Mini Review

Analysis of cytokine-driven serum amyloid A expression based on the clinical results of IL-6 blocking therapy: a new cis-acting mechanism of STAT3

Keisuke Hagihara¹), Teppei Nishikawa²), Atsumi Matsumura³), Jian Song⁵), Toshio Tanaka¹) and Kazuyuki Yoshizaki¹,2,*)

¹) Department of Respiratory Medicine, Allergy, Rheumatic disease, Osaka University Graduate School of Medicine, Osaka, Japan
²) Health Care Center, Osaka University, Osaka, Japan
³) Osaka University Graduate School of Medicine, Medical Science, Osaka, Japan

Anti-cytokine therapies have been used for rheumatoid arthritis (RA) and their efficacy has been previously reported. Serum amyloid A (SAA) is an acute phase protein and known as a precursor of amyloid fibril in AA amyloidosis. IL-6 blocking therapy normalized the serum levels of SAA, in RA patients, while anti-TNF α or IL-1 therapy did not. From the clinical point of view, the exact identification of the induction mechanism of SAA is important. We found that IL-6 plays a critical role in the synergistic induction of the SAA gene by combining with proinflammatory cytokines. Moreover, we discovered that STAT3 plays an essential role in cytokine-driven SAA gene expression even though no typical STAT3 response element (RE) is located in its promoters. STAT3 and NF-κB p65 first form a complex following IL-1 and IL-6 stimulation, after which STAT3 interacts with non-consensus sequences at a 3′-site of NF-κB RE on the SAA gene promoter. STAT3 then recruits p300, which coordinates the interaction of NF-κB p65, STAT3 and C/EBP β in the synergistic induction of the SAA gene. Our findings have a direct bearing on a better understanding of inflammatory status and are expected to contribute to the development of a therapeutic strategy for AA amyloidosis.


Correspondence should be addressed to:
Kazuyuki Yoshizaki, Department of Respiratory Medicine, Allergy, Rheumatic disease, Osaka University Graduate School of Medicine, 2-2 Yamada-Oka, Suita, Osaka 565-0871, Japan. Phone: +81-6-6879-3831, Fax: +81-6-6879-3839, e-mail: kyoshizaki@hpc.cmc.osaka-u.ac.jp

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Introduction

Anti-cytokine therapies have been used for rheumatoid arthritis (RA) and their efficacy has been previously established in some clinical trials¹²). Serum amyloid A (SAA), an acute phase protein, and mainly produced in the liver, is well known as a precursor of amyloid A proteins in AA (secondary) amyloidosis,
which is a serious complication of RA\textsuperscript{30}. The human SAA family consists of SAA1, SAA2, and SAA4. The first two, also known as acute phase SAAs, dramatically increase by as much as 1000 times during inflammation\textsuperscript{41}. It has further been reported that the SAA2 gene is induced by nuclear factor \( \kappa B \) (NF-\( \kappa B \)) and CAAT enhancer-binding protein \( \beta \) (C/EBP \( \beta \)) in response to stimulation by interleukin-1 (IL-1) together with interleukin-6 (IL-6) (IL-1 + IL-6)\textsuperscript{31}.

However, this induction model does not fully explain certain clinical results, which showed that anti-IL-6 therapy, but not anti-tumor necrosis factor \( \alpha \) (TNF \( \alpha \)) or IL-1 therapy, normalized the serum level of SAA\textsuperscript{1-2}. For this reason, we investigated the exact induction mechanism of SAA by proinflammatory cytokines, and especially focused on the SAA1 gene, which is reportedly as a main amyloidogenic factor in AA amyloidosis\textsuperscript{42}. We previously discovered a new \textit{cis}-acting mechanism of signal transducers and activators of transcription 3 (STAT3)\textsuperscript{43,44}. The study reported here constitutes a new clinical research approach, and has a direct bearing on a better understanding of the pathogenesis of inflammatory status.

**IL-6 plays a critical role in the synergistic induction of the SAA gene by proinflammatory cytokines**

We first established an SAA isoform real-time quantitative RT-PCR assay system to examine the clinical results of anti-IL-6 receptor antibody therapy\textsuperscript{45}. We then used this assay system to examine the effects of various combinations of proinflammatory cytokines, and found that IL-6 and IL-1 or IL-6 and TNF \( \alpha \), but not IL-1 and TNF \( \alpha \), synergistically induced SAA1 gene expression. These results showed that IL-6 is necessary for the synergistic induction of SAA gene by combining with IL-1, and TNF \( \alpha \) (Fig.1A). Next, we confirmed this finding with the aid of specific inhibitors, namely, the anti-IL-6 receptor monoclonal anti-body (anti-IL-6R Mab), the IL-1 receptor antagonist (IL-1 ra), and the anti-TNF \( \alpha \) monoclonal anti-body (anti-TNF \( \alpha \) Mab).

