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Serum amyloid A-induced IL-6 production by rheumatoid synoviocytes

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Abstract In this study, we investigated the role of serum amyloid A protein (SAA) in the production of interleukin-6 (IL-6) using rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS). Recombinant SAA stimulation induced the production of pro-inflammatory cytokine, IL-6, from RA-FLS. The signaling events induced by SAA included the activation of the mitogen-activated protein kineases, p38 and JNK1/2 and the activation of nuclear factor-kappa B (NF-κB). Inhibitor studies have shown SAA-induced IL-6 production to be down-regulated by NF-κB inhibition and partially inhibited by p38 or JNK inhibitors. Our findings demonstrate that SAA is a significant inducer of IL-6, which is critically involved in RA pathogenesis.

Keywords: Interleukin-6; Nuclear factor-kappa B; Rheumatoid arthritis; Serum amyloid A protein

1. Introduction

Serum amyloid A protein (SAA) is an acute-phase reactant found predominantly in the plasma high density lipoprotein fraction. SAA is released to the circulation in response to pro-inflammatory cytokines and the blood concentration of SAA can be increased by as much as 1000-fold over the basal level [1]. The liver is a major source of acute-phase SAA and inflammatory cytokines, such as interleukin-6 (IL-6), TNF-α and IL-Iβ, are potent inducers of SAA expression by hepatocytes [2]. However, the extra-hepatic expression of SAA has also been documented [3].

A number of immunomodulatory roles of SAA have been reported to show cytokine-like properties [4]. SAA has been demonstrated to be a chemoattractive ligand for the human *N*-formyl peptide receptor-like 1 (FPRL1), a transmembrane G-protein-coupled receptor [5]. In vitro studies have provided the evidence that SAA can act as a chemoattractant for monocytes, leukocytes and T lymphocytes [6]. Furthermore, it has been reported that SAA significantly stimulates the secretion of the pro-inflammatory cytokines, TNF-α, IL-8 and IL-Iβ, by cultured human neutrophils [7–9]. These findings indicate the important regulatory role of SAA in the inflammatory and immune responses.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovitis and the destruction of articular structures [10]. An important feature of RA synovial fibroblasts is an increased production of matrix metalloproteinases (MMPs) which degrade articular matrix components [11]. RA synovial fibroblasts also produce pro-inflammatory cytokines, which play a pivotal role in RA pathogenesis [12]. Elevated SAA levels are observed in the sera, synovial fluids and inflamed synovium of RA patients [13,14]. O'Hara et al. also demonstrated overexpressed SAA and FPRL1, the receptor for SAA, in inflamed rheumatoid synovial tissues [15]. We and other investigators have previously demonstrated the de novo synthesis of SAA from RA-FLS in vitro and the increased production of matrix metalloproteinase from SAA-stimulated RA-FLS [16,17]. SAA may be involved in the pathogenesis of RA; however, it remains unclear whether SAA is involved in the overproduction of pro-inflammatory cytokines of rheumatoid synovium.

The aim of this study was to investigate the ability of SAA to induce pro-inflammatory cytokine production from RA-FLS, which plays a central role in the RA pathogenesis. Our data suggest that SAA appears to participate in the IL-6 overproduction that occurs in the RA synovium.

2. Materials and methods

2.1. Reagents

Recombinant human SAA was purchased from Peprotech (Rocky Hills, NJ). Cycloheximide and actinomycin D were purchased from Sigma (St. Louis, MD). PD98059, SB203580, SP600125 and BAY11-7082 were obtained from Calbiochem (San Diego, CA).

2.2. Patients

All RA patients fulfilled the American College of Rheumatology criteria for RA. Synovial tissue samples were obtained from five patients with RA during synovectomy. Synovial fibroblasts were isolated from the synovial tissues by enzymatic digestion. The isolated synovial fibroblasts were used at the third or fourth passages for subsequent experiments. All experiments were performed with the protocol approved by the ethics committee of Nagasaki Medical Center.

