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IL-17 induces the production of IL-16 in rheumatoid arthritis

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Abbreviations: FLS, fibroblast-like synoviocytes; GMCSF, granulocyte-macrophage colony stimulating factor; OA, osteoarthritis; PBMC, Peripheral blood mononuclear cells; PGE2, Prostaglandin E2; RA, rheumatoid arthritis; TLRs, Toll-like receptor

Abstract

The purpose of this study was to investigate the expression of IL-16 in the rheumatoid synovium and the role of inflammatory cytokines and Toll-like receptor (TLR) ligands in IL-16 production by fibroblast-like synoviocytes (FLS) of rheumatoid arthritis (RA) patients. Immunohistochemical staining was performed with a monoclonal antibody to IL-16 in synovial tissues from patients with RA and likewise in patients with osteoarthritis (OA). FLS were isolated from RA synovial tissues and stimulated with IL-15, IL-1β, IFN-γ, and IL-17. The IL-16 mRNA level was assessed by semiquantitative RT-PCR and real time (RT) PCR and a comparison was made between IL-16 mRNA levels produced by RA-FLS and OA-FLS. Production of IL-16 was identified by a western blot assay, and IL-16 production after stimulation by specific ligands of TLR2 and TLR4 was assessed by RT-PCR. While immunohistochemical staining demonstrated strong expression of IL-16 mRNA in synovial tissues from patients with RA, similar findings were not present in the OA group. Moreover, mRNA expression of IL-16 by RA-FLS increased after treatment with IL-17 but not with IL-15, IL-1β, and IFN-γ. Specifically, IL-17 increased IL-16 mRNA level by RA-FLS and peripheral blood mononuclear cells in a dose-dependent manner. However, IL-17 did not stimulate IL-16 production in OA-FLS. Peptidoglycan, a selective TLR2 ligand, also increased production of IL-16 by RA-FLS dose-dependently, whereas LPS, a selective TLR4 ligand, had no such stimulatory effect. The results from our data demonstrate that IL-17 and TLR2 ligands stimulate the production of IL-16 by RA-FLS.

Keywords: interleukin-16; interleukin-17; rheumatoid arthritis; synovial membrane; Toll-like receptors

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder that is characterized by synovial hyperplasia, neoangiogenesis, and progressive destruction of cartilage and bone (Weyand and Goronzy, 1997; Koch, 1998). T cells comprise a large proportion of the inflammatory cells infiltrating the RA synovium responsible for such changes, and thus is the reason RA is considered a Th-1-associated disease. T cell activation and migration into the synovium is important for the initiation and perpetuation of the disease (Weyand and Goronzy, 1997).

IL-16 is a 17 kDa chemoattractant identified initially by its actions on T cells (Center *et al.,* 1997). Recent studies show that IL-16 contributes to the regulatory process of $CD4^+$ immune cell recruitment and activation at sites of inflammation through the binding of the D4 region of CD4 on lymphocytes (Wu *et al.,* 1999). IL-16 is synthesized by a variety of immune (T cells, eosinophils, and dendritic cells) and nonimmune cells (fibroblasts, epithelial cells, and neuronal cells) (Cruikshank *et al.,* 2000). The principal site of IL-16 production in the joint is the $CD68^-$ fibroblast-like cells of the synovial lining (Franz *et al.,* 1998).

Franz *et al*. (1998) showed that IL-16 is produced and IL-16 mRNA is expressed in human synovial fibroblasts from patients with RA, but not in cells from patients with osteoarthritis (OA). In synovial fluid, IL-16 concentration correlates positively with chemotactic activity, and the highest concentrations

have been observed in patients with early RA (Franz *et al.,* 1998). Synovial fibroblasts from patients with RA produce high levels of IL-16 in response to RA-IgG, and this is one possible mechanism through which T cells infiltrate articular tissues in RA (Pritchard *et al.,* 2004). The expression of IL-16 increases in cultured fibroblast-like synoviocytes (FLS) stimulated by IL-1β (Sciaky *et al.,* 2000).

