EXTENDED REPORT

Alpha-1-anti-trypsin-Fc fusion protein ameliorates gouty arthritis by reducing release and extracellular processing of IL-1β and by the induction of endogenous IL-1Ra

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ABSTRACT

Objectives In the present study, we generated a new protein, recombinant human alpha-1-anti-trypsin (AAT)-IgG1 Fc fusion protein (AAT-Fc), and evaluated its properties to suppress inflammation and interleukin (IL)-1β in a mouse model of gouty arthritis.

Methods A combination of monosodium urate (MSU) crystals and the fatty acid C16.0 (MSU/C16.0) was injected intra-articularly into the knee to induce gouty arthritis. Joint swelling, synovial cytokine production and histopathology were determined after 4 h. AAT-Fc was evaluated for inhibition of MSU/C16.0-induced IL-1β release from human blood monocytes and for inhibition of extracellular IL-1β precursor processing.

Results AAT-Fc markedly suppressed MSU/C16.0-induced joint inflammation by 85–91% ($p<0.001$). Ex vivo production of IL-1β and IL-6 from cultured synovia were similarly reduced (63% and 65%, respectively). The efficacy of 2.0 mg/kg AAT-Fc in reducing inflammation was comparable to 80 mg/kg of plasma-derived AAT. Injection of AAT-Fc into mice increased circulating levels of endogenous IL-1 receptor antagonist by fourfold. We also observed that joint swelling was reduced by 80%, cellular infiltration by 95% and synovial production of IL-1β by 60% in transgenic mice expressing low levels of human AAT. In vitro, AAT-Fc reduced MSU/C16.0-induced release of IL-1β from human blood monocytes and inhibited proteinase-3-mediated extracellular processing of the IL-1β precursor into active IL-1β.

Conclusions A single low dose of AAT-Fc is highly effective in reducing joint inflammation in this model of acute gouty arthritis. Considering the long-term safety of plasma-derived AAT use in humans, subcutaneous AAT-Fc emerges as a promising therapy for gout attacks.

INTRODUCTION

Several studies demonstrate that the inactive interleukin (IL)-1β precursor can be processed into an active cytokine independently of caspase-1 (reviewed in refs. 1–3). The proteinases that that are responsible for such caspase-1-independent conversion of IL-1β are primarily found in neutrophils, which are the main inflammatory cells in gouty arthritis. The neutrophil serine proteases proteinase-3 (PR3), neutrophil elastase and cathepsin G cleave the precursor within a few amino acids of the caspase-1 site at N-terminal 117.[1] Dipeptidyl peptidase I-deficient mice are unable to activate neutrophil serin proteases. However, treatment with a specific caspase-1 inhibitor provides protection against IL-1β-mediated cartilage damage caused by chronic destructive joint inflammation.[2] These data support the concept that targeting IL-1β release by dual inhibition of caspase-1 and serine proteinases would be of therapeutic value in neutrophil-rich inflammation.

The major natural inhibitor of serine proteases is alpha-1-anti-trypsin (AAT). Serum levels in healthy subjects range from 1 to 3 mg/mL, but rise during infections and inflammatory diseases. In addition to the inhibition of serine proteinases, AAT possesses a broad spectrum of anti-inflammatory and immunomodulatory properties[4 5] independent of protease inhibition.[6] AAT inhibits caspase-1, caspase-3 and caspase-8; downregulates toll-like receptor (TLR)2 and TLR4 expression on immune cells; and is incorporated in lipid rafts, which inhibits cell activation and increases the expression of angiopoietin-like protein-4.[7–11] In addition, peripheral blood mononuclear cells (PBMCs) from human subjects deficient for AAT produce higher levels of inflammatory cytokines when stimulated ex vivo.[12]