Fig.1B shows that anti-IL-6R Mab, but not IL-1 ra or anti-TNF \( \alpha \) Mab, eliminated the synergistic induction of SAA in response to triple stimulation of IL-6, IL-1, and TNF \( \alpha \). Almost the same results were obtained when three typical hepatic cell lines, HepG2, Hep3B, and PLC/PRF/5, were used. We show here representative data for HepG2 cells. These results indicate that IL-6 plays a critical role in the synergistic induction of SAA gene by proinflammatory cytokines.

However, the SAA gene is thought to be synergistically induced by NF-\( \kappa B \) and C/EBP \( \beta \) in response to IL-1 and IL-6, respectively. Since IL-6 has two main signal transduction pathways, the MAPK and Jak-STAT pathways, which regulate C/EBP \( \beta \) and STAT3\textsuperscript{46}, we used Jak2 inhibitor-AG490 to determin
mine whether the Jak-STAT pathway is involved in the expression of the SAA gene. Suprisingly, AG490 reduced the synergistic induction of SAA1 to 30%* (Fig.1C). These findings therefore indicate that the Jak-STAT pathway plays an important role in the synergistic induction of the SAA gene. It has further been reported that STAT3 binds to a \( \gamma \)-interferon activation sequence (GAS) like sequence (-TTTNNGAA)*. The C-reactive protein (CRP) gene, a major acute phase protein activated by IL-6, has a STAT3 response element (RE) (-TTCCCGAA) in its promoter*.

However, no STAT3 RE has been found in the human SAA1 promoter*.

**STAT3 is essential for the synergistic induction of human SAA genes via the NF-\( \kappa \)B RE containing region after formation of a complex with NF-\( \kappa \)B p65**

To examine the effect of STAT3 on the SAA1 promoter activity, pEF-BOS dominant negative STAT3 Y705F (dn STAT3) or pEF-BOS wild type STAT3 (wt STAT3) was co-transfected with the pGL3-SAA1 promoter luciferase construct (-796/+24) (pGL3-SAA1) into HepG2 cells*. dn STAT3 completely inhibited the transcriptional activity of pGL3-SAA1 even after stimulation with IL-1+6, and wt STAT3 augmented the transcriptional activity of pGL3-SAA1 three times more than that resulting from IL-6 and IL-1+6 stimulation (Fig.2A). These results indicate that STAT3 plays an essential role in the transcriptional augmentation of SAA1 genes.

Since no typical STAT3 consensus sequence has been identified in the human SAA1 promoter*, it became necessary to determine how STAT3 is involved in the transcriptional activation of SAA gene by using 5' deletion mutants of the SAA1 promoter. We observed that the promoter activity of SAA1 genes was partly reduced by deletion of C/EBP\( \beta \) RE and completely eliminated by deletion of NF-\( \kappa \)B RE (data not shown)*. To examine the possibility that STAT3 might act on the transcriptional activity of SAA via the C/EBP\( \beta \) and NF-\( \kappa \)B RE-containing regions, wt STAT3 was co-transfected with a pGL3-SAA1, pGL3-SAA1 C/EBP\( \beta \) RE deleted mutant (\( \Delta \) C/EBP\( \beta \) RE), and a pGL3-SAA1 NF-\( \kappa \)B RE deleted mutant (\( \Delta \) NF-\( \kappa \)B RE). As seen in Fig.2B, the co-expression of wt STAT3 increased the transcriptional activity of pGL3-SAA1 and pGL3-SAA1 \( \Delta \) C/EBP\( \beta \) RE almost three-fold. However, co-expression of wt STAT3 did not enhance the transcriptional activity of pGL3-SAA1 \( \Delta \) NF-\( \kappa \)B RE. These results suggest that STAT3 is involved in the transcriptional activity of SAA, most likely via the NF-\( \kappa \)B RE containing region. In the case of rat \( \gamma \) fibrinogen, the CTGGGAATCCC sequence
was found to be responsible for transactivation by both STAT3 and NF-κB(10). Furthermore, it has been reported that TCC was necessary for NF-κB binding and that the CTGGGAA sequence was needed for STAT3 binding because of the loss of transcriptional activity in an AGATCTATCCCT mutant. To investigate whether STAT3 binds to the CAGGGAC sequence of the NF-κB RE in the SAA1 promoter region, we created two mutated constructs, pGL3-SAA1 NF-κB RE M1 (AGATCTATTTCC) and M2 (CAGGGACTTGTAC). We expected that STAT3 would bind to NF-κB RE M2, but not to NF-κB RE M1. However, neither transfection with NF-κB RE M1 nor with NF-κB RE M2 resulted in transcriptional activity even when wt STAT3 was co-expressed (Fig.2B). These findings suggest that STAT3 does not bind to the NF-κB RE, which mediates the transcriptional activity of the human SAA1 gene. We confirmed these results with a gel shift assay using a wild type SAA1 oligonucleotide (-196/-73) probe (wt SAA1 probe) or SAA1 mutated the NF-κB RE M2 oligonucleotide (SAA1 mt NF-κB RE M2 probe) which contains the C/EBP β and NF-κB RE in the human SAA1 promoter. Two major nucleoprotein complexes were detected after cytokine stimulation using the wt SAA1 probe, but formation of the complex was diminished when the SAA1 mt NF-κB RE M2 probe was used (data not shown)(7). This result was consistent with that for the deletion mutants of SAA1 promoter (Fig.2B) and indicated that NF-κB RE on the SAA1 promoter is essential for the transactivation of SAA1.