In inflammatory arthritis, IL-17 plays key roles in the propagation of joint inflammation, cartilage destruction, and bone erosion (Lubberts *et al.,* 2005). IL-17RA knockout mice exhibit reduced serum chemokine concentration and concomitantly reduced neutrophil migration to bone. IL-17 activates NF-κB and stimulates the production of several inflammatory mediators, including IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), and prostaglandin E2 (PGE_2) in synoviocytes (Lubberts *et al.,* 2005; Miossec, 2004; Stamp *et al.,* 2004; Hwang *et al.,* 2004). IL-17 is released mainly by memory T cells and is associated particularly with the severity of synovial inflammation of patients with RA (Cho *et al.,* 2004; Hwang *et al.,* 2004).

Toll-like receptors (TLRs), phylogenetically conserved receptors that recognize pathogen- associated molecular patterns, are important because of their special function in linking innate immunity to adaptive immunity (Akira *et al.,* 2001; Takeda and Akira, 2005). TLR2 and TLR4 are expressed in inflamed RA synovium, and the expression of these receptors is associated with the presence of inflammatory cytokines (Seibl *et al.,* 2003; Radstake *et al.,* 2004). We recently reported that activation of TLR2 by its specific ligand mediates the upregulation of CXCL8, vascular endothelial growth factor, and IL-8 (Cho *et al.,* 2007). We also observed that the combined engagement of TLR2 and TLR4 upregulates IL-15 in RA-FLS (Jung *et al.,* 2007), which may contribute to the maintenance and perpetuation of synovitis in patients with RA.

We now report, for the first time, that IL-17 markedly increases the production of IL-16 and that stimulation of TLR2 by its specific ligands synergistically enhances the production of IL-16 in FLS from patients with RA.

Materials and Methods

Reagents

Antibodies to IL-1β, IL-15, IL-16, IL-17, and IFN-γ were obtained from R&D Systems (Minneapolis, MN). Peptidoglycan (PGN) and LPS were obtained from Sigma-Aldrich (St Louis, MO).

Isolation of synovial fibroblasts

FLS were isolated by enzymatic digestion of synovial tissues obtained from patients with RA undergoing total joint replacement surgery.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood by Ficoll-Hypaque (SG1077) density gradient centrifugation

Hematoxylin and eosin staining

The tissue slides were prepared as described below, deparaffinized with three changes of xylene of 5 min each, rehydrated in a graded series of ethanol (100-70%), and washed in tap water. The sections were stained with hematoxylin for 5 min and washed in running tap water for 5 min. Tissue slides were counterstained with eosin for 15 s to 2 min, depending on the age of the eosin and the depth of the counterstaining desired. The slides were dehydrated in 95% and 100% alcohol with two changes of 2 min each or until the excess eosin was removed. Slides were treated with xylene (two changes of 2 min each) and mounted in permount or balsam.

Immunohistochemistry of synovial tissues

Immunohistochemical staining for IL-16 was performed with sections of synovium. Briefly, synovium was obtained from patients with RA or OA, fixed with 4% paraformaldehyde solution overnight at 4°C, dehydrated with alcohol, washed, embedded in paraffin, and sectioned into slices 7μm thick. Sections were depleted of endogenous peroxidase activity by adding methanolic H_2O_2 and blocked with normal serum for 30 min. The sections were incubated overnight at 4°C with polyclonal anti-human IL-16 antibody (R&D Systems); incubated with the secondary antibody, biotinylated anti-rabbit IgG, for 20 min; incubated with streptavidin-peroxidase complex (Vector, Peterborough, UK) for 1 h; and incubated with 3,3'-diaminobenzidine (Dako, Glostrup, Denmark) for 5 min. The sections were counterstained with hematoxylin. Samples were photographed with an Olympus photomicroscope (Tokyo, Japan).

Expression of IL-16 mRNA determined by RT-PCR and real-time PCR

mRNA was extracted using RNAzol B (Biotex Laboratories, Houston, TX) according to the manufacturer's instructions. Reverse transcription of 2 μg aliquots of total mRNA was carried out at 42°C using the Superscript reverse transcription system (TaKaRa,

Shiga, Japan). PCR amplification of cDNA aliquots was performed by adding 2.5 μM dNTPs, 2.5 U *Taq* DNA polymerase (TaKaRa), and 0.25 μM of sense and antisense primers. The following sense and antisense primers for each molecule were used (5'→3'): IL-16, 5'-ATGCCTGACCTCAACTCCACT (sense) and 5'-GCCACCCAGCTGCAAGATTTC (antisense); and GAPDH, 5'-CGATGCTGGGCG-TGAGTAC (sense) and 5'-CGTTCAGCTCAGGG-ATGACC (antisense). Reactions were processed in a DNA thermal cycler (PerkinElmer Cetus, Wellesley, MA) through 35 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 30 s for IL-16; and 25 cycles at 94°C for 30 s, at 56°C for 30 s, and at 72°C for 30 s for GAPDH.

