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Mechanism and Clinical Significance of IL-6 Combined with TNF-α or IL-1 for the Induction of Acute Phase Proteins SAA and CRP in Chronic Inflammatory Diseases

Soken-Nakazawa J Song and Kazuyuki Yoshizaki*

Division of Biological and Molecular Science, Department of Organic Fine Chemicals, The Institute of Scientific and Industrial Research, Osaka University, Japan

***Corresponding author:** Kazuyuki Yoshizaki, Division of Biological and Molecular Science, Department of Organic Fine Chemicals, The Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan, Tel: +81-6-6877-5737; E-mail: kyoshi@sanken.osaka-u.ac.jp

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Abstract

Serum amyloid A (SAA) and C-reactive protein (CRP) are two major acute phase proteins whose serum concentrations increase in response to inflammation, though the exact mechanism underlying this induction remains unknown. Dysregulated production of interleukin (IL)-6 plays a pathogenic role in various inflammatory diseases such as rheumatoid arthritis, Castleman's disease and systemic juvenile idiopathic arthritis. IL-6 blocking therapy with an anti-IL-6 receptor antibody (tocilizumab) can ameliorate clinical symptoms and abnormal laboratory findings, and completely normalize CRP and SAA levels in rheumatoid arthritis patients. Conversely, therapy for blocking tumor necrosis factor (TNF)-α, another key pathogenic factor in rheumatoid arthritis, barely lowers CRP and SAA to within their normal range, suggesting that IL-6 and TNF-α play different roles in the induction of acute phase protein expression. To clarify the different pathogenic roles and mechanisms of IL-6 and TNF-α or IL-1 in the induction of CRP and SAA in chronic inflammatory diseases, we investigated the transcriptional regulation mechanisms of SAA and CRP using hepatoma-derived cell lines *in vitro*, and related these mechanisms to the different effects of IL-6 and TNF-α blocking therapies on serum SAA and CRP in rheumatoid arthritis patients *in vivo*. We propose transcriptional regulation models for inflammatory cytokine-induced SAA and CRP expression that explain why IL-6 plays an essential role in the induction of SAA and CRP in the presence of inflammation.

Keywords: SAA; CRP; IL-6; Inflammatory disease; Rheumatoid arthritis

Introduction

Dysregulated production of inflammatory cytokines, particularly tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, is implicated in the pathogenesis of chronic immuno-inflammatory disorders. Rheumatoid arthritis (RA) is a typical chronic inflammatory autoimmune disease, characterized by persistent synovitis and progressive destruction of cartilage and bone in multiple joints [1]. Patients may develop systemic inflammatory manifestations, including increased levels of acute phase proteins (APPs) such as C-reactive protein (CRP) and serum amyloid A (SAA), in addition to local inflammation of the joints [2-4]. The exact etiology and pathogenesis of RA are not yet fully understood, but pro-inflammatory cytokines, particularly TNF-α, IL-6 and IL-1, are known to play important roles in RA pathogenesis [5,6]. Treatments with anti-cytokine agents such as infliximab (anti-TNF-α), tocilizumab (anti-IL-6 receptor) and anakinra (anti-IL-1) have been shown to effectively ameliorate disease activity, inhibit joint destruction [4,7] and significantly reduce CRP and SAA levels in RA patients. Importantly, tocilizumab has been shown to be more effective than the TNF-α inhibitor at normalizing CRP and SAA levels and other inflammatory parameters in RA patients [3,7-9]. This indicates that IL-6 and TNF-α make functionally different contributions to abnormal laboratory findings such as induced APP expression. However, the exact roles of these cytokines in inflammatory diseases remain speculative and largely unknown. To better understand the pathogenic roles of cytokines in different abnormal laboratory findings *in vivo* and the different clinical effects of IL-6 and TNF blockages, we first investigated the transcriptional mechanisms by which the APPs SAA and CRP are induced by IL-6 and TNF-α or IL-1 *in vitro*.