We assumed that STAT3 forms a complex with NF-κB and contributes to the transcriptional augmentation of the human SAA gene. To examine the validity of this hypothesis, we performed IP-Western of STAT3 and NF-κB(7). Fig.2C clearly shows that STAT3 is associated with NF-κB p65 following IL-1+6 treatment, but that no specific band of NF-κB p50 was detected (data not shown). These findings are consistent with those reported by Betts et al, namely, that overexpression of NF-κB p65 but not p50 enhanced the transcriptional activity of human SAA2 in a dose-dependent manner(5). This indicates that crosstalk between STAT3 and NF-κB p65 contributes to the transcriptional augmentation of SAA by IL-1+6 stimulation.

**STAT3 acts on the SAA1 promoter by means of a newly discovered cis-acting mechanism**

We next investigated how STAT3 contributes to the formation of the transcriptional complex comprising NF-κB, C/EBP β and STAT3. STAT3 is reportedly associated with p300(11), which suggests the possibility that heteromeric complex formation of
STAT3, NF-κB p65 and p300 may be involved in the transcriptional activity of human SAA gene. To examine this possibility, we performed a Chromatin immunoprecipitation (ChIP) assay using chromatin isolated from HepG2 cells. STAT3 and p300 were strongly recruited to the SAA1 promoter region (-226/+24) in response to IL-6 or IL-1+6 and weakly recruited by IL-1. NF-κB p65 was recruited by IL-1 or IL-1+6 and only slightly recruited so by IL-6 (Fig.3A). These results provide clear evidence that STAT3 forms a transcriptional complex with NF-κB p65 and p300 on the SAA promoter region. A subsequent luciferase assay using pGL3-SAA1 (-226/+24) co-transfected with p300 wt in pCMV3 (wt p300) and wt STAT3 demonstrated that co-expression of wt p300 alone did not augment the luciferase activity of pGL3-SAA1 (-226/+24), but that co-expression of wt p300 with wt STAT3 dramatically enhanced the luciferase activity in a dose-dependent manner (Fig.3B). This suggests that STAT3 interacts with p300 in the transcriptional activation of the human SAA gene.

However, the question remained how STAT3 binds to the promoter region of the SAA1 gene because no typical STAT3 RE seems to be located there. It is likely that STAT3 either binds to the promoter region of the SAA gene indirectly, or obtains binding affinity for an unknown DNA sequence in a complex with NF-κB p65. In an attempt to answer this question, we performed DNA affinity chromatography using an wt SAA1 probe. Nuclear extracts specifically interacting with a biotinylated wt SAA1 probe were collected with the aid of streptavidin-Dynabeads with a magnet and transcriptional factors were analyzed by Western blotting. NF-κB p65 and STAT3 were both pulled down by the wt SAA1 probe from the nuclear extracts of HepG2 cells after IL-1+6 stimulation (Fig.3C). We next used the SAA1 mt NF-κB RE M2 probe, which has no binding affinity for NF-κB p65, to examine the possibility that NF-κB p65 has an effect on the binding affinity of STAT3 to the SAA1 promoter region. As shown in Fig.3C, neither NF-κB p65 nor STAT3 was detected by this probe, indicating that the interaction between STAT3 and NF-κB p65 is essential for the binding affinity of STAT3 to the wt SAA1 probe. We assumed that the formation of the heteromeric complex of STAT3 and NF-κB p65 gives STAT3 binding affinity to the SAA1 promoter region.