The expression of IL-16 mRNAs was determined by real-time PCR with SYBR Green I. For quantitative analysis of IL-16 mRNAs, a LightCycler (Roche Diagnostics Mannheim, Germany) was used. The relative expression levels in the samples were calculated from the IL-16 levels normalized to the endogenously expressed housekeeping gene (GAPDH). Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 0 s (hold time) at 95°C, 15 s at 65°C, and 0 s (hold time) at 95°C. The rate of temperature change was 20°C/s, except in the final step, in which it was 0.1°C/s. The crossing point (C_p) was defined as the maximum of the second derivative from the fluorescence curve.

Western blot analysis

FLS were incubated with 1-10 ng/ml of IL-17 for 48 h, and whole-cell lysates were prepared from about 2 $\times 10^5$ cells by homogenization in the lysis buffer and centrifuged at 14,000 rpm for 15 min. The protein concentration in the supernatant was determined using the Bradford method (Bio-Rad, Hercules, CA). Protein samples were separated on 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). For western hybridization, the membrane was preincubated with 0.5% skim milk in TTBS buffer (0.1% Tween 20 in Tris-buffered saline) at room temperature for 2 h. The primary antibody to IL-16 (R&D Systems) or β-actin, diluted 1 : 1,000 in 5% BSA-0.1% Tween-20-TBS, was added and incubated for overnight at 4°C. The membrane was washed four times with TTBS, and HRP-conjugated secondary antibody was added and incubated for 1 h at room temperature. After TTBS washing, hybridized bands were detected using the ECL detection kit and Hyperfilm-ECL reagents (Amersham Pharmacia).

Statistical analysis

Data are expressed as the mean ± SD. The data were compared between pairs of groups using Student's *t* test for independent samples and Student's paired *t* test for matched pairs. Correlation coefficients were determined using Spearman's rank correlation test.

Results

Expression of IL-16 in RA synovium

IL-16 expression in joint tissue sections of RA patients was assessed by staining with anti-IL-16 monoclonal antibody or with irrelevant primary isotype-specific antibodies as a negative control. OA synovium was examined as a related control. Immunohistochemical staining revealed pronounced expression of IL-16 in the synovial lining, the sublining, and the perivascular region of the RA synovium, and at sites of lymphocyte

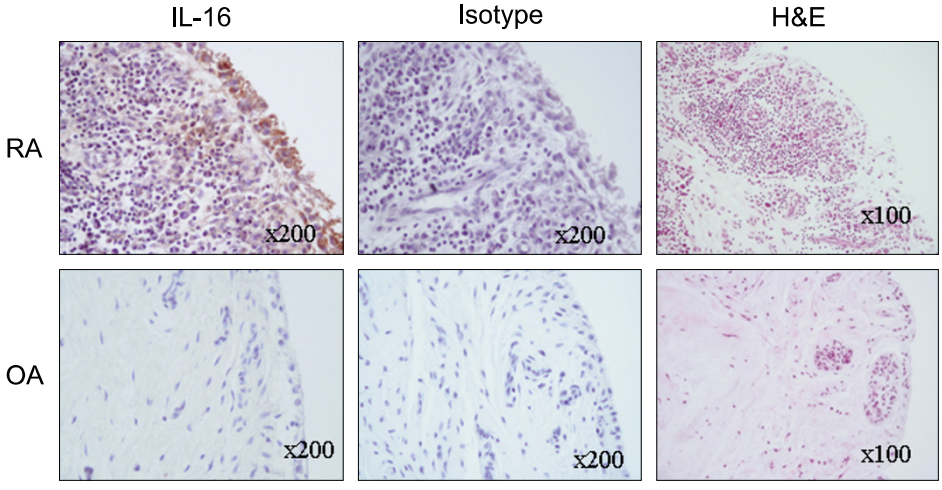

Figure 1. IL-16 expression increases significantly more in RA synovium than in OA synovium. Immunohistochemical detection of IL-16 in the synovium of patients with RA and OA. All tissues were counterstained with hematoxylin (original magnification 100, 200×).

infiltration. In contrast, minimal IL-16 staining was observed in OA synovium (Figure 1).