Mechanism of Cytokine-Induced SAA Expression *In Vitro*

APPs are mainly produced in hepatocytes. To study mechanisms of SAA induction by pro-inflammatory cytokines, SAA mRNA was analyzed in hepatoma-derived cell lines (HepG2 and Hep3B) following cytokine stimulation. We used real-time PCR to quantify the effects of IL-6, TNF-α and IL-1β on SAA mRNA expression. As shown in Figure 1A, stimulation with IL-6, but not with TNF-α or IL-1, significantly induced SAA mRNA. The combination of IL-6+TNF-α or IL-6+IL-1 induced SAA mRNA to a level greater than that stimulated by any one cytokine, though the combination of TNF-α+IL-1 had no such effect. These results suggest that, of the three cytokines, IL-6 is an essential factor while TNF-α and IL-1 are supplementary factors for the induction and augmentation of SAA mRNA [10]. Moreover, SAA induction was not further enhanced by stimulation with all three cytokines compared with stimulation by IL-6+TNF-α or IL-6+IL-1 (Figure 1A). This indicates that the IL-6 signal transduction pathway is both essential and different from that of TNF-α or IL-1, and that TNF-α and IL-1 may use a common signal transduction pathway.

Until recently, it was thought that the SAA promotor contained the C/EBPβ response element (RE) and the NF-κB RE, but not the STAT3 RE [11]. However, we have identified a non-consensus STAT3 binding site (named STAT3 NCBS) in the SAA promotor from a set of

sequence candidates, using a new method of statistical analysis for protein-DNA complex structures [12]. Previously, we have shown that activated transcriptional factor NF-κB/STAT3/C/EBPβ-recruited p300 forms a heteromeric complex on the SAA promoter, which is essential for the augmentation of SAA mRNA expression following stimulation by IL-6+TNF-α or IL-6+IL-1 (Figure 1B) [13], Our findings further demonstrated that STAT3 binds to the predicted site (STAT3 NCSB) for SAA expression when induced by IL-6+IL-1 [12].

Based on these findings, we proposed model for the complex formation which is shown in Figures 1B and 1E. Here, SAA induction is initiated by the activation of STAT3 stimulated by IL-6 (Figure 1C), then activated STAT3 binds with IL-1-activated NF-κBp65 for enhanced induction (Figure 1B). In the case where NF-κB is activated by TNF-α or IL-1, SAA mRNA is not significantly induced compared with an unstimulated control, because of the absence of IL-6-induced STAT3 activation (Figures 1D and 1E). Clinical data has indicated different roles for IL-6 and TNF-α or IL-1 in the induction of SAA production. Our *in vitro* results provide a mechanism for explaining this difference, using a transcriptional regulation model of IL-6 combined with TNF-α or IL-1 for the induction of SAA expression.

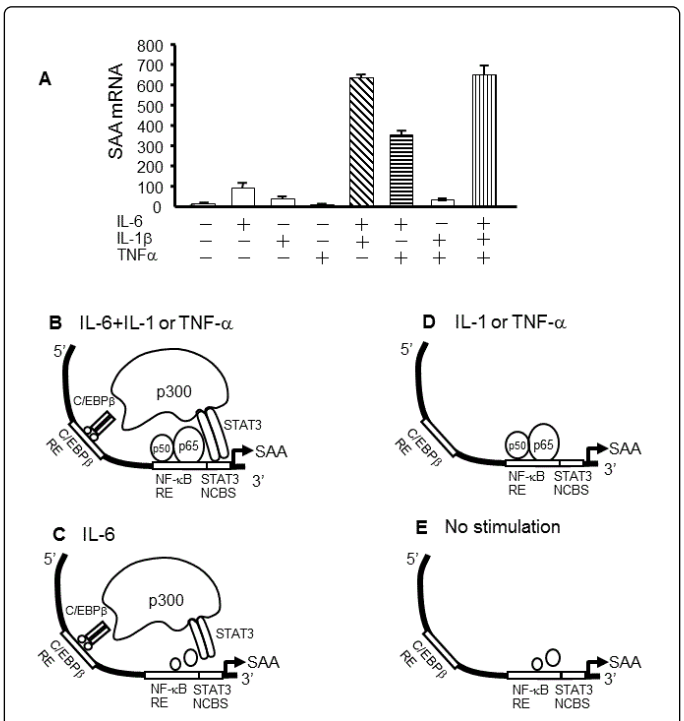

Figure 1: Synergistic effect and mechanism of cytokine-induced of SAA expression. (**A**) SAA mRNA expression induced by IL-6, TNF-α, IL-1 and their combination in Hep3B cells. SAA mRNA was assayed by quantitative real-time PCR. (**B**) Formation of transcription factor complex on SAA promoter induced by IL-6+IL-1 or TNF-α. (**C**) Activation of SAA transcription induced by IL-6. (**D**) Activation of SAA transcription induced by IL-1 or TNF-α. (**E**) No cytokine control.

