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Anti-Cytokine Therapy for AA Amyloidosis

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1. Introduction

Amyloid A (AA) amyloidosis is a serious complication of chronic inflammatory diseases, including rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), inflammatory bowel disease (IBD), familial Mediterranean fever (FMF), and others [1]. Several reports suggest a prevalence of about 3 to 6% in rheumatoid arthritis patients [2-5], about 11 to 13% in FMF patients [6,7], and about 1 to 3% in IBD patients [8]. Serum amyloid A (SAA) is well known as a precursor of amyloid A proteins in AA amyloidosis. Insoluble amyloid fibril deposition is derived from the extracellular aggregation of proteolytic fragments of SAA. Human SAA family proteins are apolipoproteins of high-density lipoprotein molecules. Acute phase SAA consists of SAA1 and SAA2, which are mainly produced by pro-inflammatory cytokines in the liver such as interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6), and dramatically increase, by a magnitude of up to 1000 times during inflammation [9, 10]. Long-term overproduction of the SAA protein is a key component of the resultant pathogenic cascade [1]. The physiological roles of the various SAA isotypes remain unclear, but analysis of AA amyloid deposits has shown that SAA1 is the main amyloidogenic factor [11] and SAA1 genotypes are involved in the development of AA amyloidosis [12, 13]. In fact, it is reported that serum levels of SAA are associated with relative risk of death in AA amyloidosis patients. Relatively favorable outcomes are reported in patients with SAA concentrations remaining in the low-normal range (<4 mg per liter) [14, 15]. In Figure 1, the suppression of SAA levels by anti-cytokine therapy that may lead to clinical amelioration of symptoms, prevention of progressive organ deterioration, or recovery from damage caused by amyloid A deposits in the pathogenic cascade of AA amyloidosis is schematically represented. Anti-cytokine therapies have been used for rheumatoid arthritis (RA) and other chronic inflammatory diseases.

es, and, as noted, their efficacy has been established in several clinical trials [16], although the best choice of biologic for AA amyloidosis remains controversial.

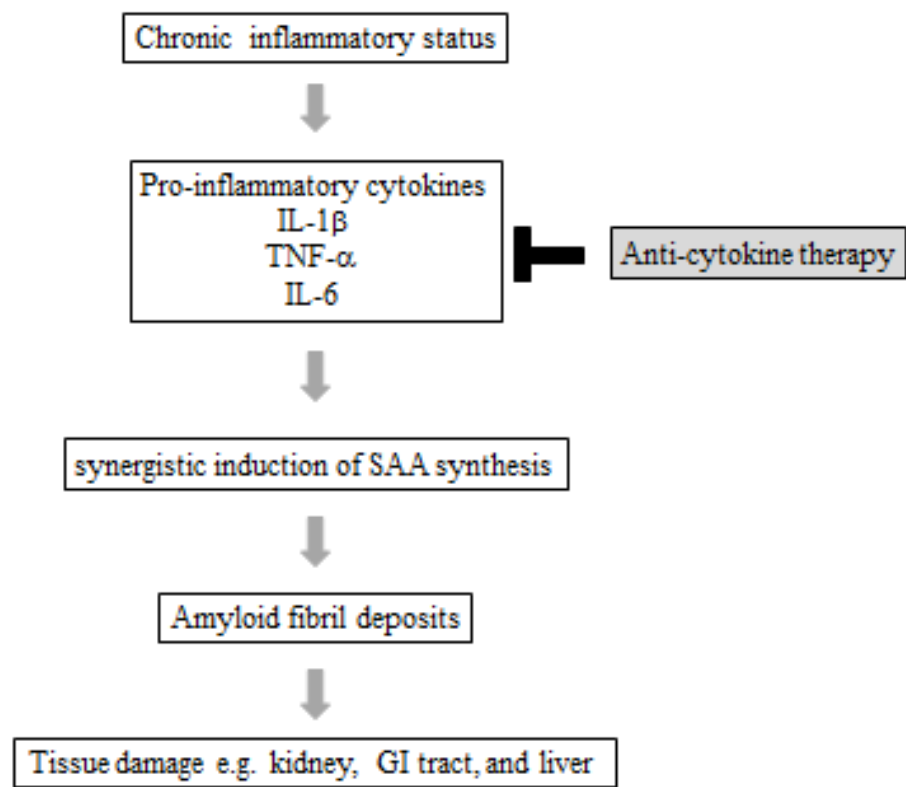


Figure 1. Anti-cytokine therapy for the pathogenesis of AA amyloidosis

In this chapter, we outline the clinical effect of anti-cytokine therapy for AA amyloidosis. We summarize animal models of AA amyloidosis association with pro-inflammatory cytokines, and finally, we show results elucidating the cytokine-driven induction mechanism of SAA. The formation of a transcriptional complex with signal transducer and activator of transcription 3 (STAT3) and nuclear factor κ B (NF- κ B) p65 play a critical role in the synergistic induction of SAA by IL-1, TNF- α , and IL-6. These results provide a rationale for IL-6 blocking therapy as a highly reasonable candidate to normalize the serum levels of SAA in the treatment of AA amyloidosis.

2. Clinical effect of anti-cytokine therapy for AA amyloidosis

In The European League Against Rheumatism (EULAR) recommendations 2010 for the management of RA, the efficacy and safety of biologics were reviewed in patients with RA. We summarize the biologics against TNF- α , IL-1, and IL-6 in Table 1. Five anti-TNF drugs are available, but golimumab and certolizumab have not been reported in the treatment of AA amyloidosis, and we found only 1 report of treatment with adalimumab in a patient with AA amyloidosis complicating JIA [17].

Anti-TNF drugs	Characteristic	Dosage
Infliximab (IFX)	Chimeric (mouse/human)mAb IgG1	div. once 8 weekly 3-10mg/kg
Adalimumab (ADA)	Human mAb, IgG1	s.c. once biweekly 40-80mg/body
Etanercept (ETN)	Fusion protein of TNF receptor 2 and IgG1 Fc component	s.c. once weekly 25-50mg/body
Golimumab (GLM)	Human mAb, IgG1	s.c. once monthly 50-100mg/body
Certolizumab (CZP)	Humanized Fab' fragment conjugated to a polyethylene glycol	s.c. once monthly 400 mg /body
IL-1 receptor antagonist		
Anakinra (ANA)	non-glycosylated version of human IL-1RA	s.c. once or twice daily 100mg/body
Anti-IL-6 receptor antibody		
Tocilizumab (TCZ)	Humanized mAb, IgG1	div. once 4 weekly 8mg/kg