Increased expression of IL-16 by IL-17 stimulation and additive upregulation by TLR2 stimulation

FLS obtained from four patients with RA and four patients with OA were evaluated for mRNA expression by semiquantitative RT-PCR (Figure 2A) and real-time PCR (Figure 2B). Basal gene expression of IL-16 was stronger in RA-FLS than in OA-FLS (Figure 2A). In one representative sample of RA-FLS, IL-16 mRNA expression was strongly induced after incubation with IL-17 (20 ng/ml). However, IL-15 (10 ng/ml), IL-1β (10 ng/ml), or IFN-γ (10 ng/ml) did not affect the expression of IL-16 mRNA (Figure 2A). The mRNA expression of IL-16 relative to GAPDH was higher when FLS were cultured with IL-17 than when cultured with IL-15, IL-1β, or IFN-γ (Figure 2B). The mRNA expression of IL-16 by RA-FLS ($n = 4$) was strongly upregulated by IL-17 in a dose-dependent manner. The expression of IL-16 mRNA was evaluated by RT-PCR (Figure 3A and B) and realtime PCR (Figure 3C and D). Compared with untreated cells, relative mRNA expression of IL-16/GAPDH was: for 1 ng/ml of IL-17, 2.1 ± 0.5; for 10 ng/ml of IL-17, 6.0 ± 1.1; and for 20 ng/ml of IL-17, 13 ± 2.1. $^{*}P < 0.05$, $^{**}P < 0.01$ (Figure 3A).