Mechanism of Cytokine-Induced CRP Expression *In Vitro*

CRP is also induced by pro-inflammatory cytokines involved in inflammation. As for SAA production in hepatocytes, we found that stimulation with IL-6 but not IL-1 can induce CRP mRNA, while stimulation by the combination of IL6+IL-1 further enhanced CRP mRNA induction (Figure 2A). These findings suggest that IL-6 is an essential factor and IL-1 a supplementary factor for CRP expression [14]. To understand the transcriptional mechanism and to examine whether the CRP induction mechanism differs from that of SAA, we investigated the synergistic induction mechanism of the CRP gene by IL-6 and IL-1 in hepatocytes using the same experimental methods as listed above for the SAA gene. An analysis of RE of transcription factors in the CRP promoter region suggests that the STAT3 RE and the hepatocyte nuclear factor (HNF-1) RE, but not the NF-κB RE, are present (Figure 2B) [15,16].

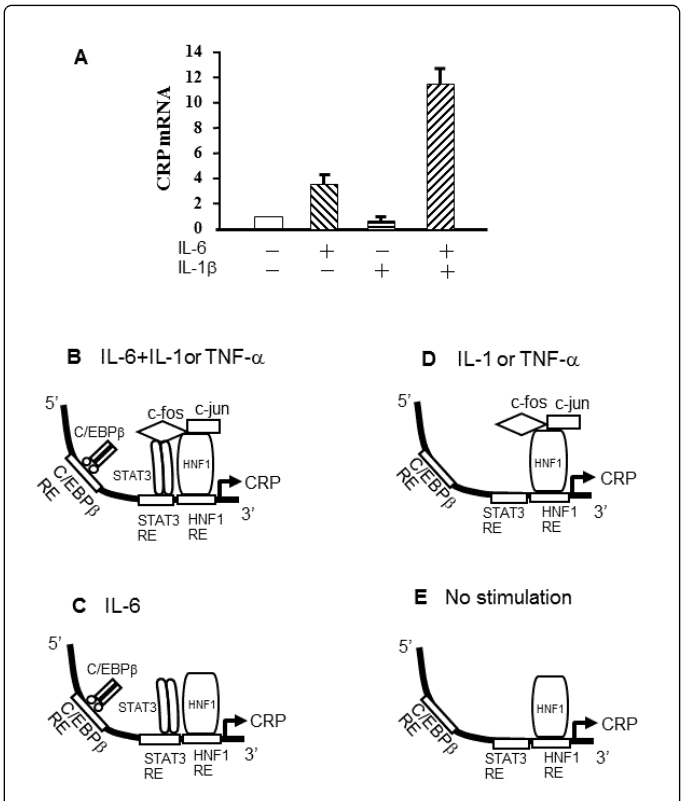

Figure 2: Synergistic effect and mechanism of cytokine-induced of CRP expression. (**A**) CRP mRNA expression induced by IL-6, TNF-α, IL-1 and their combination in Hep3B cells. CRP mRNA was assayed by quantitative real-time PCR. (**B**) Formation of transcription factor complex on CRP promoter induced by IL-6+IL-1 or TNF-α. (**C**) Activation of CRP transcription induced by IL-6. (**D**) Activation of CRP transcription induced by IL-1 or TNF-α. (**E**) No cytokine control.