Table 1. Examples of biologics for inflammatory cytokines

2.1. Anti-TNF therapy for AA amyloidosis

Several studies have reported that the efficacy of various anti-TNF drugs in the treatment of patients with AA amyloidosis, and infliximab (IFX) and etanercept (ETN) have been used in many of them (Table 2). In 2002, Elkayam et al. first reported successful treatment of an AA amyloidosis patient with IFX. A 67-year-old woman with RA developed moderately active disease and significant proteinuria. AA amyloidosis was diagnosed by a renal biopsy. After 14 weeks with IFX the patient's SAA decreased from the pre-therapy level of 29 mg/L to 4.5 mg/L. In addition, clinical remission of the nephrotic syndrome was observed as along with stabilization of amyloid deposits confirmed by ¹²³I-labeled SAP scintigraphy after 1 year [18]. In 2003, Verschueren et al. reported that a 26-year-old man with JIA and IBD associated with spondyloarthropathy (HLA B27+) developed significant proteinuria. AA amyloidosis was diagnosed by a renal biopsy. IFX improved the proteinuria after 9 months, but amyloid deposits in renal specimens remained almost the same in the mesangium and in the subendothelial and subepithelial spaces after IFX therapy [19]. Ortiz-Santamaria et al. reported the clinical effect of IFX on 6 patients with AA amyloidosis (5 patients with related RA and 1 with ankylosing spondylitis (AS)). Three patients were withdrawn from the therapy in the first 2 months, 2 because they required hemodialysis and 1 because of an anaphylactic reaction. Serum creatinine levels and proteinuria stabilized in 1 patient and improved in 2 patients during treatment with IFX [20]. Gottenberg et al. reported that 15 patients with AA amyloidosis and renal involvement were treated with TNF inhibitors. Baseline characteristic

of the 15 patients were different (RA 5, AS 6, JIA 1, psoriatic arthritis (PA) 1, adult Still's disease (ASD) 1, and Chronic infantile neurologic cutaneous and articular (CINCA) syndrome 1). Ten patients received IFX, 4 received ETN, and 1 underwent both types of treatment. Frequency of diarrhea was markedly reduced in 2 of the 3 patients with digestive tract amyloidosis, while amyloidosis progressed in 7 patients and was stabilized in 5 patients. This retrospective study suggested only the possibility that TNF inhibitors were effective for AA amyloidosis [21].

Authors	Design	Disease	Drugs	Organ dysfunction	Results
Elkayam et al. 2002 [18]	Case report	RA 1	IFX	Kidney	Proteinuria improved
Verscueren et al. 2003 [19]	Case report	IBD+AS 1	IFX	Kidney	Proteinuria improved
Ortiz-Santamaria et al. 2003 [20]	Case series	RA 5, AS 1	IFX 6	Kidney	3 patients withdrawn, stabilized in 1 patient and improved in 2 patients
Gottenberg et al. 2003 [21]	Retrospective study	RA 5, AS 6, JIA1, PA1, ASD1 CINCA 1	IFX10 ETN4 Both1	Kidney	Progressed in 7 patients and stabilized in 5 patients
Smith et al. 2004 [22]	Case report	RA 1	ETN	Kidney	Proteinuria improved
Ravindran et al. 2004 [23]	Case report	RA with Felty's syndrome 1	ETN→ IFX	kidney	Proteinuria improved
Matyas et al. 2004 [28]	Case report	FMF 1	IFX	Kidney	Proteinuria improved

Table 2. Anti-TNF drugs in the treatment with AA amyloidosis patients

Studies of ETN in AA amyloidosis patients are fewer than those of IFX. In 2004 Smith et al. reported that ETN improved proteinuria in a patient with renal amyloidosis complicating RA for 3 years [22]. In 2004, Ravindran et al. published a report of a case of RA with secondary Sjögren's syndrome and Felty's syndrome complicated by AA amyloidosis and nephrotic syndrome, which was treated with ETN for 1 year and IFX for 1 year. After 1 year, the patient's urinary protein was 6 g over 24 h. The patient changed to monotherapy with IFX. Marked reductions in proteinuria as well as a sustained stabilization of renal function were observed. In addition, a regression of AA amyloid, as quantified by ^{125}I -labeled SAP scintigraphy was established [23]. It seems that IFX therapy might be more effective than ETN therapy for AA amyloidosis. In fact, while ETN treatment of patients with AA amyloidosis produced a decrease in SAA, there were no significant changes in serum creatinine or proteinuria [24]. In 2009, Kuroda et al. reported the effects of TNF inhibitors on 14 patients with

AA amyloidosis associated with RA. Four patients were treated with IFX and 10 with ETN. Twenty-four hour urinary protein excretion was significantly decreased in 3 patients, stable in 6, and increased in 3 after initiation of anti-TNF therapy. The gastroduodenal biopsies from 9 patients showed significant reductions in amyloid deposits, which were no longer detectable in 2 patients [25].

In 2010, Nakamura et al. evaluated the efficacy of ETN treatment in 14 patients with RA complicated by AA amyloidosis. The AA amyloidosis improved and stabilized after 89.1 ± 27.2 weeks. Proteinuria decreased from 2.24 to 0.57 g/day ($p < 0.01$) and SAA fell from 250 to 26 mg/L. Diarrhea secondary to gastrointestinal AA amyloidosis was less, but serum creatinine levels did not improve.

Authors	Design	Disease	Drugs	Organ dysfunction	Results
Bosca et al. 2006 [29]	Case report	Crohn's disease 1	IFX	Kidney	Proteinuria improved
Perry et al. 2008 [24]	Case series	Inflammatory arthritis 9	ETN	Kidney	No significant changes in serum creatinine or proteinuria
Kuroda et al. 2009 [25]	Case series	RA 14	IFX 10 ETN 4	Kidney GI tract	Proteinuria improved in 3 patients, remained the same in 6 patients, and increased in 3 patients. Gastroduodenal amyloid deposit reduced in 9 patients.
Nowak et al. 2009 [17]	Case report	JIA 1	ADA	Kidney	Proteinuria improved
Nakamura et al. 2010 [26]	Case series	RA 14	ETN	kidney	After 89.1 ± 27.2 weeks, proteinuria decreased and diarrhea was less. Serum creatinine level did not improve.
Fernandez-Nebro et al. 2010 [27]	Multicenter, controlled, dynamic prospective cohort study	RA 21, SA 8, PA 4, JIA 1, aSD 1, APS 1	IFX 29 ETN 7	Kidney 94% Liver 8% GI tract 11%	Proteinuria reduced by 59.7 % during the first 24 months. Serum creatinine level did not improve. The level of acute phase reactants diminished but did not reach the normal level.

Table 3. Anti-TNF drugs in the treatment with AA amyloidosis patients

In 2010, Fernandez-Nebro et al. reported a multicenter, controlled, dynamic prospective cohort study of 36 patients with AA amyloidosis who were treated with either IFX (29) or ETN (7). As external controls, 35 non-amyloid patients (RA 18, SA 11, PA 5, JIA 1, aSD 0, APS 0) treated with TNF drugs were extracted from the *Base de Datos de Productos Biológicos de la Sociedad Española de Reumatología* registry. Long-term anti-TNF treatment reduced the median levels of proteinuria by 59.7% during the first 24 months, while both mean serum creatinine levels and creatinine clearance levels remained stable. Serum levels of CRP decreased, but

did not reach the normal level. In a multivariate Cox regression analysis, the duration of amyloidosis and the level of proteinuria were independent predictors of anti-TNF treatment failure, and the level of proteinuria was the only predictor of mortality in AA amyloidosis. The number of infections was 3 times higher in AA amyloidosis patients [27]. In addition, it has been reported that anti-TNF treatment is effective for clinical improvement in AA amyloidosis associated with FMF [28] or Crohn’s disease [29]. Taking these findings together, treatment of AA amyloidosis with TNF inhibitors is promising, although anti-TNF therapy does not always lead to the better clinical outcomes or normalize serum levels of SAA in AA amyloidosis patients. For instance, in an open phase I/II trial of IFX, Elliott et al. reported that IFX reduced serum SAA levels in patients with RA from 245 mg/L to 58 mg/L after 1 week of treatment and to 80 mg/L after 2 weeks [30]. In 1999, Charles et al. reported that IFX therapy after 24 weeks decreased SAA levels from 378 mg/L to 56 mg/L, but did not normalize the levels as in the above report [31].