On the basis of our results and the results obtained with rat γ fibrinogen, we focused our attention on the 3′-site of NF-κB RE (CAGGGACTTTCCCGAGG) as a candidate for the STAT3 binding site, because sequences contiguous to NF-κB RE may have influenced the binding affinity of STAT3. On the assumption that mutation of this site could influence the binding affinity of STAT3 but not of NF-κB p65, we created a SAA1 mt NF-κB RE M3 probe (CAGGGACTTTCCCGATGATCTA). As expected, the specific bands of STAT3 from the nuclear extracts of HepG2 cells after IL-1+6 stimulation were markedly reduced by the SAA1 mt NF-κB RE M3 probe compared to the effect obtained with the wt SAA1 probe, although the specific bands of NF-κB p65 were observed almost as intact as those seen in

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**Fig.4 Effects of anti-cytokine therapy on the cytokine-driven transcriptional activity of the human SAA gene**

(A) Cytokine stimulation caused the formation around the NF-κB RE of a heteromeric complex with STAT3 and NF-κB p65. STAT3, which is assumed to interact with the 3′-site of NF-κB RE, recruits the co-activator p300, which then coordinate the interaction of NF-κB p65, STAT3, and C/EBPβ thus resulting in the increased transcriptional activity of the human SAA gene. (B) anti-TNFα or IL-1 therapy reduces the activity of the NF-κB signaling pathway, although the transcriptional complex remains. (C) anti-IL-6 therapy inhibits the activation of STAT3 and C/EBPβ, and eliminates the formation of the transcriptional complex on the SAA promoter.
with the wild type (Fig.3C). These results support our hypoth-
thesis that the binding affinity of STAT3 for the human SAA pro-
moter region is the result of the formation of a heteromeric com-
plex comprising STAT3 and NF-κB p65.

Our overall data shows that we were able to demonstrate that
STAT3 acts on the human SAA promoter via a newly discov-
ered cis-acting mechanism, that is, the formation of a heteromeric
complex containing STAT3, NF-κB 65 and p300. We also cre-
atated a schematic model to explain the synergistic induction of
the human SAA gene by IL-1+6 stimulation (Fig.4A). At the
same time, this schematic model also explains the effect of an-
cytokine therapy on the transactivation of SAA. Anti-TNFα or
IL-1 therapy reduced but did not eliminate the NF-κB signaling
pathway, because this pathway is activated by various stimuli
e.g. toll-like receptors, and other cytokines and so on[23]. As a
consequence, the transcriptional complex on the SAA promoter
persisted after Anti-TNFα or IL-1 therapy (Fig.4B). On the other
hands, IL-6 family cytokines, such as oncostatin M, IL-11, and
LIF, except for IL-6, have little effect on the production of acute
phase protein[13]. IL-6 blocking therapy inhibits the activation of
STAT3 and C/EBP β, and also prevents the formation of the
transcriptional complex on the SAA promoter (Fig.4C).

SAA is the precursor of the amyloid A protein in AA amyloi-
dosis, and we previously observed that humanized anti-IL-6 re-
ceptor antibody therapy normalized serum levels of SAA and
improved the clinical symptoms of AA amyloidosis in some
Castleman’s disease patients[14]. Furthermore, Gilmore et al re-
ported that normalization of the serum SAA level can lead to
amyloid regression in patients with AA amyloidosis[5]. From the
clinical point of view, our findings in this context therefore con-
stitute an aid for the development of a new therapeutic strategy
for AA amyloidosis which uses normalization of the serum SAA
level.

Conclusion

The clinical results of anti-IL-6 therapy for RA[7] or Castleman’s
disease patients[14] prompted us to investigate the exact mecha-
nism by which proinflammatory cytokines induce SAA. This
investigation enabled us to demonstrate that STAT3 plays an
essential role in the synergistic induction of SAA by IL-1+6
stimulation via a new cis-acting mechanism. Our results further
suggest that the serum SAA level is affected by the intensity of
the interaction between STAT3 and NF-κB p65. NF-κB is a
key regulator of pro-inflammatory cytokines, matrix metallo-
proteinases, inducible nitric oxide synthase, cyclooxygenase 2,
and other pro-inflammatory genes[23]. The interaction between
STAT3 and NF-κB p65 may thus contribute to the expression
of these pro-inflammatory genes. Our study constitutes a novel
clinical research approach “from the bedside to bench”, findings
which are expected to aid understanding of inflammatory status
and the development of a therapeutic strategy for AA amyloidosis.

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