It has been shown previously that over-expression of c-Fos dramatically enhances CRP promoter activity by IL-1 and IL-6 despite the CRP gene having no AP-1 RE in its promoter. An augmentative effect of c-Fos requires the presence of the STAT3 and HNF-1 RE. Through further analysis of the CRP transcriptional mechanism using

immunoprecipitation, western blot analysis, the super-shift assay and chromatin immunoprecipitation assay, we demonstrated that activated c-Fos/STAT3/HNF-1α forms a complex on the CRP gene promoter after stimulation with IL-6+IL-1, which is essential for synergistic induction. We used these findings to develop a transcriptional regulation model (Figure 2B) for the induction and augmentation of CRP by stimulation with IL-6+IL-1 or TNF-α (Figure 2A) [14]. The proposed model suggests that IL-6-activated STAT3 binds to HNF-1, which is continuously activated and bound to the HNF-1 RE, resulting in the induction of CRP expression (Figures 2A and 2C). IL-1-activated c-Fos (Figure 2D) augments CRP mRNA induction by binding to the complex of IL-6-activated STAT3 and HNF-1 (Figure 2B). However, IL-1-activated c-Fos and c-Jun are not able to induce CRP mRNA expression without IL-6-activated STAT3 (Figure 2D) [14,17,18].

Clinical significance of IL-6 combined with IL-1 or TNF-α in the induction of acute phase proteins SAA and CRP in RA and other inflammatory diseases

Having demonstrated that IL-6 plays a central role in cytokine-induced SAA and CRP expression in hepatocytes at a transcriptional level, we then evaluated the effects of IL-6, IL-1 and TNF-α blockades on SAA mRNA expression in Hep3B cells in the presence of IL-6 together with TNF-α and IL-1β. Tocilizumab treatment eliminated the combined induction effect of IL-6, TNF-α and IL-1β, so that the SAA expression level was normalized (Figure 3). IL-1 receptor antagonist treatment resulted in the partial inhibition of SAA, while SAA expression remained elevated following anti-TNF-α antibody treatment [10]. Similar results were observed for CRP using the same experimental methods (data not shown).

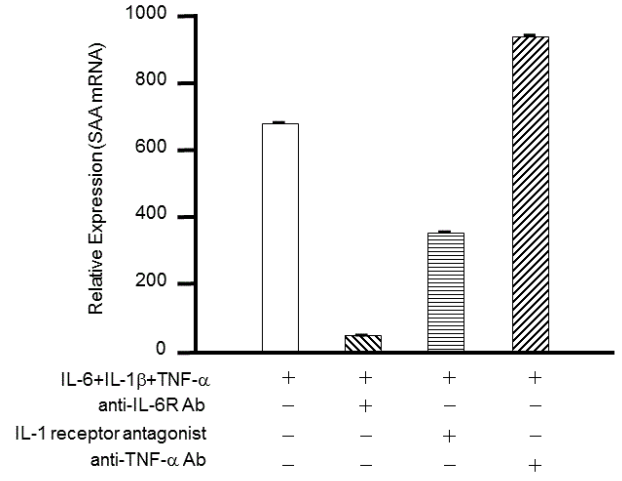

Figure 3: Effects of anti-IL-6R Ab, anti-TNF-αAb and IL-1 receptor antagonist on SAA mRNA expression induced by a stimulation combination of IL-6, TNF-α and IL-1β in Hep3B cells. SAA mRNA was assayed by quantitative real-time PCR three hours after the stimulation.

These results further confirm our finding that IL-6 is an essential factor and TNF-α and IL-1 are supplementary factors for the induction and augmentation of SAA and CRP expression. To determine whether our mechanism for cytokine-induced SAA and CRP production can explain clinical manifestations and abnormal laboratory findings, we investigated the effects of IL-6 and TNF-α blocking treatments on serum SAA and CRP levels in RA patients, and compared the clinical effects with our proposed models of transcriptional regulation for SAA and CRP. We found that anti-IL-6 treatment inhibited the activation of STAT3 and C/EBPβ and eliminated the formation of a transcriptional complex on the SAA or CRP promoter (Figures 4 and 5). However, the transcription complex on the promoter remained after anti-IL-1 or anti-TNF-α treatment (Figures 4C and 5C). This mechanism provides an explanation for the clinical findings of elevated serum SAA and CRP (right-hand bar of Figures 4A and 5A) being reduced to normal levels in RA patients treated with tocilizumab (right-hand bar of Figures 4B and 5B). It also explains why blocking TNF-α barely lowered CRP and SAA levels to within their normal range (right-hand bar of Figures 4C and 5C).