2.2. Anti-IL-1 therapy for AA amyloidosis

IL-1 is a key pro-inflammatory that contributes to pathogenesis of RA. The IL-1 receptor antagonist anakinra (ANA) was thought to be a promising drug for RA, however, it has been reported to be less effective than other biologics for this disease [16]. It has been reported that ANA is effective for Muckle–Wells syndrome caused by a mutation in the gene encoding the protein [32], and we also found several reports that ANA is effective for AA amyloidosis complicating familial cold autoinflammatory syndrome [33], FMF [34], FMA and Behcet’s disease [35], and cryopyrin-associated periodic syndrome (CAPS) [36]. In all cases, ANA normalized serum levels of SAA and dramatically improved proteinuria in renal amyloidosis. Unfortunately, these diseases are very rare, thus use of ANA in AA amyloidosis remains limited.

Authors	Design	Disease	Drugs	Organ dysfunction	Results
Thornto et al. 2007 [33]	Case report	FCAS 1	ANA	Kidney	Proteinuria improved
Moser et al. 2009 [34]	Case report	FMF 1	ANA	Kidney	Good outcome after transplantation
Bilginer et al. 2010 [35]	Case report	FMF and Behçet's disease 1	ANA	Kidney	Proteinuria improved
Ait-Abdesselam et al 2010 [36]	Case report	CAPS 1	ANA	Kidney	Proteinuria improved

Table 4. Anti-IL-1 drugs in the treatment with AA amyloidosis patients

2.3. Anti-IL-6 therapy for AA amyloidosis

IL-6 blocking therapy is effected by tocilizumab (TCZ), which is a humanized anti-IL-6 receptor monoclonal antibody of the IgG1 class. TCZ has been used for the treatment of RA [37], JIA [38], and multicentric Castleman's disease [39]. In the EULAR recommendations 2010 for the management of RA, TCZ was noted for demonstrating efficacy in patients who failed treatment with TNF inhibitors (level of evidence 1B) [16]. In 2003, it was reported that 15 patients with active RA were treated with TCZ biweekly for 6 weeks in an open label phase I/II trial [40]. Serum levels of C-reactive protein and SAA were completely normalized at 6 weeks after TCZ therapy, which identified anti-IL-6 therapy as a promising treatment for AA amyloidosis. In 2006, Okuda et al. first reported successful treatment with TCZ in JIA complicated with AA Amyloidosis [41] in a 26-year-old woman with JIA who initially developed severe intractable diarrhea in 2001, after which AA amyloidosis was confirmed by GI tract and mucosal biopsy. In 2003, the patient presented with proteinuria, and amyloid deposits in the kidneys were confirmed by renal biopsy. At the same time, the patient developed steroid induced glaucoma.

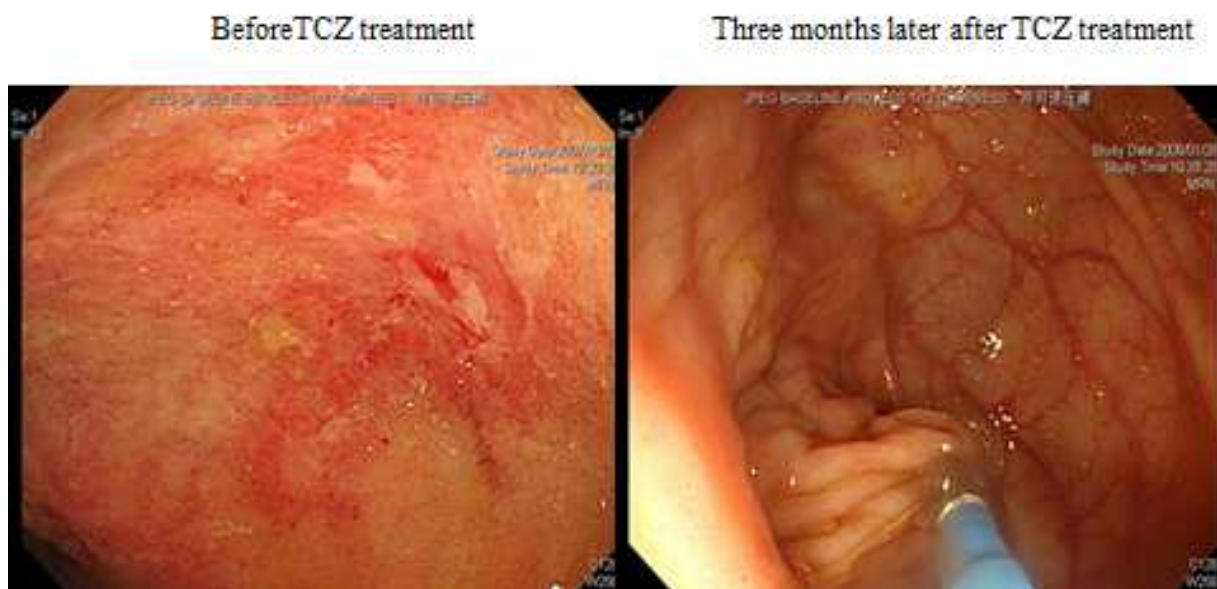


Figure 2. Results of endoscopic examination before and after TCZ therapy. Before TCZ therapy the appearance of the mucosa in the colon was edematous and reddish. After 3 months of TCZ therapy, no abnormality was observed. Quoted from : Nishida S., et al. Ann Rheum Dis. 2009 [49] unpublished data.

In patients who showed severe disease activity despite aggressive treatment with MTX at a dosage of 15 mg/week and prednisolone at a dosage of 10 mg/day, TCZ immediately normalized the serum levels of SAA, from 242.7 $\mu\text{g/mL}$ to 2.49 $\mu\text{g/mL}$ after the first dose. Gastrointestinal symptoms such as diarrhea and abdominal pain disappeared after 1 month and proteinuria improved after 2 months. Moreover, gastrointestinal biopsy specimens showed dramatic regression of AA protein deposits. We also experienced successful treatment of AA amyloidosis with TCZ for a 50-year-old woman with RA, who had failed TNF inhibitor including ETN and IFX, and had developed severe diarrhea and weight loss. AA amyloid de-

posits were confirmed by colon biopsy. TCZ administration immediately normalized serum levels of SAA, stopped the diarrhea, and diminished the disease activity of RA. Notably, 3 months after TCZ treatment, amyloid A protein deposits had completely disappeared (fig.2 and fig.3) [42].