In the present study, we developed AAT-Fc, a new recombinant form of human AAT fused with the Fc domain of human IgG1, and evaluated its ability to suppress gouty arthritis, a classic IL-1β-mediated disease.[13–15] Based on several trials, IL-1β is clearly the causal cytokine in acute gout attacks. Reduced clinical manifestations and number of attacks have been demonstrated for the IL-1 receptor antagonist (IL-1Ra) anakinra, the IL-1 trap rilonacept and the human monoclonal anti-IL-1β canakinumab. In the present study, experimental gouty arthritis was elicited by intra-articular instillation of monosodium urate (MSU) crystals mixed with palmitic acid (MSU/C16.0). Following the intra-articular injection, there is joint swelling, influx of inflammatory cells and synovial cell cytokine production. We compared the effects of plasma-derived AAT to AAT-Fc on joint swelling, release of MSU/C16.0-induced IL-1β from human blood monocytes, PR3-dependent processing of the IL-1β precursor and the in vitro as well as in

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vivo induction of IL-1Ra. We also studied gouty arthritis in transgenic mice expressing human AAT. We conclude that AAT-Fc could be a safe and novel therapy for the treatment of MSU-mediated inflammation.

MATERIALS AND METHODS[i]

Animals

Male C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Human AAT transgenic mice (AAT-tg) were a kind gift from A. Churg.[16] Mice (either sex) were used at 10–12 weeks.

MSU/C16.0-induced gouty arthritis

Joint inflammation was induced by intra-articular injection of 300 µg MSU crystals mixed with 200 µM C16.0/bovine serum albumin (BSA) in 10 µL phosphate buffered saline (PBS) into the right knee joint of naive mice. Four hours after intra-articular injection, macroscopic joint swelling was determined. Synovial tissue was isolated and either cultured for 2 h in tissue culture medium at 37°C or transferred directly into 200 µL Triton×100 (0.5% in PBS). In addition, knee joints were removed for histology. The MSU-induced peritonitis model (3 mg MSU intraperitoneally) was used to validate AAT-Fc treatment of the gouty arthritis model.

AAT treatment of gouty arthritis

Mice were injected intraperitoneally with 50 µg AAT-Fc fusion protein 2, 24, 48 or 72 h prior to induction of gouty arthritis. Plasma-derived AAT (pd-AAT) (Prolastin C, Grifols, Barcelona, Spain) was purchased from the University of Colorado Pharmacy. Recombinant IL-1Ra (anakinra, SOBI, Stockholm, Sweden) was injected intraperitoneally 2 h before gouty arthritis induction. IvIg (Gamunex, Grifols) or BSA (Sigma-Aldrich, St-Louis, USA) was used as negative control in doses ranging from 50 µg to 2 mg.

RESULTS

Intra-articular versus systemic treatment of gouty arthritis with human AAT

To explore the anti-inflammatory efficacy of AAT in gouty arthritis, we investigated whether human AAT suppresses acute joint inflammation elicited by MSU/C16.0 when administered locally or systemically. As shown in figure 1A, B, an intra-articular dose of 2 µg AAT-Fc alone did not induce joint inflammation or cell influx into the joint cavity. Next, we injected MSU/C16.0 intra-articular together with 2 µg AAT-Fc and observed a marked suppression of joint inflammation (figure 1A, B). Local IL-1β and IL-6 production from synovial tissue extracts was also decreased (see online supplementary figure S1). We next investigated the ability of systemically administered AAT to suppress MSU/C16.0-induced intra-articular inflammation. Human plasma-derived AAT was administered intraperitoneally at a dose of 2 mg per mouse (80 mg/kg), which is comparable to the dose infused into humans treated weekly for AAT deficiency. Two hours later, MSU/C16.0 was injected intra-articular. After an additional 4 h, the joints were examined and the degree of inflammation was scored. As shown in figure 1C, there was significant reduction in joint inflammation. Consistent with these findings, the number of inflammatory cells in the joint cavity was also reduced (figure 1D). In addition, both IL-1β and KC (murine homolog of IL-8) levels were decreased in AAT-treated mice compared with the vehicle control group (figure 1E, F).