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Abbreviations: FLS, fibroblast-like synoviocytes; FPRL1, *N*-formyl peptide receptor-like 1; IL-6, interleukin-6; MAPKs, mitogen-activated protein kineases; NF-κB, nuclear factor-kappa B; RA, rheumatoid arthritis; SAA, serum amyloid A protein

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2.3. Measurement of cytokine secretion

RA-FLS (5×10^4) were seeded in 24-well plates containing RPMI plus 10% FCS for 24 h. Following 24 h of incubation in serum-free RPMI, the cells were stimulated with SAA (0–1 μM) for 24 h. Cell-free supernatants were collected by centrifugation at $400 \times g$ for 5 min and assayed for IL-6 with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from RA-FLS using the RNeasy total RNA isolation protocol (Qiagen, Crauley, UK). cDNA was prepared with Superscript reverse transcriptase (Invitrogen, Grand Island, NY). Specific FPRL1 were designed to generate a 1.1-kb product: sense primer 5′-CACCAGGTGCTGCTGGCAAG-3′and anti-sense primer 5′-AATATCCCTGACCCATCCTCA-3′. The amplification of the IL-6 transcripts was accomplished on a Light Cycler (Roche Diagnostics, Mannheim, Germany) using specific primers. The housekeeping gene fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for verification of equal loading.

2.5. Analysis of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kineases (MAPKs) activation

For measurement of FPRL1, IκB-α and MAPKs by a Western blot analysis, serum-starved RA-FLS seeded in 6-well plates were stimulated with SAA (1 μM) and the cells were washed by ice-cold PBS and lysed with a lysis buffer (1% Nonidet P 40, 50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 10 μg/mL aprotinin and 10 μg/mL leupeptin) for 20 min at 4 °C. Insoluble material was removed by centrifugation at $15000 \times g$ for 15 min at 4 °C. The supernatant was saved and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An identical amount of protein (50 μg) for each lysate was subjected to 10% SDS–polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. Western blot analysis using the primary antibodies against FPRL1 (Santa Cruz Biotechnology, Santa Cruz, CA), IκB-α and phospho-specific or pan anti-MAPKs antibodies (Biosource, Camarillo, CA) was performed with an ECL Western blotting kit (Amersham, Little Chalfont, UK).

NF-κB nuclear translocation in RA-FLS was detected by immunocytochemistry. Cells grown in 8-chamber culture slides (Lab Tech, Rockville, MD) were incubated with SAA (1 μM) for 30 min and then washed in PBS. Cells were fixed in 4% paraformaldehyde for 20 min and blocked in 0.01% H_2O_2 for 10 min. Incubation with rabbit polyclonal anti-NF-κB p65 (1:100, Santa Cruz Biotechnology) was performed for 1 h then with HRP-conjugated anti-Rabbit IgG (Dako Japan, Kyoto, Japan) for 30 min and ENVISON plus (Dako) was used for detection. The slides were washed in PBS, then mounted and assessed by microscopy.

2.6. Preparation of nuclear extracts

The cells were washed with cold phosphate buffered saline (PBS), harvested, and pelleted, then resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KC1, 0.6% Nonidet P-40, 1 mM EDTA, 1 mM PMSF and 1 mM DTT) and incubated on ice for 15 min. After centrifugation at 14000 rpm for 15 min, the supernatant cytoplasmic protein was collected. The remnants were resuspended in buffer B (20 mM PMSF and 1 mM DTT) and incubated on ice for 30 min, and stirred discontinuously every 10 min, and then were centrifuged at 14000 rpm for 15 min, and the supernatant nuclear extract was collected. All proteins were stored at −80 °C. The protein concentration was determined by a Bio-Rad protein assay kit.

2.7. p65 activity detection

The p65 subunit activity was detected using a Trans AM™ NF-κB p65 Kit (Active Motif, Cambridge, MA, USA). The kit contained a 96-well plate on which had been immobilized an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′). The active form of NF-κB contained in the nuclear extract specifically binds to this oligonucleotide. The primary antibodies used to detect NF-κB recognize an epitope on p65 that was accessible only when NF-κB was activated and bound to its target DNA. An HRP-conjugated secondary antibody provided a sensitive colorimetric readout that was easily quantified by spectrophotometry. The nuclear extracts (1 μg/well) isolated from SAA-treated RA-FLS were analyzed using this kit.

3. Results

3.1. FPRL1 gene expression of RA-FLS

In order to determine whether RA-FLS are able to respond to SAA, mRNA and protein expressions of SAA receptor, FPRL1, were investigated. As shown in Fig. 1, FPRL1 mRNA (A) and protein (B) expressions were detected in SAA-stimulated or unstimulated RA-FLS.