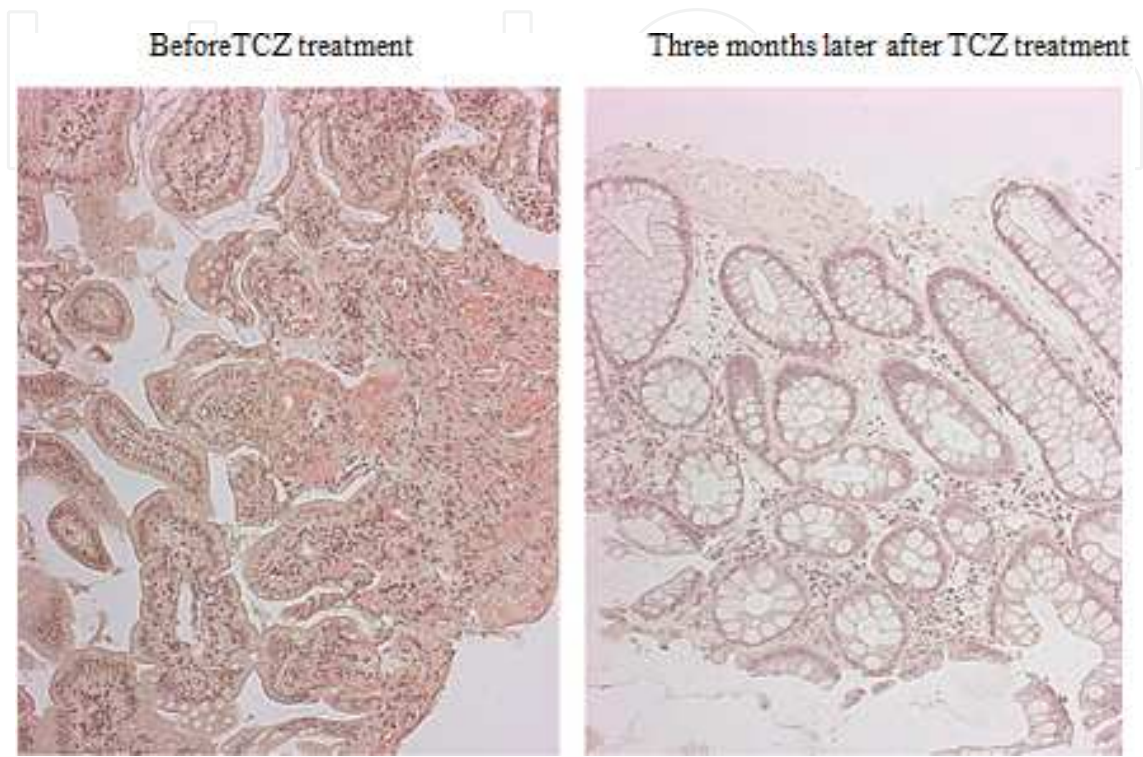


Figure 3. Results of colon biopsy before and after TCZ therapy massive amyloid deposits had disappeared 3 months later after TCZ treatment. Quoted from : Nishida S., et al. Ann Rheum Dis. 2009 [49] unpublished data.

	Design	Disease	Drugs	Organ dysfunction	Results
Okuda et al. 2006 [41]	Case report	JIA 1	TCZ	Kidney GI tract	Proteinuria improved GI symptoms disappeared
Nishida et al. 2009 [42]	Case report	RA 1	ETN,IFX → TCZ	GI tract	Severe diarrhea improved
Sato et al. 2009 [43]	Case report	RA 1	TCZ	GI tract	Severe diarrhea improved
Inoue et al. 2010 [44]	Case report	RA 1	TCZ	GI tract	Paralytic ileus improved
Kishida et al. 2011 [45]	Case report	JIA with HB 1	TCZ	Kidney GI tract	Proteinuria improved Amyloid deposits in GI tract disappeared

Table 5. Anti-IL-6 drugs in the treatment with AA amyloidosis patients

In 2009, Sato et al. reported that TCZ relieved severe diarrhea in AA amyloidosis associated with RA. A 53-year-old woman with RA went into hypovolemic shock because of severe watery diarrhea. AA amyloidosis was confirmed by colon biopsy. A 60 mg dosage of prednisolone therapy and glucocorticoid pulse therapy with 1 g dosage of methylprednisolone did not ameliorate the severe diarrhea. After TCZ administration, the life-threatening diarrhea lessened within about 6 h. However, the patient developed a perforation of the small intestine 2 days after TCZ administration. After successful surgery, administration of TCZ resumed and reduced AA amyloid deposits [43]. In 2010, Inoue et al. reported that TCZ resolved paralytic ileus related to AA amyloidosis of the GI tract associated with RA. After 3 courses of TCZ treatment, a colon biopsy revealed no amyloid deposition [44]. In addition, Kishida et al. reported that TCZ therapy improved proteinuria and amyloid deposits in duodenal mucosa for a patient with adult-onset Still's disease complicated by AA amyloidosis [45]. These dramatic effects of TCZ on AA amyloidosis provide material for bedside-to-bench research and have indicated that TCZ might be more promising in the treatment of AA amyloidosis than anti-TNF or IL-1 inhibitors, although further clinical studies are needed to further evaluate its efficacy and safety, and the question remains as to how TCZ immediately normalizes the SAA levels.

Next, we summarize animal models of pro-inflammatory cytokines associated with AA amyloidosis.

3. AA amyloidosis model mice

AA amyloidosis is the most common form of systemic amyloid disease induced in animals [46]. Mice especially have been used to induce amyloidosis. Several strains (CBA/J, C57B1/6J, C3H/HeJ, BALB/cJ) are susceptible [47]. Animal models are considered pivotal for the study of genetic risk factors. The murine model of AA amyloidosis faithfully reproduces the pathogenesis of its human counterpart. Of the major substances used to induce amyloid in animal species, casein has been preferred [47]. Silver nitrate [48], Freund adjuvant [49], and lipopolysaccharide [50] injection also induce AA amyloidosis. Several drugs have been evaluated using the above animal models. For example, in clinical trials, tenidap treatment reduced levels of CRP and SAA in RA patients [51], and in 1996, Husebekk et al. reported that tenidap inhibited amyloid deposits in an AA amyloidosis model using CBA/J mice induced by complete Freund adjuvant [52]. However, they did not examine the levels of IL-1, TNF- α , and IL-6.

Triptolide isolated from *Tripterygium wilfordii* has anti-inflammatory effects on adjuvant-induced arthritis in rats and on immune cells including T cells, B cells, and monocyte [53, 54]. Cui et al. reported that triptolide inhibited splenic amyloid deposition in both rapid and chronic induction models of AA amyloidosis induced by casein in ICR mice. Triptolide also immediately decreased SAA and IL-6 levels without changes in IL-1 or TNF- α . They suggested that triptolide inhibits experimental murine amyloidosis via suppression of IL-6 [55].

Mihara et al. examined whether the anti-IL-6 receptor antibody MR16-1 inhibited the development of AA-amyloidosis in a transient and chronic mouse model using C57BL/6 mice induced by amyloid enhancing factor (AEF) and complete Freund adjuvant. In the transient model, administration of MR16-1 before the injection of AEF and adjuvant completely prevented amyloid deposition and normalized SAA production. A chronic model was induced by AEF injection into IL-6 transgenic mice. One week later MR16-1 was injected intravenously. MR16-1 decreased amyloid deposition even when injected 1 week after AEF injection, although MR16-1 only partially inhibited SAA and IL-6 levels in IL-6 transgenic mice [56]. Mice that constitutively express the human interleukin 6 (huIL6) proteins from a heritable transgene (H2-Ld-IL-6) begin to develop severe systemic AA amyloidosis. These mice were observed in a hunched posture and moribund state when they were as young as 3 to 5 months of age. The result suggested the possibility that AA amyloidosis is due to genetic rather than environmental factors [57]. In a search, we found no report indicating that the effects of TNF inhibitors in AA Amyloidosis model mice had been examined. IL-1 receptor antagonist partially decreased the mRNA of SAA in C57BL/6 mice using silver nitrate [58]. These studies in mouse models have provided strong evidence to support a pivotal role for IL-6 in the induction of SAA.

