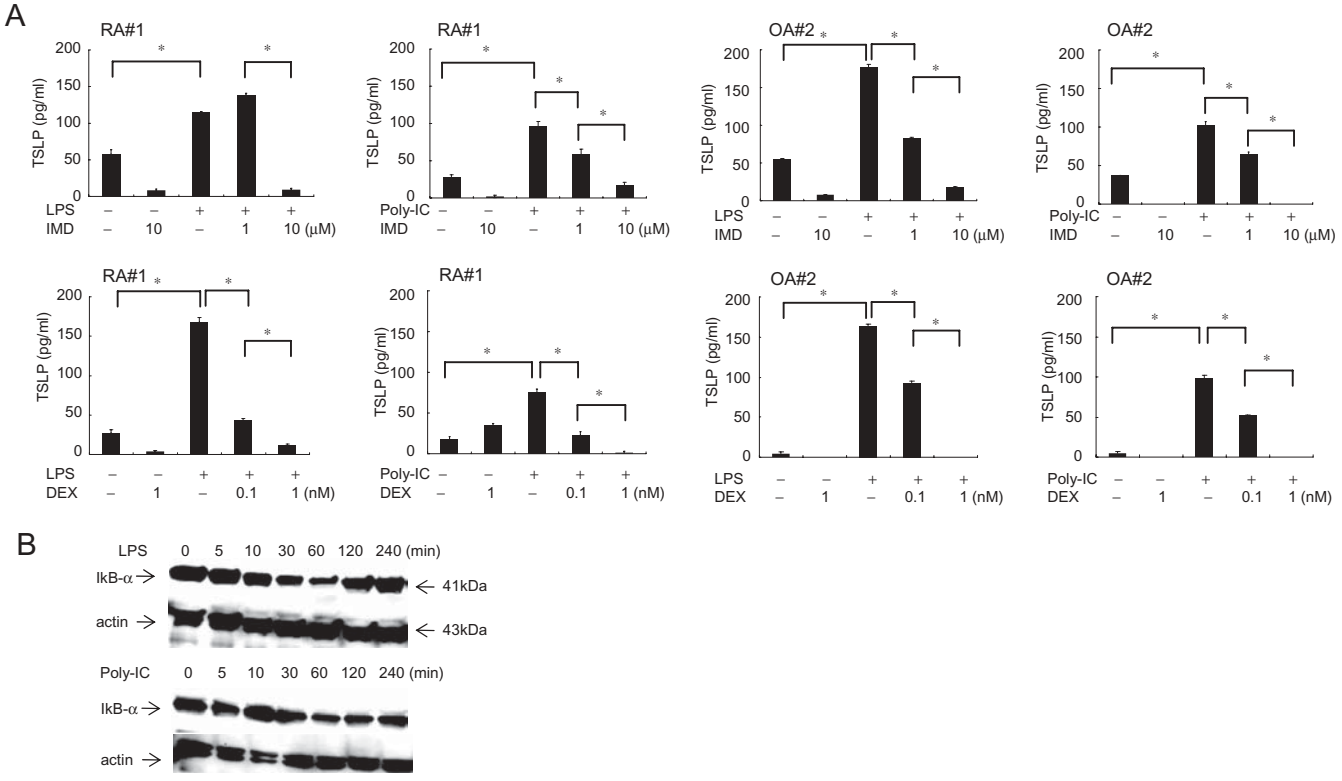

Fig. 3A,B. LPS- and poly-IC-induced TSLP expression is mediated via a nuclear factor (NF)-κB pathway in RA and OA synovial fibroblasts. **A** RA and OA synovial fibroblasts (2.5×10^5 cells/well) were cultured with 1 μg/ml LPS or 10 μg/ml poly-IC in the presence or absence of 10 μM IMD-0354 or 0.1 nM dexamethasone (*DEX*) for 24 h. The culture supernatants were then collected and the TSLP concentrations were measured by ELISA. **B** RA synovial fibroblasts (2.5×10^5 cells/well) were cultured with 1 μg/ml LPS or 10 μg/ml poly-IC for the indicated times. The cell lysates were then subjected to a Western blot analysis with specific antibodies for IκB-α and β-actin. Representative results of four independent experiments using synovial fibroblasts derived from 2 RA and 2 OA patients are shown