3.2. SSA induces secretion of IL-6 by RA-FLS

In order to evaluate whether SSA can induce increased cytokine production, the levels of IL-6 secretion in SAA-stimulated FLS were determined by ELISA. SAA stimulation induced a significant IL-6 production by FLS in a dose-dependent manner (Fig. 2A). RA-FLS were stimulated with 1 μM SAA, which is a physiological concentration in rheumatoid synovial fluids [18], or TNF-α (50 ng/ml) and secreted IL-6 was measured by ELISA. IL-6 synthesis by 1 μM SAA was higher in comparison to TNF-α-induced IL-6 synthesis in RA-FLS (Fig. 2B).

In order to investigate the mechanism for SAA-induced IL-6 production, we treated RA-FLS with the transcription inhibitor ActD and the protein synthesis inhibitor CHX. The treated cells were then stimulated with SAA. Both ActD and CHX inhibited SAA-induced IL-6 production (Fig. 2C). These results suggest that transcription and de novo protein synthesis are required for SAA-induced IL-6 production. The potential effects of contaminating LPS in the SAA preparation were examined. SAA was incubated with polymyxin B, an amphophilic cyclic polycationic peptide that forms a complex with LSP preventing LPS-induced cytokine production, before it was applied to RA-FLS. Polymyxin B did not alter SAA-induced IL-6 secretion. Under the same experimental conditions, polymyxin B inhibited the LPS-induced IL-6 secretion (Fig. 2C).

We next determined the level of IL-6 mRNA in SAA-stimulated FLS. Reverse transcription of total RNA was followed by real-time PCR with specific primers for human IL-6 and

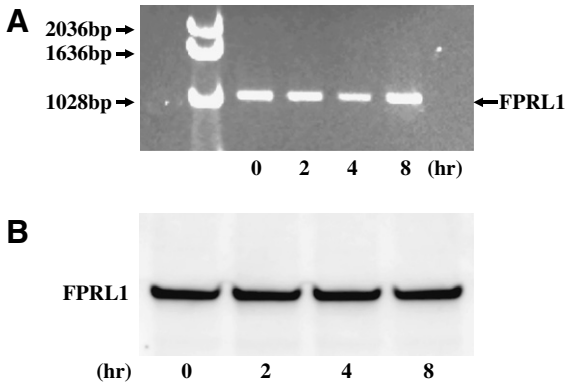

Fig. 1. Formyl peptide receptor-like1 (FPRL1) mRNA and protein expressions in RA-FLS. Total RNA and cellular extracts from SAA-treated (2–8 h) or untreated RA-FLS were analyzed by RT-PCR (A) using primers specific for FPRL1 and anti-FPRL1 Western blot (B). Three experiments were performed using different RA-FLS and a representative result is shown.