Next we describe the molecular mechanisms of the synergistic induction of SAA by IL-1, TNF- α , and IL-6.

4. Molecular mechanisms of serum amyloid A transcription

The former transcription model reported that the SAA2 gene is induced by NF- κ B and CAAT enhancer-binding protein β (C/EBP β) in response to stimulation by IL-1 together with IL-6 [59]. However, this induction model does not fully explain clinical results (fig. 4). Even if IL-6 signal transduction is inhibited, activation of NF- κ B signal still remains. However, anti-IL-6 therapy, but not anti-TNF [20, 21, 25-27] or IL-1 therapy [16], normalized the serum levels of SAA in AA amyloidosis patients [41-45]. It remains unclear whether STAT3, which is the main transcription factor of IL-6 signal transduction, plays a role in the transcriptional activation of the SAA gene. We investigated the exact induction mechanism of SAA by proinflammatory cytokines, and especially focused on the SAA1 gene, which is reported as a main amyloidogenic factor in AA amyloidosis [9].

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

We first established SAA isoforms via real time quantitative RT-PCR assay to examine various combination effects of proinflammatory cytokines. IL-6 and IL-1 or IL-6 and TNF- α induced synergistic expression of the SAA1 gene, but not IL-1 and TNF- α (fig. 5A). We confirmed the above results using each specific inhibitor in a triple stimulation with IL-6, IL-1, and TNF- α . Only Anti-IL-6R monoclonal antibody (Mab) completely inhibited the synergistic induction of both SAA1 and SAA2 mRNA (fig. 5B). We obtained almost the same

results with 3 typical hepatic cell lines, HepG2, Hep3B, and PLC/PRF/5. These results were in good agreement with clinical results of anti-cytokine therapy in inflammatory diseases that indicated that IL-6 plays a pivotal role in the synergistic induction of the SAA1 gene. Next, we sought to identify the signal transduction mechanism to activate the SAA1 gene. IL-6 signal transduction has 2 pathways. One is a MAPK-C/EBP β pathway and the other is a JAK-STAT pathway [62]. The JAK2 inhibitor AG490, but not the MEK1/2 inhibitor U0126, repressed the SAA1 gene expression in response to IL-1 β + IL-6 (fig. 5C) [60]. These data suggested that the JAK-STAT pathway plays an important role in SAA gene induction.

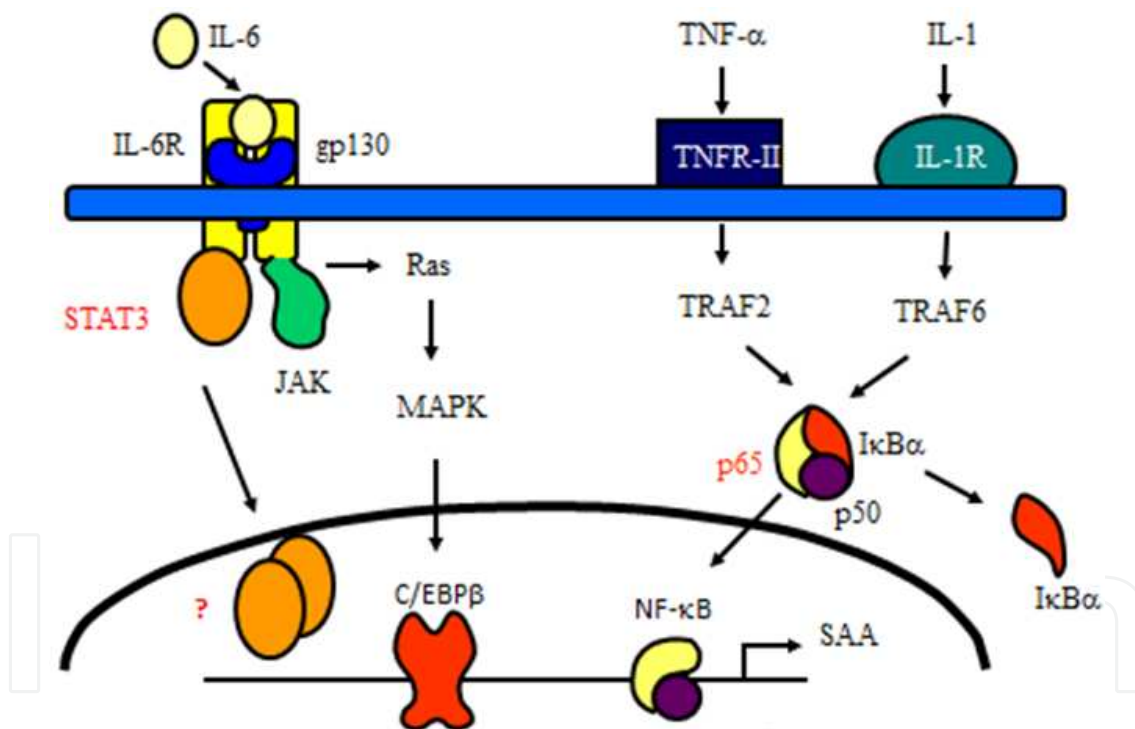


Figure 4. Former transcription model of a human SAA gene expression induced by proinflammatory cytokines; Betts et al. J. Biol Chem. 1993 [59]