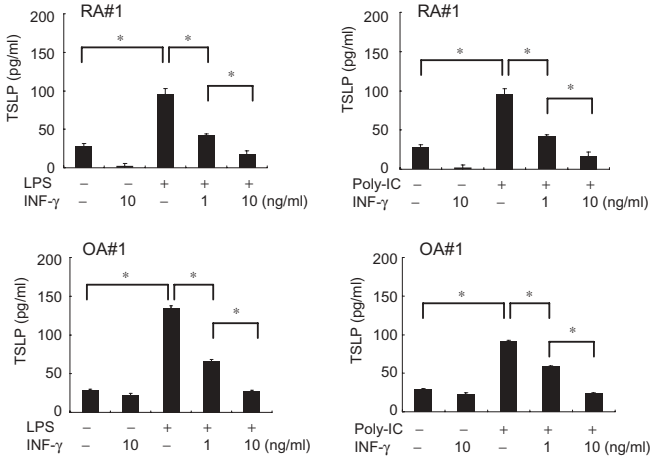

Fig. 4. Interferon-γ (*IFN*-γ) inhibits LPS- and poly-IC induced TSLP expression in RA and OA synovial fibroblasts. RA and OA synovial fibroblasts (2.5×10^5 cells/well) were cultured with 1 μg/ml LPS or 10 μg/ml poly-IC in the presence or absence of the indicated doses of IFN-γ for 24 h. The culture supernatants were then collected and the TSLP concentrations were measured by ELISA. Representative results of four independent experiments using synovial fibroblasts derived from 2 RA and 2 OA patients are shown. Values represent the mean ± SD. $^*P < 0.05$ compared with the corresponding control

in inflammatory arthritis based on our previous findings.[12] However, the factors that regulate TSLP expression remain largely unknown. This study shows that LPS and poly-IC upregulated TSLP production via a NF-κB pathway in RA and OA synovial fibroblasts whereas dexamethasone and IFN-γ downregulated it (Figs. 1, 3, and 4). The current results thus reveal a novel regulatory mechanism that controls TSLP production in a positive and negative fashion in synovial fibroblasts.

Not only RA- but also OA-derived synovial fibroblasts upregulated TSLP production upon LPS and poly-IC stimulation (Fig. 1), suggesting that the LPS- and poly-IC-induced TSLP production in synovial fibroblasts is not specific to RA and OA, but largely depends on ligand stimulation. Synovial fibroblasts express several TLRs including TLR3 and TLR4.[14,15] It is therefore likely that LPS and poly-IC stimulate synovial fibroblasts to produce TSLP via TLR4 and TLR3, respectively. However, it remains to be determined whether the newly discovered pathways for detecting dsRNA (RIG-1 and MDA)[22] are involved in poly-IC-induction of TSLP in synovial fibroblast.

IMD-0354 and dexamethasone almost completely inhibited the LPS- and poly-IC-induced TSLP production in RA and OA synovial fibroblasts, suggesting that a NF-κB pathway is critical for the TSLP production in synovial

fibroblasts (Fig. 3). Most recently, several groups have shown that the NF-κB pathway plays an important role in the regulation of TSLP expression in human bronchial epithelial cells and keratinocytes,[23–25] which is consistent with the current results. It remains uncertain whether LPS and poly-IC directly activate a NF-κB pathway via TLRs or activate this pathway secondarily by inducing proinflammatory cytokines such as TNF-α, IL-1, or IL-18 in synovial fibroblasts.

The LPS- and poly-IC-induced TSLP production in RA synovial fibroblasts was inhibited by IFN-γ (Fig. 4). Bogiatzi et al. recently reported that TNF-α and IL-1α induced TSLP production in human skin explants in synergy with Th2 cytokines (IL-4 or IL-13), which was not inhibited by IFN-γ.[25] Thus, negative regulation of TSLP production by IFN-γ may differ in different cell types.

The basal levels of TSLP production in some RA and OA synovial fibroblasts were inhibited by IMD-0354, dexamethasone, and IFN-γ (Figs. 3 and 4). We found that cultured RA or OA synovial fibroblasts spontaneously produce low levels of TNF-α (data not shown). We thus speculate that constitutively produced endogenous TNF-α might induce TSLP in synovial fibroblasts in an autocrine manner, which was blocked by these reagents.

In summary, we demonstrated that TSLP secretion of RA and OA synovial fibroblasts was positively and negatively regulated by TLRs/NF-κB pathway and IFN-γ/dexamethasone, respectively. These results reveal a novel regulatory mechanism of TSLP production in the synovial joints. Because exogenous or endogenous molecules that can activate TLRs are suggested to be present in RA joints,[16] the LPS, and poly-IC dependence of TSLP production might explain why TSLP elevation was observed in RA, not OA, synovial fluid specimens.[12] To further elucidate the precise roles of TSLP in RA or OA will be an interesting and important issue for future investigation.

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