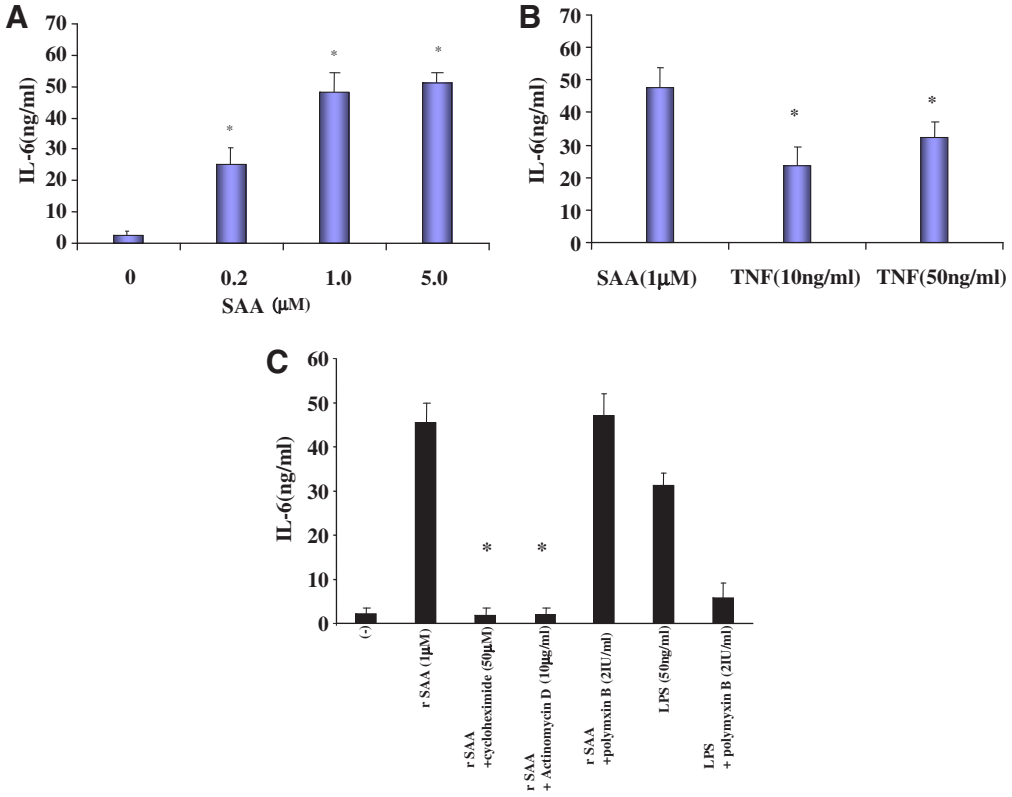

Fig. 2. SAA stimulates IL-6 protein synthesis in RA-FLS. (A) RA-FLS were stimulated with various concentrations of recombinant SAA (0–5 μM) as indicated for 24 h under serum-free conditions. IL-6 protein in the conditioned media was determined by ELISA. Data represent the means of four independent experiments run in triplicate ± S.D. $^{*}P < 0.0001$ compared to untreated RA-FLS. (B) RA-FLS were stimulated with SAA (1 μM) or TNF-α (50 ng/ml) and secreted IL-6 was measured by ELISA. Data are means of three different RA-FLS run in triplicate ± S.D. $^{*}P < 0.001$ compared to SAA-stimulated RA-FLS. (C) RA-FLS were pretreated for 1 h with or without actinomycin D (10 μg/ml), cycloheximide (50 μM) or polymyxin B (2 IU/ml). The cells were stimulated with SAA (1 μM) or lipopolysaccharide (LPS, 50 ng/ml) for 24 h. Secreted IL-6 was measured by ELISA. Data are means of three different RA-FLS run in triplicate ± S.D. $^{*}P < 0.0001$ compared to SAA-treated RA-FLS.

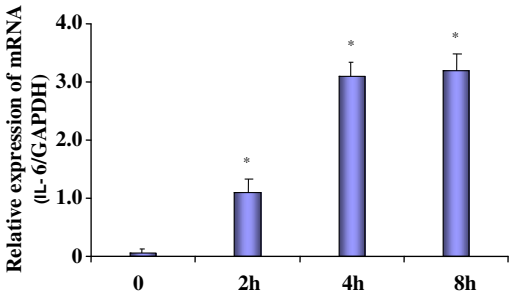

Fig. 3. SAA stimulates IL-6 mRNA expression in RA-FLS. RA-FLS were treated with 1 μM recombinant SAA for various times as indicated. IL-6 and GAPDH mRNA expression was determined by real-time PCR method. Data are means of three different RA-FLS run in triplicate ± S.D.

the housekeeping gene, GAPDH. As shown in Fig. 3, elevation of the IL-6 mRNA was observed after 2 h of SAA stimulation. This SAA-induced IL-6 mRNA expression reached a plateau after 4 h.

3.3. Role of NF-κB MAPKs and NF-κB in SAA-induced synthesis of IL-6

In order to understand the signaling mechanism of SAA-induced IL-6 production in RA-FLS, we determined the activation of MAPKs and NF-κB. SAA-induced phosphorylation of p38 and JNK 1/2 within 10 min of stimulation (Fig. 4A and B). Because the expression of the gene for IL-6 requires the activation of NF-κB [19], we also examined whether SAA can activate this transcription factor in RA-FLS. Because NF-κB activation is often accompanied by IκB-α degradation, we examined the cytosolic IκB-α level in SAA-stimulated RA-FLS. IκB-α started to degrade 10 min after SAA stimulation (Fig. 5A). As shown in Fig. 5B, nuclear translocation of the p65 subunit of NF-κB was demonstrated in SAA-stimulated RA-FLS similar to that in IL-1β-stimulated RA-FLS (positive control). The above results suggested the involvement of MAPKs and NF-κB in the SAA-induced synthesis of IL-6 in RA-FLS. To ascertain this, the cells were pretreated with the specific inhibitor SB203580 (p38 kinase), PD98059 (ERK1/2 kinase), SP600125 (JNK kinase) and Bay11-7082 (NF-κB). As shown in Fig. 6, the inhibition of NF-κB signaling blocked the SAA-induced IL-6 secretion without affecting cellular viability. SB203580 and SP600125 partially inhibited the SAA-induced IL-6 production. These results indicate the presence of converging pathways involving NF-κB and MAPK in the IL-6 production by SAA.