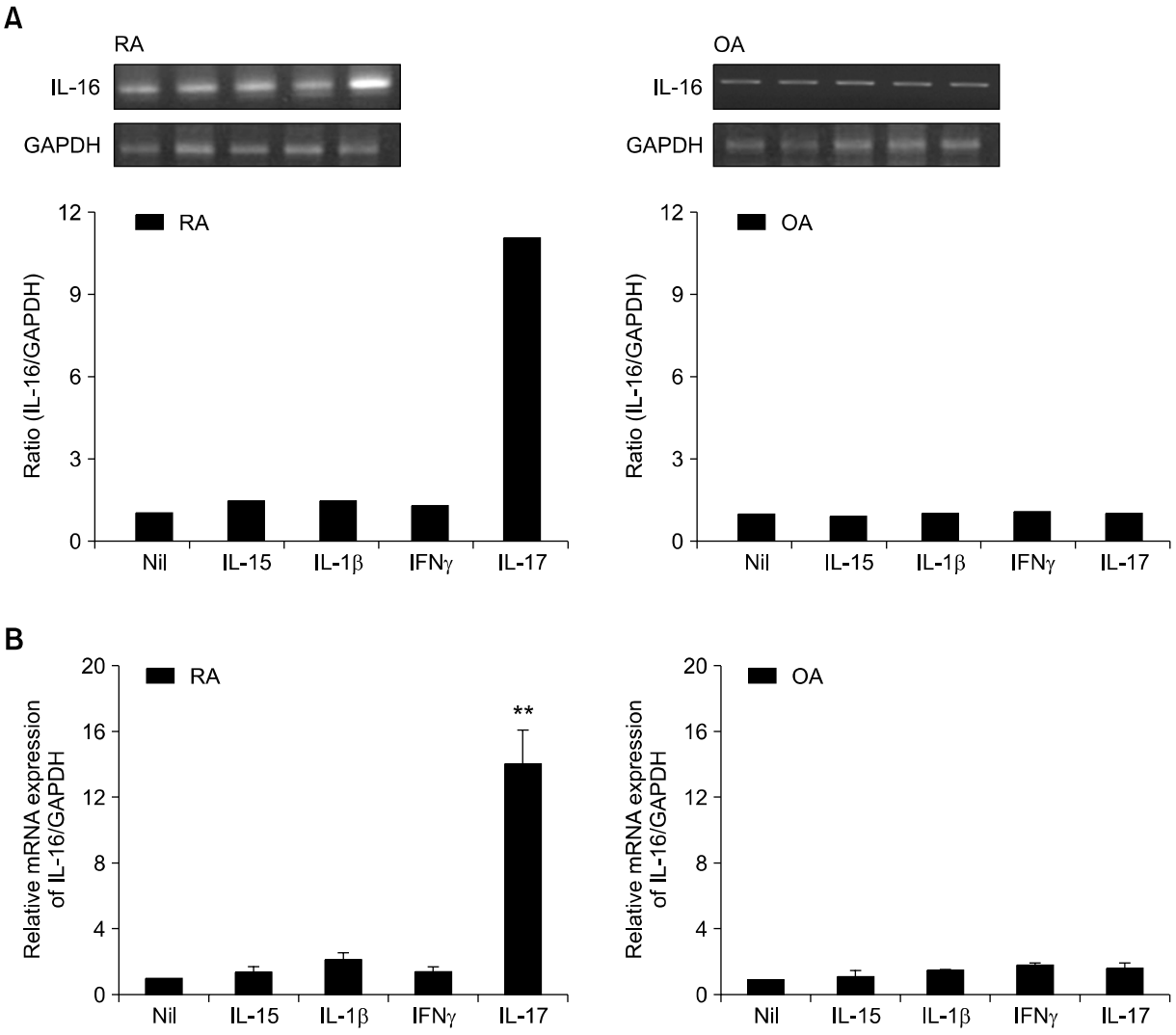

Figure 2. IL-16 mRNA expression is upregulated by IL-17 stimulation in RA-FLS, but not in OA-FLS. (A) IL-16 mRNA expression induced by IL-17 in RA-FLS. FLS were cultured with cytokines (IL-15, IL-1β IFN-γ or IL-17) for 12 h. The expression of IL-16 mRNA was evaluated by RT-PCR. The optical density was normalized to the band for GAPDH. (B) The expression of IL-16 mRNA was evaluated by real-time PCR. $^{**}P < 0.01$ compared with Nil (untreated control cells).