Comparison of plasma-derived AAT and AAT-Fc fusion protein

We next compared plasma-derived AAT (Prolastin C) with recombinant AAT-Fc. Mice were given an intraperitoneal injection of plasma-derived AAT (2 mg) or AAT-Fc (50 µg). After

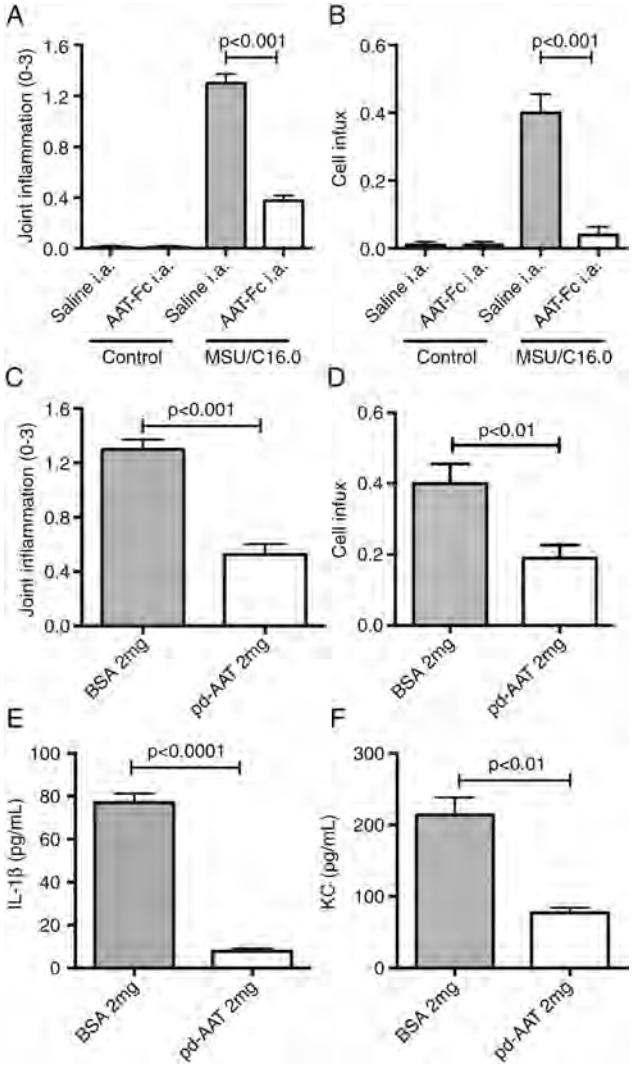

Figure 1 Intra-articularly (i.a.) application of AAT-Fc versus plasma derived AAT. (A) C56/Bl6 mice were i.a. injected with 6 µl of either saline, AAT-Fc (2 µg), MSU/C16.0 (300 µg/200 µm) or MSU/C16.0 (300 µg/200 µm) plus AAT-Fc (2 µg). After 4 h, joint inflammation was scored macroscopically after the skin was removed. 5 mice per group, 10 joints were injected, right and left. (B) The number of inflammatory cells in the joint cavity and synovial lining were determined. 10 joints per groups were scored. (C) Systemic treatment of plasma derived (pd) AAT. Mice were pre-treated for 1 h with either 2 µg BSA or 2 µg pd-AAT. Thereafter gouty arthritis was induced by i.a. injection of MSU/C16.0 (300 µg/200 µm). Joint inflammation was determined by macroscopic scoring. N=5 mice per 10 joints were injected. (D) Cell influx. (E,F) IL-1β and KC release of synovial tissue explants. Synovial tissue specimens were cultured for 2 h in RPMI. Murine IL-1β and KC were determined by ELISA. Data are expressed as mean ± SD. Mann-Whitney U-test was used for statistical analysis. The experiment was repeated once with similar results.