The DNA binding activity of p65, a subunit of NF-κB, was analyzed by Trans AM™ NF-κB p65 kit. The DNA binding activity of p65 was increased by SAA treatment in RA-FLS. This SAA-induced p65 activity was completely inhibited by

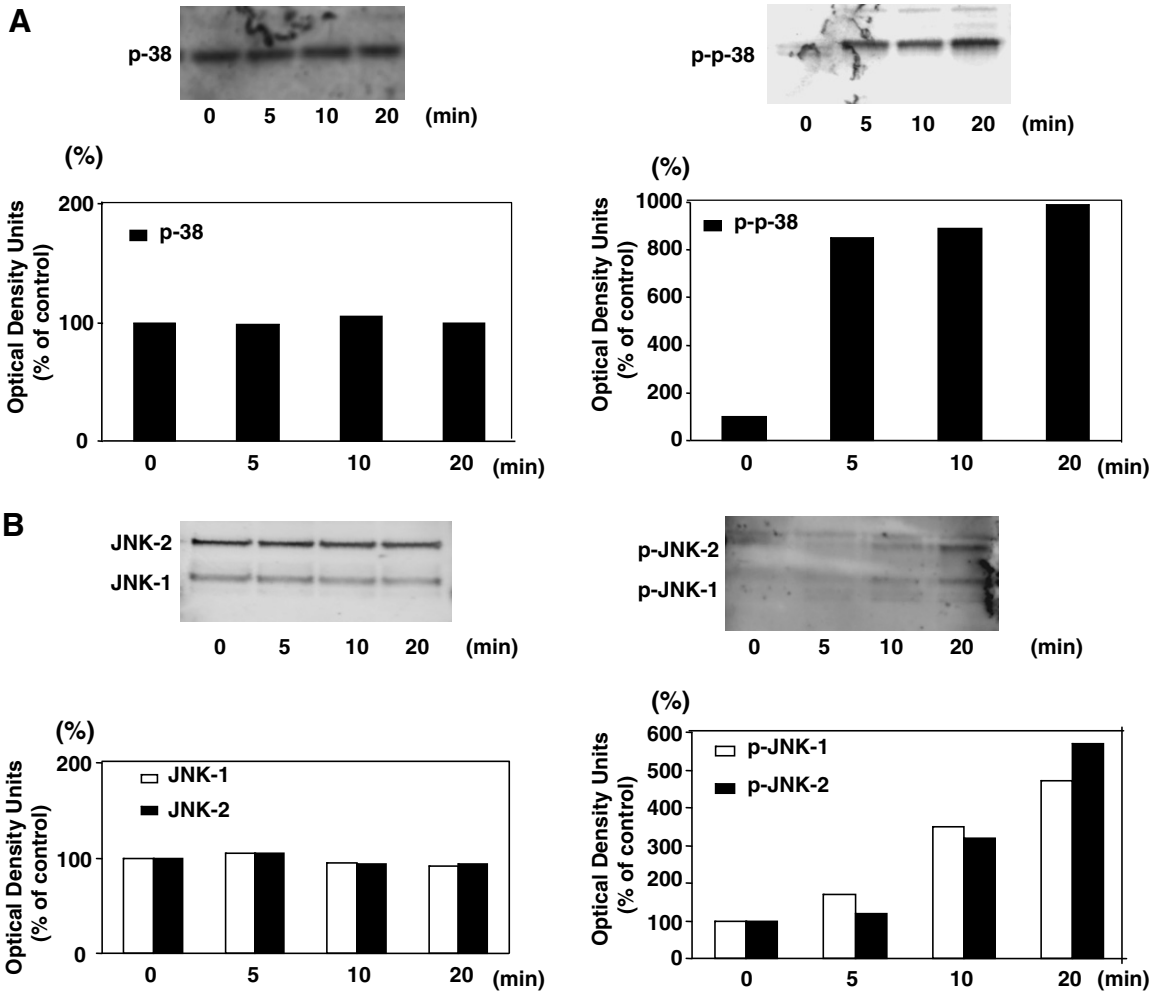

Fig. 4. Phosphorylation of p38 and JNK in SAA-treated RA-FLS. RA-FLS were stimulated with 1 μM SAA for indicated times. Phosphorylation of p38 (A) and JNK1/2 (B) were determined by Western blotting using specific antibodies against pan or phospho-MAPKs. JNK-I or p-JNK-1 indicate the pan or the phosphorylated protein levels of JNK-1, respectively. The quantitation of each protein band is shown in the lower panel. Optical density unit was assigned the ratio of the each band against control band (0 min, un-stimulated RA-FLS). Three experiments were performed using different RA-FLS and a representative result is shown.

BAY-11-7082 pretreatment. However, pretreatments with SB203580 or SP600125 inhibited SAA-induced p65 activity to a lesser degree (Fig. 7). These results indicate that these MAPKs may independently contribute to the SAA-induced IL-6 production without affecting the NF-κB pathway.

4. Discussion

In this study, we investigated the effects of SAA on the production of IL-6, which is implicated in RA pathogenesis [20], using RA-FLS. We observed that SAA induced the concentration-dependent IL-6 production. Moreover, this effect of SAA on IL-6 production was associated with the accumulation of IL-6 specific mRNA, thus suggesting that SAA-induced IL-6 production at the transcription