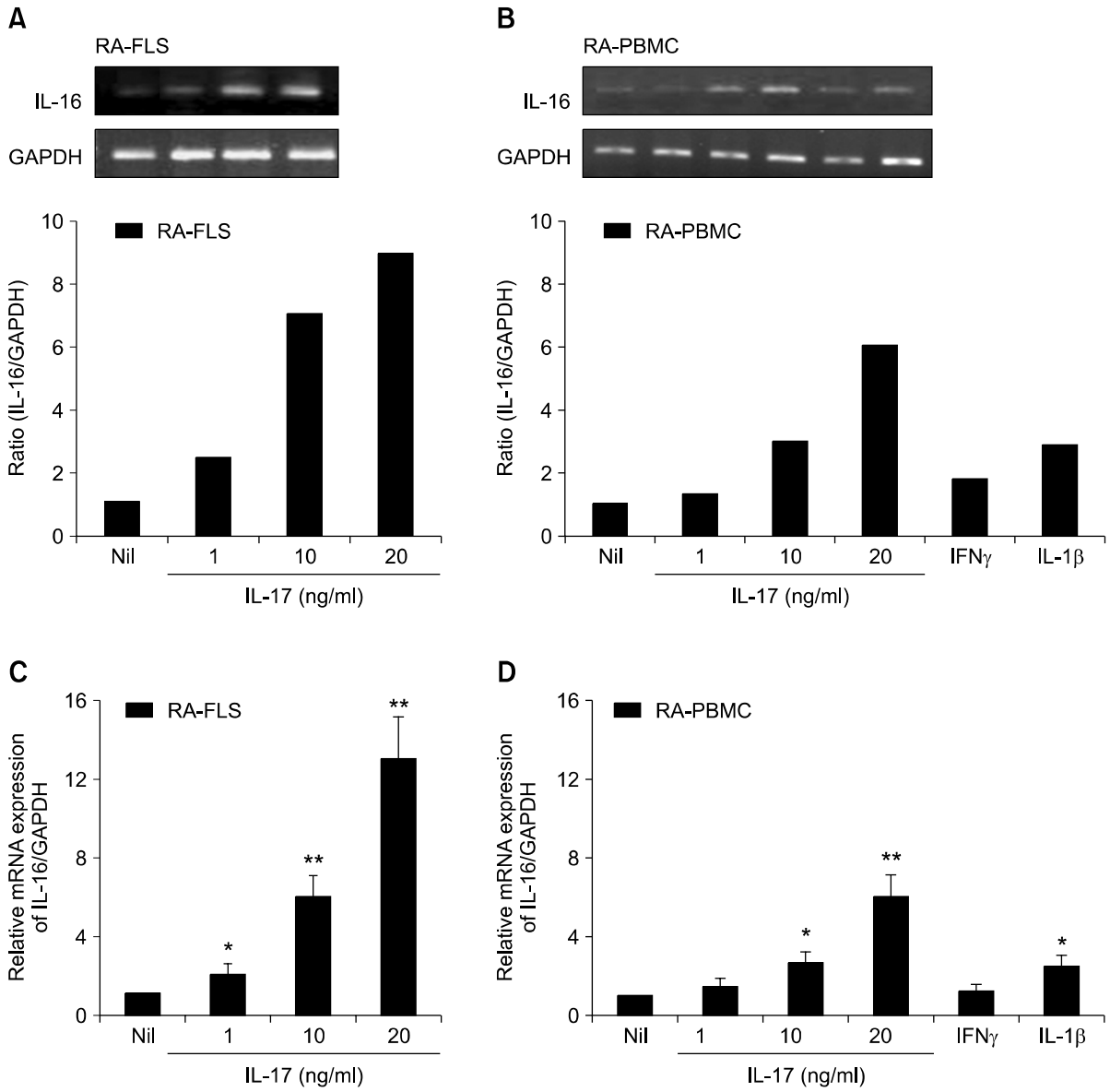

Figure 3. IL-16 mRNA expression in RA-FLS and RA-PBMC is upregulated by IL-17 in a dose-dependent manner. (A, B) IL-16 mRNA expression was induced by IL-17 in RA-PBMC. FLS were cultured with IL-17 (1-20 ng/ml) for 12 h. RA-PBMC were cultured with IL-17 (1-20 ng/ml), IFN-γ (10 ng/ml), or IL-1β (10 ng/ml) for 12 h. The expression of IL-16 mRNA was evaluated by RT-PCR. The optical density was normalized to the band for GAPDH. (C, D) The expression of IL-16 mRNA was evaluated by real-time PCR. *$P < 0.05$, **$P < 0.01$ compared with Nil (untreated control cells).

The relative mRNA expression of IL-16 by PBMC from five patients with RA ($n = 5$) was also upregulated by IL-17 in a dose-dependent manner. Compared with untreated cells, relative mRNA expression of IL-16/GAPDH was: for 1 ng/ml of IL-17, 1.5 ± 0.3; for 10 ng/ml of IL-17, 2.7 ± 0.5; for 20 ng/ml of IL-17, 6 ± 1.1. *$P < 0.05$, **$P < 0.01$ (Figure 3B). IFN-γ did not increase the expression of IL-16 in RA-FLS (data not shown) or RA-PBMC, but IL-1β increased the expression of IL-16 in RA-PBMC (2.5 ± 0.5 compared with untreated cells) (*$P <$ 0.05). The representative samples of RA-FLS and RA-PBMC show a dose-dependent increase in IL-16 mRNA expression after incubation with IL-17 (Figure 3). The dose-dependent increase in the expression of IL-16 stimulated by IL-17 was assessed with western blot analysis (Figure 4).

The production of IL-16 by RA-FLS ($n = 6$) was strongly upregulated by PGN in a dose-dependent manner (1 μg/ml PGN, 3 ± 0.5; 10 μg/ml PGN, 7.9 ± 1.1 μg/ml compared with untreated FLS) (*$P <$ 0.05, **$P < 0.01$, each dose compared with untreated

FLS) (Figure 5). The augmented production of IL-15 in response to TLR2 stimulation was regulated at the mRNA level. IL-16 gene expression did not change in response to LPS. The stimulatory effect of IL-17 on IL-16 production was augmented by PGN in a dose-dependent manner (FLS treated with 10 ng/ml IL-17 only, 6 ± 1.21 μg/ml; PGN + 10 ng/ml IL-17, 13 ± 2.5; 10 μg/ml PGN + 10 ng/ml IL-17, 17 ± 2.7 compared with untreated cells) ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$) (Figure 5).