[i]A detailed 'Materials and methods' version is provided in the online supplementary material.

2 h, all mice received an intra-articular injection of MSU/C16.0. Similar to the data shown in figure 1C, 2 mg plasma-derived AAT significantly reduced joint inflammation (figure 2A, p<0.001). However, AAT-Fc at a dose of 50 μg (40 times less) resulted in a similar reduction (p<0.001). In contrast, 50 μg plasma-derived AAT had no effect (figure 2A). The reduction in synovial extract IL-1β (figure 2B) and IL-6 levels (figure 2C) was comparable between mice treated with 2 mg plasma-derived AAT and mice treated with 50 μg AAT-Fc. Gouty arthritis attacks respond rapidly to treatment with the IL-1 receptor blocker anakinra.[17–19] Therefore, we also compared AAT-Fc treatment to treatment with anakinra. We used a high dose of 10 mg anakinra (400 mg/kg) in order to achieve total IL-1 receptor blockade. As depicted in figure 2D, 50 μg AAT-Fc reduced joint inflammation to a greater extent than total IL-1R blockade by anakinra. The reductions of synovial tissue levels of IL-1β (figure 2E) and IL-6 (figure 2F) were comparable for both treatments. To corroborate the above findings of AAT-Fc in a different MSU crystal-induced inflammation model, we applied AAT-Fc treatment on the MSU crystal-induced peritonitis model. Online supplementary figure S2A shows that AAT-Fc treatment had a similar suppressive effect as seen in the gouty arthritis model. Interestingly, again the anti-inflammatory effects of AAT-Fc treatment are comparable to high-dose IL-1Ra treatment (see online supplementary figure S2B).

Prolonged protection of AAT-Fc fusion protein in gouty arthritis

A single injection of 50 μg AAT-Fc 2 h before the intra-articular MSU/C16.0 injection results in a marked