level. This is associated with the degradation of IκB-α, NF-κB nuclear translocation and increased DNA binding activity of NF-κB. IL-6 is detectable at high levels in the sera and synovial fluids of RA patients, and the levels of IL-6 correlate with RA disease activity [21]. Furthermore, an IL-6 blockage exhibits promising efficacy in the treatment of RA [22]. These findings indicate that IL-6 is an important pro-inflammatory cytokine that is critically involved in the pathogenesis of RA. RA-FLS have been identified as significant producers of IL-6 by immunocytochemisty, in situ mRNA hybridization and by a biochemical analysis of primary cultures of synoviocytes [23,24]. In view of the pathophysiological role of IL-6 and the recognition that RA-FLS contribute to the IL-6 production, SAA seems to be implicated in rheumatoid inflammatory processes through stimulating FLS to produce IL-6. Furthermore, IL-6 is recognized as the principal inducer of most acute-phase proteins, including SAA [2]. Therefore, SAA-induced IL-6 production may enhance the sustained SAA production in an autocrine-manner which perpetuates the rheumatoid inflammation.

SAA has been recognized as an acute-phase protein, such as C-reactive protein [25]. However, recent investigations have suggested that SAA may play a role in the inflammatory process through its cytokine-like property, rather than as a passive responder [4]. For example, SAA has been shown to stimulate the secretion of the pro-inflammatory cytokines, TNF-α and IL-1β, in human neutrophils [8,9]. In RA-FLS, SAA has been