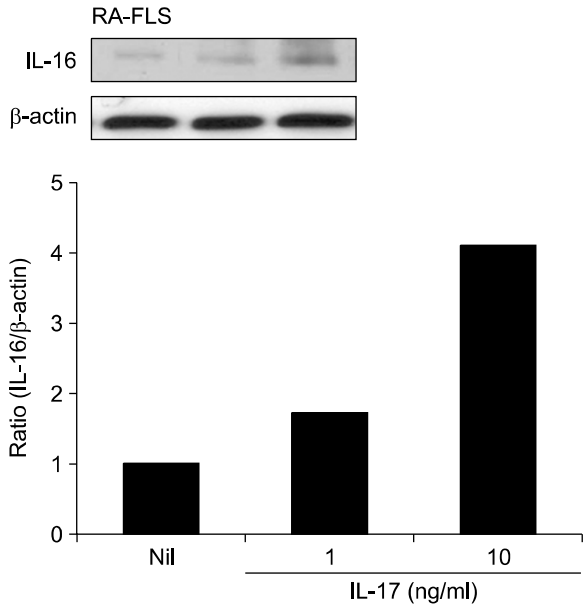

Figure 4. IL-16 protein expression increased significantly with treatment by IL-17. RA-FLS were cultured with or without IL-17 (1-10 ng/ml) for 48 h. IL-16 protein expression was measured in cell lysates by western blot analysis using goat polyclonal anti-IL-16 antibody. Results are expressed as the ratio of the densitometric intensity of the IL-16 product to that of the β-actin product. The data represent one of three independent experiments.

Discussion

We observed the spontaneous expression of IL-16 in synovial tissues from RA patients, but not in tissues from OA patients. This result agrees with previous reports (Franz *et al.,* 1998; Sciaky *et al.,* 2000 Weis-Klemm *et al.,* 2004). However, the stimuli that contribute to induce IL-16 in RA-FLS have been reported rarely. In our experiment, IL-17 and the specific ligand of TLR2 showed to be the major stimuli that induced IL-16 expression and production in RA-FLS.

IL-17 is a 17 kDa protein that is secreted predominantly by human memory T cells or mouse $\alpha\beta TCR^{+}CD4^{-}CD8^{-}$ thymocytes (Yao *et al.,* 1995; Kennedy *et al.,* 1996). IL-17 is produced spontaneously in the RA synovium, and a high concentration of IL-17 is detected in the synovial fluid of patients with RA (Chabaud *et al.,* 1999; Kotake *et al.,* 1999). In synergy with other proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ, IL-17 provokes local inflammation and amplification of inflammatory responses (Ruddy *et al.,* 2004; Albanesi *et al.,* 1999; Witowski *et al.,* 2000). IL-17 also mediates chemotaxis of neutrophils and monocytes by inducing chemoattractant molecules such as IL-8, monocyte chemoattractant protein- 1 (MCP-1), and growth-related protein-α (Witowski *et al.,* 2000; Afzali *et al.,* 2007). We observed that IL-17 but not other proinflammatory cytokines stimulated the