Figure 5

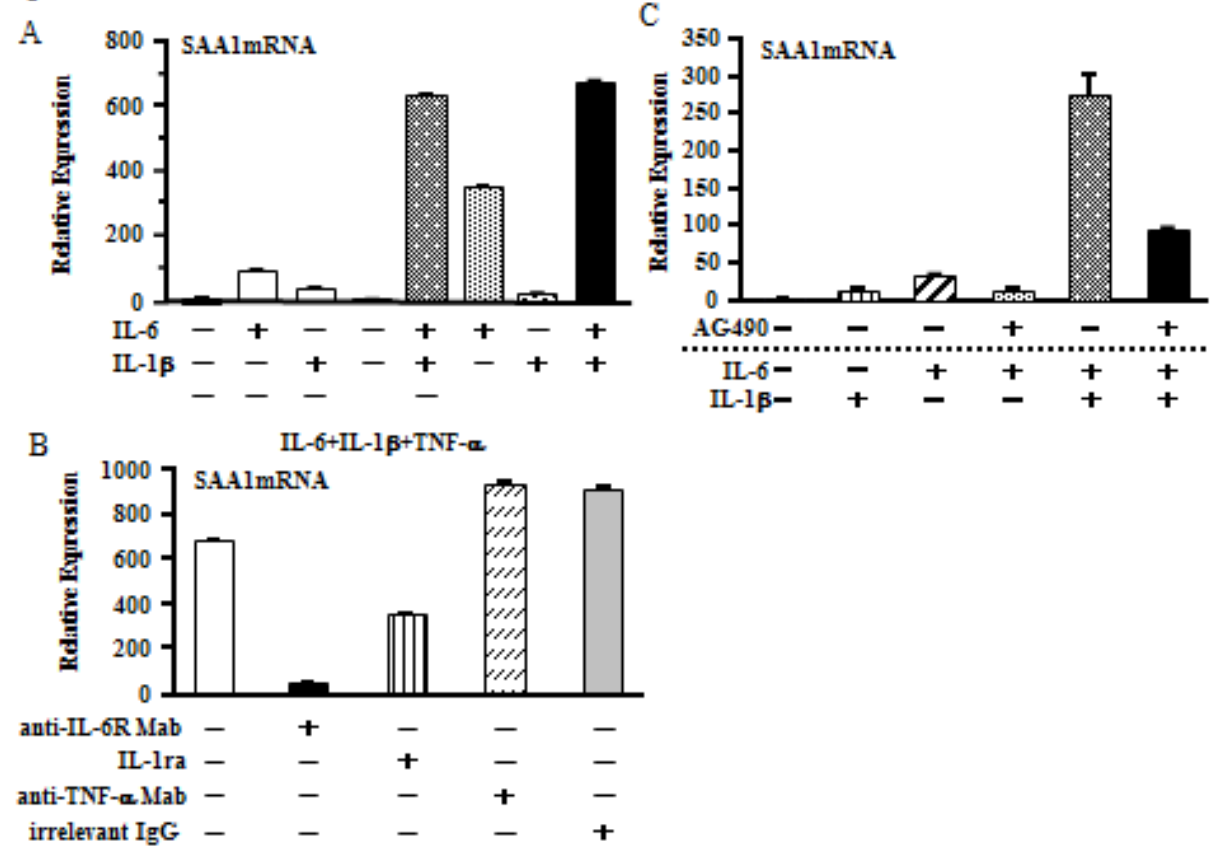


Figure 5. A) Combined effects of IL-6 (10 ng/mL), IL-1β (0.1 ng/mL), and/or TNF-α (10 ng/mL) on the induction of SAA 1. (B) Inhibitory effects of anti-IL-6R monoclonal antibody (Mab) (25 μg/μL), IL-1 receptor antagonist (Ra) (100 ng/mL), and anti-TNF-α Mab (4 μg/μL) on the synergistic induction of SAA1 generated by triple stimulation of IL-6, IL-1β, and TNF-α. Each specific reagent was incubated with HepG2 cells for 30 min prior to cytokine stimulation. (C) Effects of Jak2 kinase inhibitor-AG490 (100 μM) on synergistic induction of SAA1 mRNA. HepG2 cells were treated with AG490 or DMSO alone for 30 min before cytokine stimulation. SAA1 mRNA levels in HepG2 cells were measured by real-time quantitative RT-PCR at 6 h after cytokine stimulation. Values are reported as mean (SD) of duplicate measurements.

4.2. Essential role of STAT3 for transcriptional activity of the SAA1 gene via the NF-κB RE-containing region after formation of a complex with NF-κB p65.

STAT3 binds to a γ-interferon activation sequence (GAS) such as sequence (-TTNNNGAA), and C-reactive protein (CRP), the acute-phase protein that is active in the response to IL-6, has a STAT3 response element (RE) (-TTCCCGAA) in its promoter [61]. However, the typical STAT3 RE was not found in the human SAA1 promoter. To examine the effect of STAT3 on SAA1 promoter activity, pEF-BOS dominant negative STAT3 Y705F (dn STAT3) or pEF-BOS wild type STAT3 (wt STAT3) HepG2 cells were co-transfected with pGL3-SAA1 promoter luciferase construct (-796/+24) (pGL3-SAA1) [63]. The co-expression of dn STAT3 completely inhibited pGL3-STAT1 expression, whereas the co-expression of wt STAT3 enhanced the transcriptional activity stimulated with IL-1β + IL-6 (fig. 6A). These results indi-

cated that STAT3 plays a critical role in the transcriptional activity of the SAA1 gene. We examined the possibility that STAT3 might act on the transcriptional activity of SAA through the C/EBP β and NF- κ B RE-containing region.

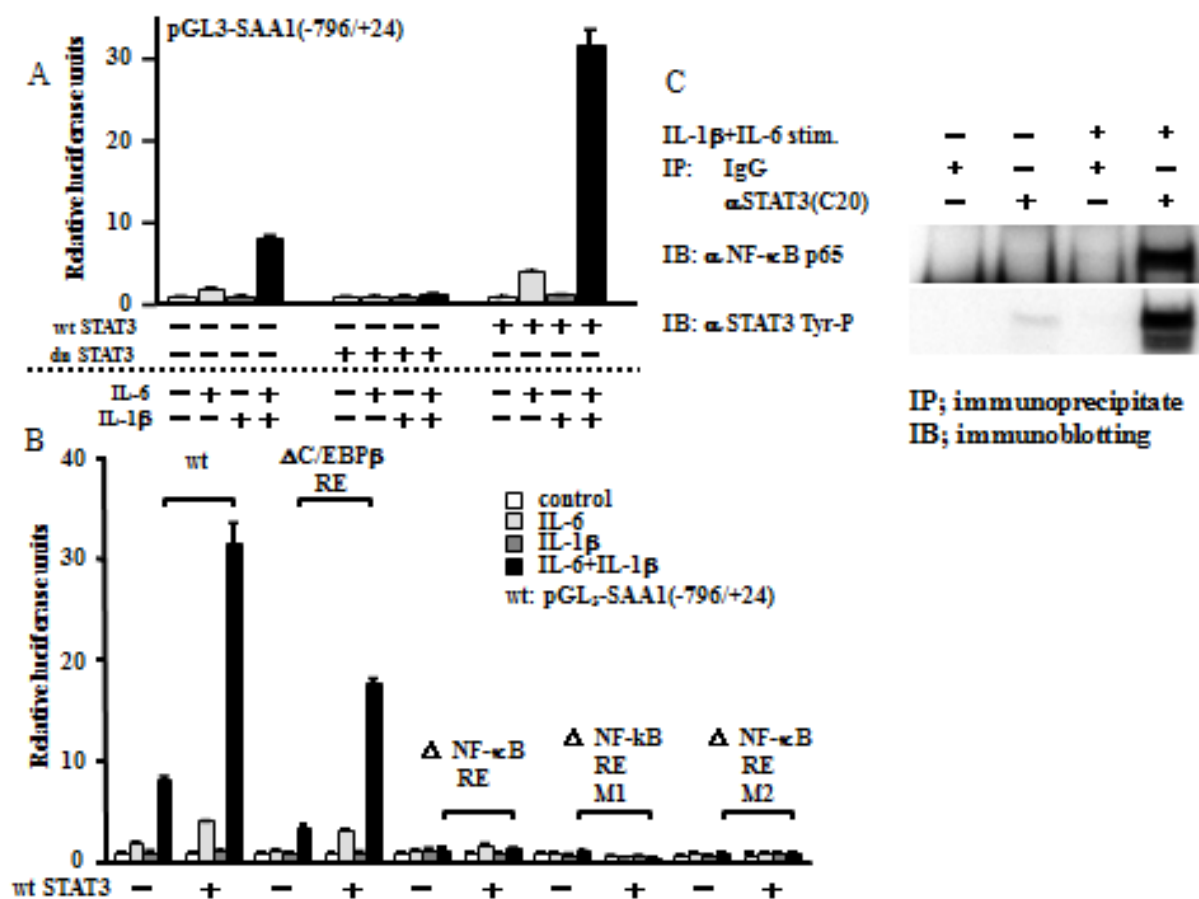


Figure 6. A) HepG2 cells were transfected with 0.5 μ g of pGL3-SAA1 (-796/+24) alone, or co-transfected with 0.5 μ g of pEF-BOS dominant negative STAT3 (dn STAT3) or pEF-BOS wild type STAT3 (wt STAT3), respectively. (B) 0.5 μ g of wt STAT3 was co-transfected with 0.5 μ g of pGL3-SAA1(-796/+24), pGL3-SAA1 Δ C/EBP β RE, pGL3-SAA1 Δ NF- κ B RE, pGL3-SAA1 NF- κ B RE M1 (AGATCTATTTC), or M2 (CAGGGACTTGTAC). Cytokine stimulation was performed with IL-6 and/or IL-1 β for 3 h. The relative luciferase activity is expressed as mean (SD) of triplicate cultures and transfections. (C) Nuclear extracts of HepG2 cells stimulated with IL-1 and IL-6 were immunoprecipitated with the anti-STAT3 C-20 antibody. Western blots were performed as shown. IP: immunoprecipitate, IB: immunoblotting.