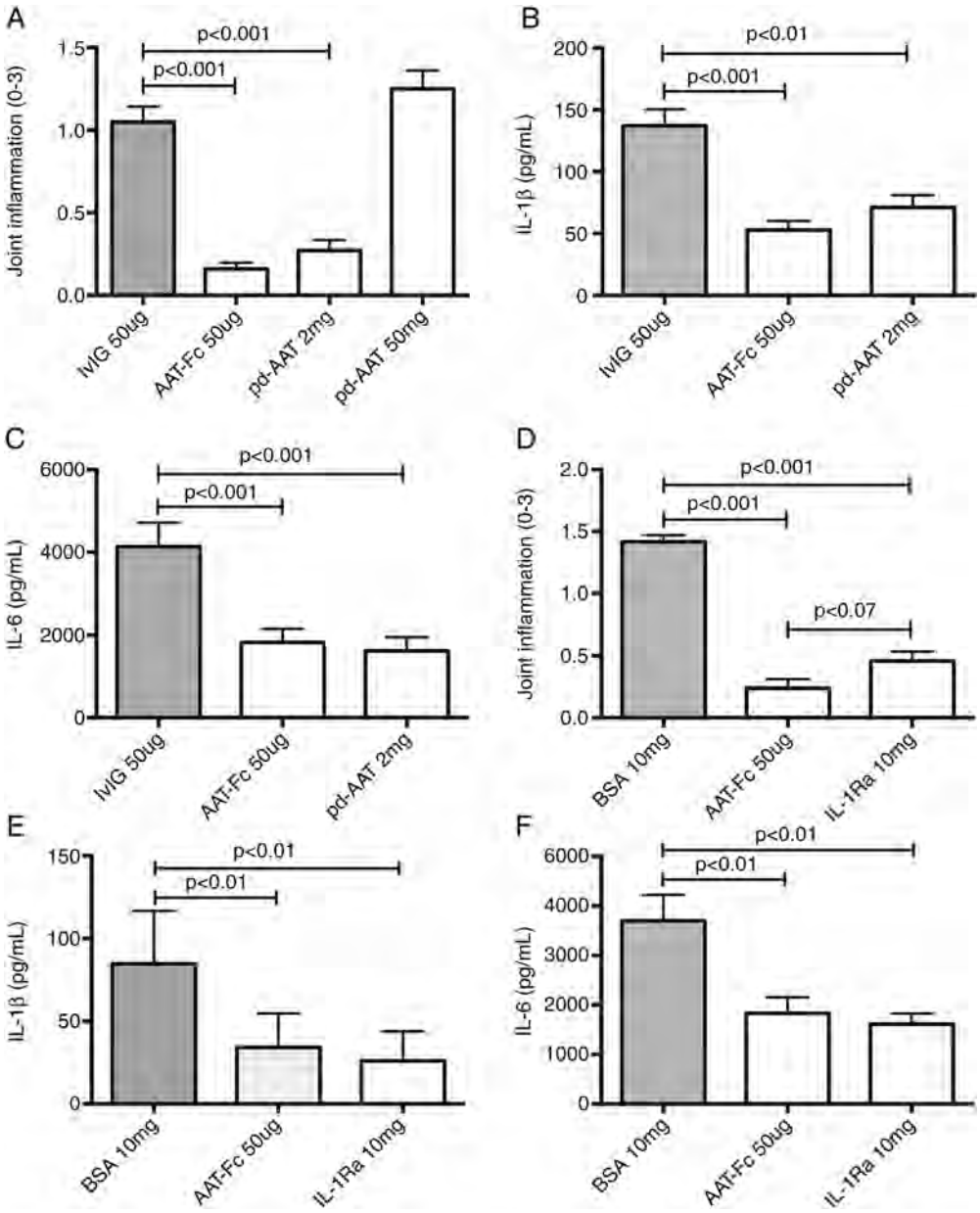

Figure 2 Systemic administration of AAT-Fc, pd-AAT and Anakinra (IL-1ra). Mice were injected intraperitoneally with either 50 μg IvIg, 50 μg AAT-Fc, 50 μg or 2 μg pd-AAT 1 h before gouty arthritis was induced by i.a. injection of MSU/C16.0 (300 μg/200 μm). (A, D) Joint inflammation determined at 4 h after induction of gouty arthritis. N=5 mice per group, 10 knee joints were examined. (B, C, E, F) Synovial tissue specimens were examined for IL-1β and IL-6 production. After isolation the explants were transferred into 200 ml Triton X (0.05% in PBS). Cytokines were measured by ELISA. N=5 explants per group. Data are expressed as mean ± SD. Mann-Whitney U-test was used for statistical analysis. These experiments were repeated once with similar results.