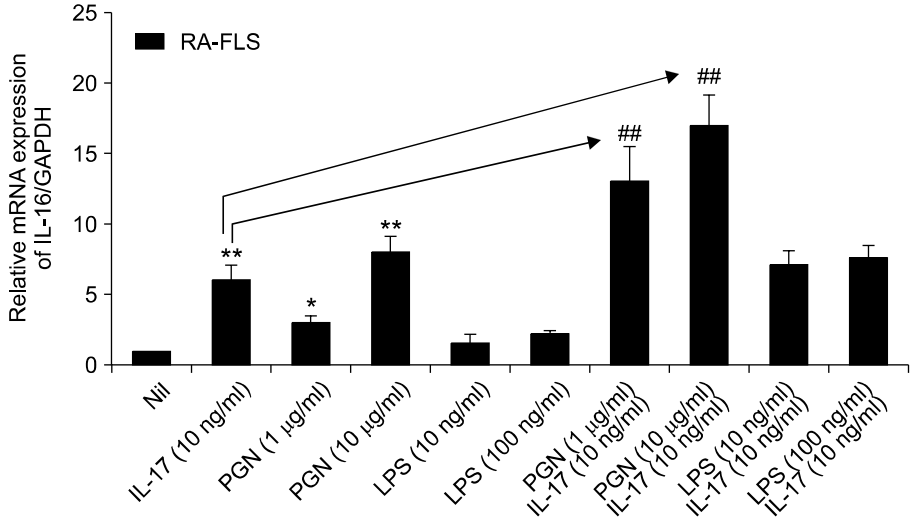

Figure 5. Increased IL-16 mRNA expression by IL-17 stimulation and additive upregulation by TLR2 stimulation. Expression of IL-16 was induced by IL-17, PGN (a TLR2 ligand), LPS (a TLR4 ligand), IL-17 + PGN or IL-17 + LPS. FLS were cultured with IL-17 (10 ng/ml), PGN (1-10 ng/ml), LPS (10-100 ng/ml), IL-17 + PGN, or IL-17 + LPS for 12 h. PGN (0.1-10 ng/ml), LPS (10-1000 ng/ml), or PGN (1 μg/ml) plus LPS (10-1000 ng/ml) for 12 h. The expression of IL-16 mRNA was evaluated by real-time PCR. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Nil (untreated control cells). $^{\#\#}P < 0.01$ compared with IL-17 stimulation.

production of IL-16. Weis-Klemm *et al*. (2004) reported that IL-1β or TNF-α do not upregulate IL-16 mRNA in FLS in RA patients. In contrast, an experiment using human orbital fibroblasts showed that IL-1β increases IL-16 synthesis (Sciaky *et al.,* 2000). We observed a partial stimulatory effect of IL-1β on IL-16 in RA-PBMC, but not in RA-FLS. We postulate that the effects of cytokines on IL-16 production differ between cell types. Our results suggest that the upregulation of IL-16 is another important function of IL-17 in RA-FLS along with its various proinflammatory roles. In addition, the stimulatory effect of IL-17 on IL-16 was observed specifically in RA-FLS, but not in OA-FLS.

TLRs are key receptors responsible for the recognition of bacterial components such as LPS, cytosine phosphate guanine (CpG) DNA, and endogenous ligands. TLR2 has been identified as a receptor for gram-positive bacterial cell wall proteins, such as PGN and bacterial lipopeptide (Underhill *et al.,* 1999; Brightbill *et al.,* 1999). TLR4 interacts with LPS (Zhang *et al.,* 1999) and with endogenous ligands such as heat-shock proteins (Ohashi *et al.,* 2000), fragments of hyaluronic acid (Termeer *et al.,* 2002), and fibronectin (Okamura *et al.,* 2001). As mentioned above, TLR expression increases in the rheumatoid synovium where it participates in the perpetuation of synovial inflammation. Our previous work demonstrated that activation of TLRs by their specific ligands could induce the production of chemokines or proinflammatory cytokines in RA-FLS (Cho *et al.,* 2007; Jung *et al.,* 2007). Those findings prompted us to test the potential role of TLR activation in the provocation of IL-16. We identified a stimulatory effect of the TLR2 ligand on IL-16 production in RA-FLS. In contrast, activation of TLR4 did not influence IL-16 expression. We also observed additive effect of PGN and IL-17 on IL-16 production.

IL-16 is detected in a number of inflammatory processes and acts as a potent chemoattractant for all peripheral immune cells expressing CD4, including $CD4^+$ monocytes (Cruikshank *et al.,* 1987), eosinophils (Rand *et al.,* 1991), and dendritic cells (Kaser *et al.,* 1999). There are conflicting data about the roles of IL-16 in inflammatory conditions. Animal studies have reported that IL-16 is an immunomodulatory mediator and that administration of IL-16 in a murine model of RA decreases the production of IFN-γ, IL-1β and TNF-α (Cruikshank *et al.,* 1996; Klimiuk *et al.,* 1999). Blaschke *et al*. (2001) reported no significant association between serum and synovial fluid IL-16 concentration and synovial tissue expression of IL-16 and clinical disease activity in patients with RA. In contrast, IL-16 contributes to airway inflammation in bronchial asthma (Cruikshank *et al.,* 1995) and to granulomatous inflammation (Wu *et al.,* 1999). IL-16 stimulates the expression and production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in human PBMC (Mathy *et al.,* 2000). In RA, the induced IL-16 may play a role in the perpetuation of synovitis by recruiting inflammatory cells, includeing T cells, into synovial tissues, although the exact role of IL-16 in RA pathogenesis needs to be investigated further.

The results in this paper demonstrate that IL-17 and activation of TLR2 increase the production of IL-16 in RA-FLS. Although the intracellular signaling cascades through which IL-17 or TLR2 ligand provoke the production of IL-16 in RA-FLS have not been defined, this finding may provide future molecular targets to treat patients with RA.

Acknowledgments

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