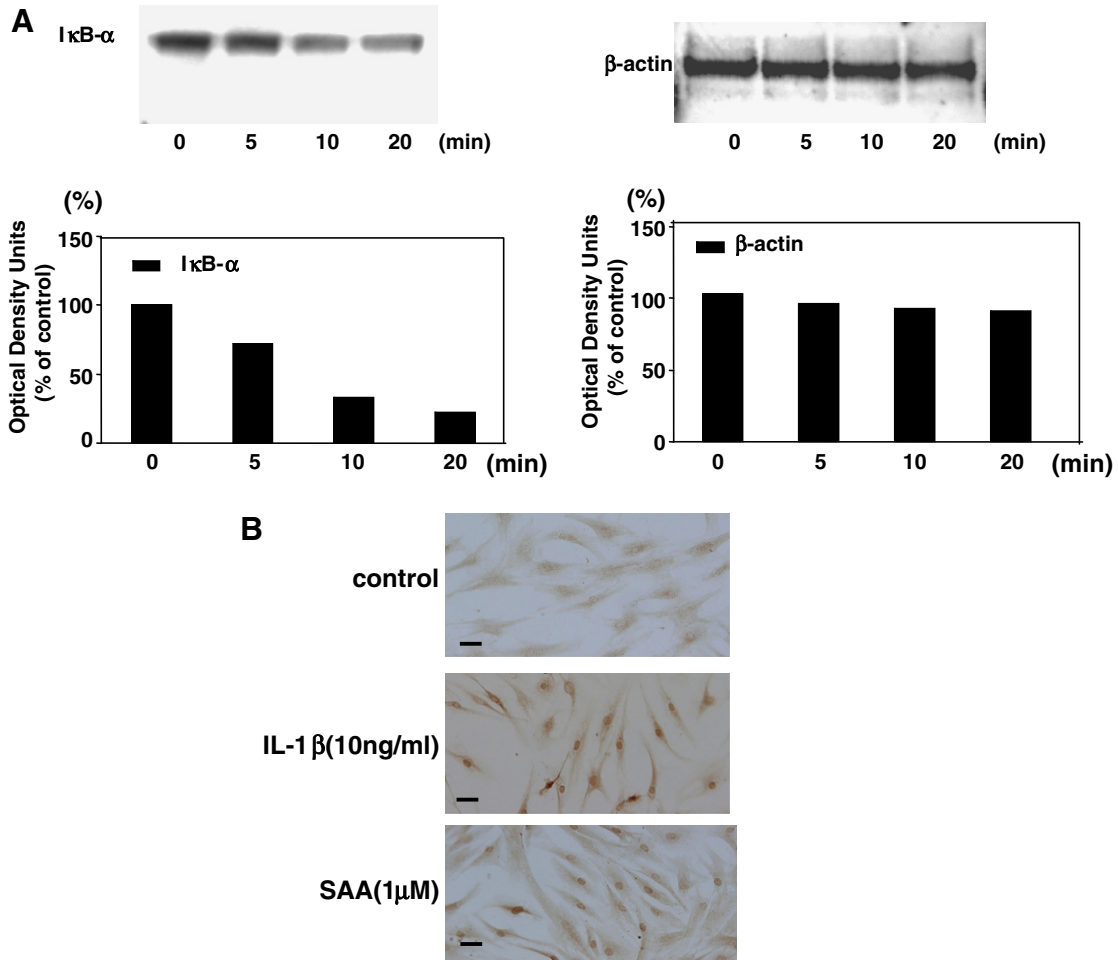

Fig. 5. SAA-induced NF-κB activation in RA-FLS. (A) RA-FLS were stimulated with 1 μM SAA for various times as indicated. Western blot analysis was used to detect the cytoplasmic level of IκB-α in RF-FLS. Time-dependent degradation of IκB-α was observed in SAA-treated RA-FLS. Equal loading of samples was determined by an antibody against β-actin. Three experiments were performed using different RA-FLS and a representative result is shown. (B) SAA-induced nuclear translocation of NF-κB RA-FLS were stimulated with IL-1β (10 ng/ml, positive control) or SAA (1 μM) for 30 min. Untreated cells were used as negative control. The nuclear translocation of NF-κB was assessed by immunocytochemical staining. Bar = 50 μm. Three experiments were performed using different RA-FLS and a representative result is shown.

demonstrated to induce the matrix metalloproteinases [16,17]. In rheumatoid synovium, the interaction between FLS and immigrated immune cells establishes a local cytokine network [26]. However, very little data are currently available regarding the function of SAA, which is highly expressed in rheumatoid synovium, in cellular interaction and the cytokine network. Our data indicate that the interaction between SAA and RA-FLS was therefore responsible for the enhanced IL-6 production. The levels of SAA were elevated in rheumatoid synovial fluids [18]. We demonstrated that RA-FLS respond to SAA by producing a pro-inflammatory cytokine, IL-6. Interestingly, IL-6 induces SAA production in rheumatoid synovial cells [27], thus providing a positive feedback loop between these molecules in rheumatoid synovium.