In our experimental results, the transcriptional activity of the SAA1 gene was partly decreased by deletion of CEBP- β RE and completely diminished by deletion of NF- κ B RE with co-expression of wt STAT3. These results suggest that STAT3 is involved in the transcriptional activity of SAA, most likely through NF- κ B RE (fig. 6B). Competitive binding of STAT3 and NF- κ B has been found in a rat γ -fibrinogen gene promoter that included a CTGGGAATCCC sequence [64]. It was reported that TCC was important for NF- κ B binding and that CTGGGAA was necessary for STAT3 binding. Based on these reports, we created 2 mutant constructs, pGL3-SAA1 NF- κ B RE M1 (AGATCTATTTC) and M2 (CAGGGACTTGTAC). We expected that STAT3 would bind to NF- κ B RE M2 but not to NF- κ B RE

M1. However, neither transfection with NF- κ B RE M1 nor M2 resulted in transcriptional activity, even when WT STAT3 was co-expressed (fig. 6B). We hypothesized that STAT3 forms a complex with NF- κ B and augments the transcriptional activity of the human SAA gene. To examine our hypothesis, we performed IP-western blot for STAT3 and NF- κ B. Figure 6C clearly shows that STAT3 is associated with NF- κ B p65 following IL-1 β + IL-6 treatment [63]. However, no specific band of NF- κ B p50 was detected. These findings were consistent with those reported by Betts et al. that overexpression of NF- κ B p65 but not p50 enhanced the transcriptional activity of human SAA2 in a dose-dependent manner [59], and demonstrating that crosstalk between STAT3 and NF- κ B p65 contributes to the transcriptional augmentation of SAA by IL-1 β + IL-6 stimulation.

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

Next, we investigated how STAT3 contributes to the formation of the transcriptional complex comprising NF- κ B, C/EBP β , and STAT3. STAT3 is reportedly associated with p300 [65], which indicates the possibility that heteromeric complex formation of STAT3, NF- κ B p65, and p300 is involved in the transcriptional activity of the human SAA gene. To examine this possibility, we performed a chromatin immunoprecipitation (Ch-IP) assay using chromatin isolated from HepG2 cells. STAT3 and p300 were clearly recruited to the SAA1 promoter region (-226/+24) in response to IL-6 or IL-1 β + IL-6, and weakly recruited by IL-1 β . NF- κ B p65 was recruited by IL-1 or IL-1 β + IL-6 and weakly recruited by IL-6 [63] (fig. 7A). When we performed a luciferase assay using pGL3-SAA1 (-226/+24) co-transfected with p300 wt in pCMV β (wt p300) and wt STAT3, we found 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. 7B). These results suggest that STAT3 interacts with p300 in the transcriptional activity of the human SAA gene by forming a transcriptional complex with NF- κ B p65 and p300 on the SAA promoter region. However, it still remains to be determined how STAT3 binds to the promoter region of the SAA1 gene, because no typical STAT3 RE has been located. To address this, we performed DNA affinity chromatography using a wt SAA1 probe. We hypothesized that the formation of a STAT3-NF- κ B p65 complex might confer STAT3 binding affinity to the SAA1 promoter. From our result and study of rat γ -fibrinogen, we focused our attention on the 3' site of NF- κ B RE (CAGGGACTTCCCCAGGGAC) as a candidate STAT3 binding site because the sequence contiguous to the NF- κ B RE might have influenced the binding affinity of STAT3. To test this hypothesis, we created SAA1 mt NF- κ B RE M3 (CAGGGACTTCCCCAGATCTA). As expected, the specific bands of STAT3 from the nuclear extracts of HepG2 cells after IL-1 β + IL-6 stimulation were decreased by the SAA1 mt NF- κ B RE M3 compared to the wt SAA1 probe, although the specific bands of NF- κ B p65 were found almost intact, as with the wt SAA1 (fig. 7C). We were able to demonstrate that STAT3 acts on the human SAA promoter via a newly discovered *cis*-acting mechanism, namely, the formation of a heteromeric complex containing STAT3, NF- κ B 65, and p300.