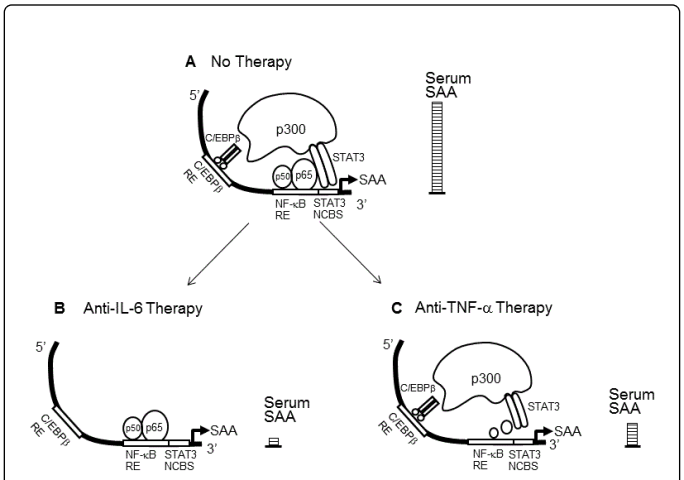

Figure 4: Effects of anti-cytokine treatment on the cytokine-induced transcriptional activity of SAA gene. (**A**) Cytokine stimulation activated transcriptional factor NF-κB/STAT3/C/EBPβ-recruited p300 forms a heteromeric complex on the SAA promoter (left panel), which is assumed to increase the SAA level in RA patients (right panel). (**B**) Effects of anti-IL-6 treatment on formation of the transcriptional complex (left panel) and production of serum SAA (right panel). (**C**) Effects of anti-TNF-α treatment on formation of the transcriptional complex (left panel) and production of serum SAA (right panel).

Thus, our proposed mechanism explains why tocilizumab is superior to the TNF-α inhibitor for reducing serum SAA and CRP levels in RA patients. Our findings from both *in vitro* and *in vivo* data have clarified the mechanism of induction and inhibition of SAA and CRP by different cytokines and their inhibitors. Although tocilizumab has similar efficacy and safety profiles to TNF-α inhibitor for RA treatment, our mechanism suggests that IL-6 and TNF-α act through different pathogenic mechanisms in RA and other inflammatory diseases [19,20]. Actually, studies have reported that increased CRP and SAA levels in alcohol users, abusers of cocaine and methamphetamine (METH), cigarette smokers and HIV-infected patients [21-24]. It has been well known that alcohol user and drug abusers develop chronic inflammation that leads to immune

dysfunction as well as neuronal impairments [24]. The knowledge we provided in this paper will be useful for further analysis of pathogenic mechanisms and the development of therapies for different inflammatory diseases using different cytokine blockers.

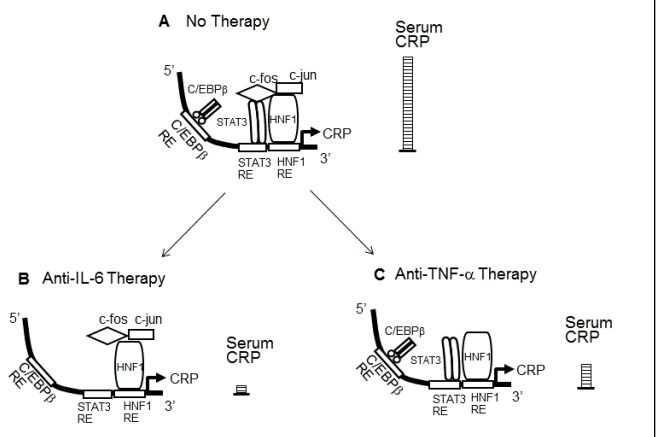

Figure 5: Effects of anti-cytokine treatment on the cytokine-induced transcriptional activity of CRP gene. (**A**) Cytokine stimulation activated transcriptional factor c-Fos, STAT3 and HNF-1α which form a complex on the CRP promoter, which is assumed to increase the CRP level in RA patients (right panel). (**B**) Effects of anti-IL-6 treatment on formation of the transcriptional complex (left panel) and production of serum CRP (right panel). (**C**) Effects of anti-TNF-α treatment on formation of the transcriptional complex (left panel) and production of serum CRP (right panel).

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