NF-κB transcription factor is centrally involved in cytokine and chemokine-driven inflammatory responses, including IL-6 induction [28]. In our study, treatment with BAY-11-7082 blocked SAA-induced IL-6 production, suggesting that the effects of SAA are mediated through NF-κB. The involvement of NF-κB in the SAA-mediated pro-inflammatory response was further confirmed by the demonstration that SAA degraded IκB-α expression and nuclear translocation of p65, a subunit of NF-κB, in RA-FLS. In addition to NF-κB, our study indicates the role of MAPK in SAA-mediated IL-6 gene expression. Indeed, the pharmacological inhibition of p38 MAPK partially suppressed the SAA-induced IL-6 production. Cross-talk of the MAPK pathway with the NF-κB pathway has also been shown in other studies [29]. However, our data indicated that the SAA-activated DNA binding activity of NF-κB was not completely inhibited when the JNK or p38 activities were blocked by SP600125 or SB20358, thus suggesting that these MAPKs may be involved in IL-6 gene induction by affecting other signaling pathways. Although the expression of IL-6 in RA-FLS has been shown to be regulated primarily by NF-κB [30], previous studies have demonstrated that the pro-inflammatory transcription factor, such as AP-1, may contribute to the activation of the IL-6 gene [31]. These findings suggest that SAA triggers a complex signaling network, thus resulting in IL-6 induction in RA-FLS.

In conclusion, we herein demonstrated that SAA is a strong IL-6 inducer in rheumatoid synovium. Our data provide valuable insight into the cellular mechanisms of how SAA partici-

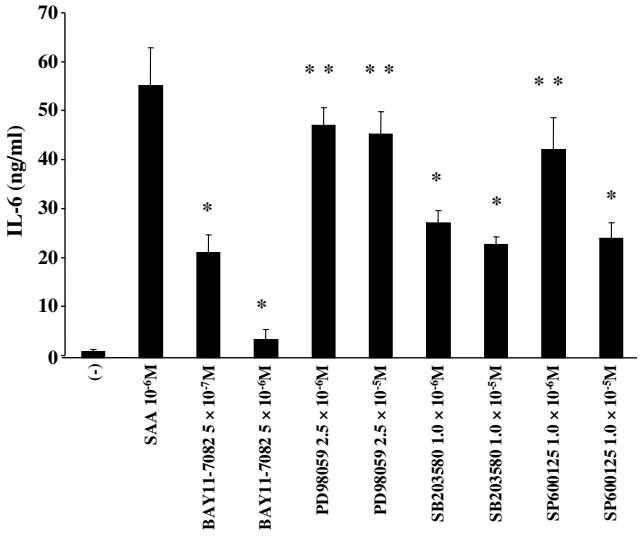

Fig. 6. NF-κB inhibition suppressed IL-6 secretion. RA-FLS in serum-free media were incubated with vehicle (DMSO, media) PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and BAY11-7082 (NF-κB inhibitor) for 1 h. Cells were then stimulated with 1 μM SAA for 24 h, after which medium was collected and IL-6 content was measured by ELISA. Data are means of three different RA-FLS run in triplicate ± S.D. $^{*}P < 0.0001$ compared to SAA-treated RA-FLS. $^{**}P < 0.01$ compared to SAA-treated RA-FLS.

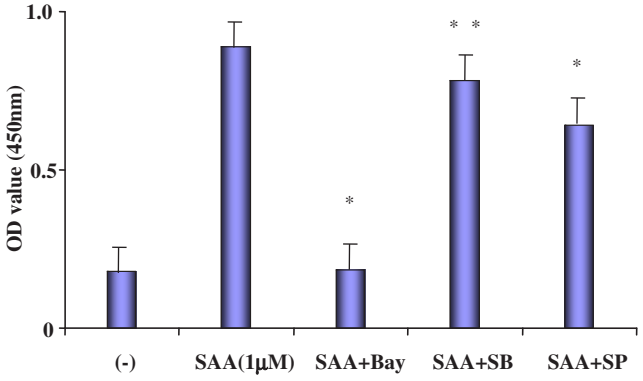

Fig. 7. DNA binding activity of p65 in SAA-stimulated RA-FLS. RA-FLS pretreated with various inhibitors were stimulated with SAA and nuclear extracts were isolated. The DNA binding activity of p65, NF-κB subunit was detected Trans AM™ NF-κB p65 kit using nuclear extracts as described in Section 2. Data are means of two different RA-FLS run in triplicate ± S.D. BAY; NF-κB inhibitor, BAY-11-7082 5 μM, SP; JNK inhibitor, SP600125 10 μM, SB; p38 inhibitor, SB203580 10 μM. $^{*}P < 0.0001$ compared to SAA-treated RA-FLS. $^{**}P < 0.01$ compared to SAA-treated RA-FLS.

pates in the cytokine network in inflamed RA synovium, thus suggesting that the SAA-mediated pro-inflammatory processes could be potential therapeutic targets in RA.

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