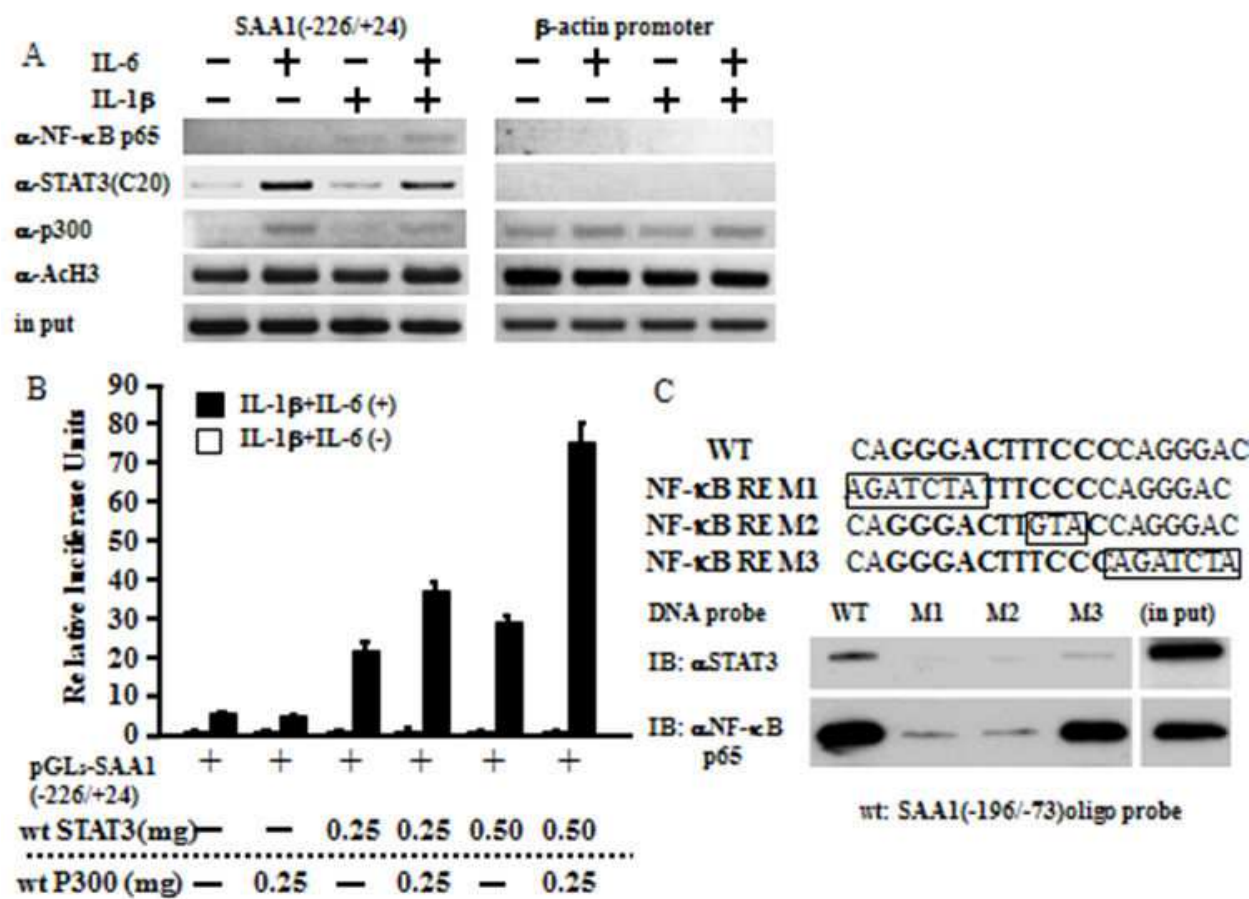


Figure 7. A) ChIP assays demonstrate recruitment patterns of STAT3, NF- κ B p65 and p300 on the SAA1 promoter (-226/+24) from HepG2 cells treated with IL-6 and/or IL-1 β for 30 min. Anti-AcH3 antibody was used as a positive control for this assay. (B) HepG2 cells were transfected with pGL3-SAA1 (-226/+24) (0.5 μ g), 0.25 μ g of p300 wild type in pCMV β (wt p300), and/or 0.25–0.5 μ g of wt STAT3. IL-1 and IL-6 stimulation was performed for 3 h. Relative luciferase activity is expressed as the mean (SD) of triplicate cultures and transfections. (C) DNA affinity chromatography was performed with 200 μ g of the nuclear extracts from HepG2 cells after cytokine stimulation. The nuclear extracts were mixed with 1 μ g of biotinylated DNA probe and 50 μ l of streptavidin-Dynabeads was added to the samples, mixed, and collected with a magnet. The trapped proteins were analyzed by western blotting.

We have formulated a schematic model to describe the synergistic induction of the human SAA gene by IL-1 β , TNF- α , and IL-6 stimulation (fig. 8A). This model explains the effect of anti-cytokine therapy on the transactivation of SAA. Anti-TNF α or anti-IL-1 therapy reduced NF- κ B signaling pathways but they were not eliminated, because the NF- κ B signaling pathway is activated by various stimulations, including toll-like receptors and other cytokines [66]. Consequently, the transcriptional complex on the SAA promoter remained after Anti-TNF α or IL-1 therapy (fig. 8B). On the other hand, IL-6 family cytokines, excluding IL-6 but including oncostatin M, IL-11, and LIF, have little influence on the production of acute phase proteins [68]. IL-6 blocking therapy inhibits the activation of STAT3 and C/EBP β , and prevents the formation of the transcriptional complex on the SAA promoter (fig. 8C). It is well known anti-TNF- α decreases serum levels of IL-6 [67], which indicates that the effect of anti-TNF- α therapy represses the serum levels of SAA by decreasing IL-6.

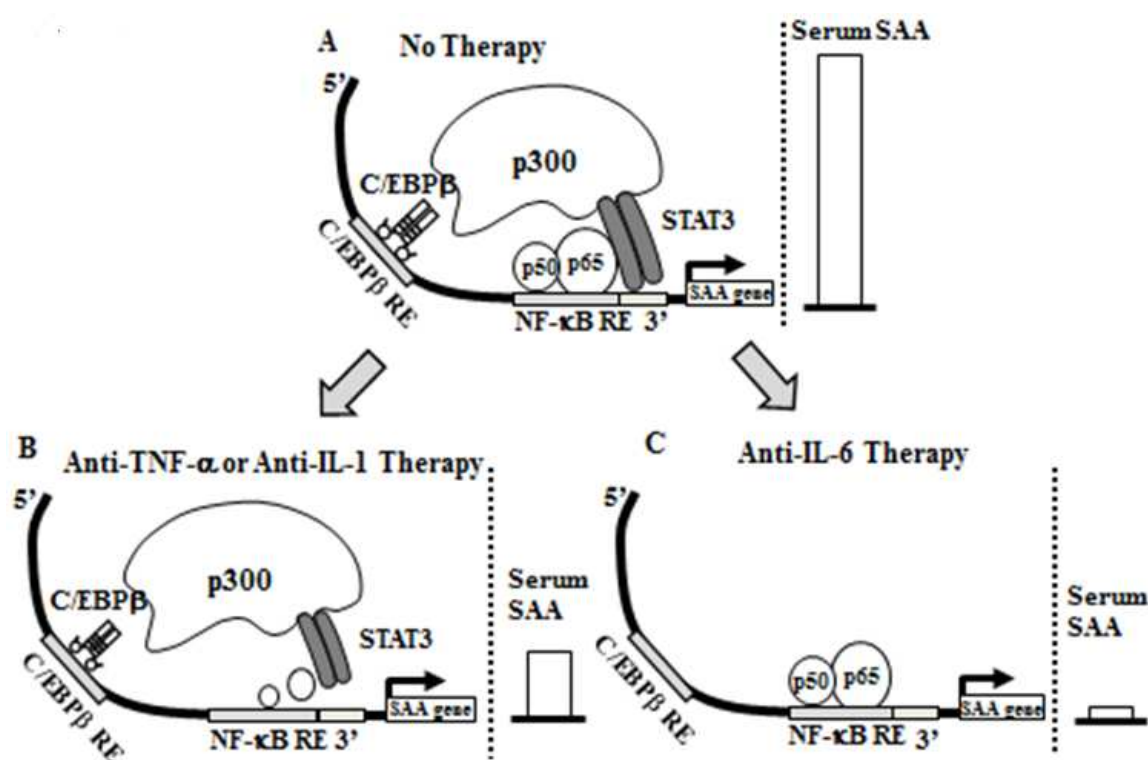


Figure 8. Effects of anti-cytokine therapy on the cytokine-driven transcriptional activity of human SAA gene. (A) Cytokine stimulation caused the formation around NF-κB RE of a heteromeric complex with STAT3 and NF-κB p65. STAT3, which is assumed to interact with the 3'-site of NF-κB RE, recruits the co-activator p300, which then coordinates the interaction of NF-κB p65, STAT3, and C/EBPβ, thus resulting in the augmentation of transcriptional activity of human SAA gene. (B) anti-TNFα or IL-1 therapy reduces the activity of NF-κB signaling pathway, however, transcriptional complexes are still remained. (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.

5. Conclusion

Patients with AA amyloidosis whose SAA concentrations remain in the low-normal range [14, 15] have been reported to have a better prognosis. From the clinical point of view, our findings indicate that anti-IL-6 therapy is the most rational and promising therapy for AA amyloidosis patients via normalization of SAA level. In addition, our study is a new clinical research approach 'from bedside to bench' and directly leads to a better understanding of the pathogenesis of several inflammatory diseases.

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