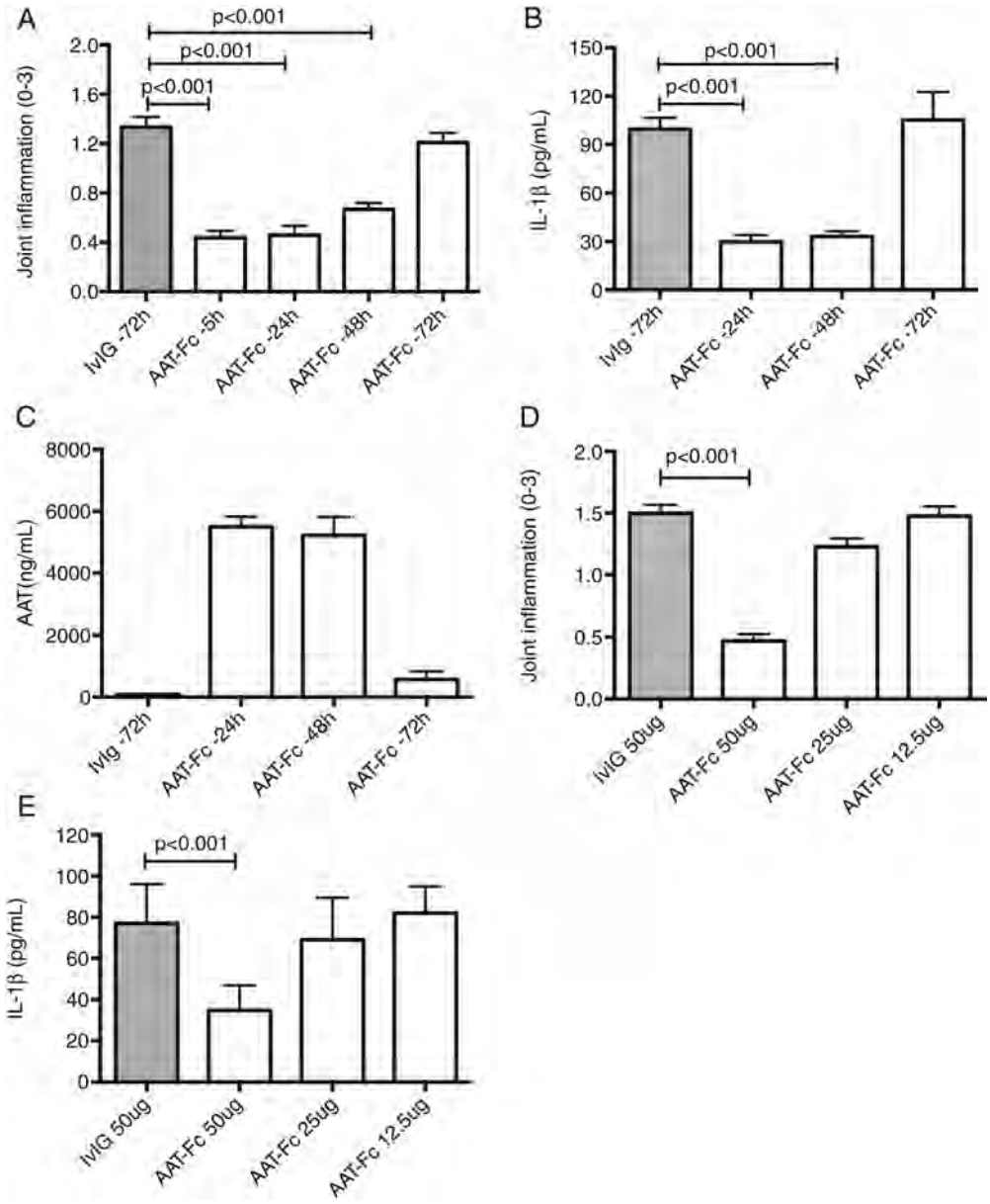

Figure 3 Time and dose dependent suppression of gouty arthritis by AAT-Fc. (A) C57/Bl6 mice were injected with 50 µg of AAT-Fc or 50 µg IvIg at several time points before induction of gouty arthritis. Joint swelling was scored 4 h after injection of MSU/C16.0. (B) IL-1β levels in synovial tissue specimens. (C) Serum human AAT-Fc levels at different time points after i.p. injection of AAT-Fc. (D) Dose-response of AAT-Fc. Mice were injected with of AAT-Fc or IvIg 1 h before induction of gouty arthritis. (E) Synovial tissue specimens were examined for IL-1β. For details see figures 1 and 2. Data are expressed as mean ± SD. Mann-Whitney U-test was used for statistical analysis. These experiments were repeated once with similar results.

reduction in joint inflammation as well as cytokine production. AAT-Fc is comprised of two molecules of AAT (MW=55 kDa) plus the Fc domain of IgG (MW=172 kDa). Therefore, AAT-Fc blood levels should be more constant compared with the monomeric plasma-derived AAT. For that reason, we administered 50 µg AAT-Fc 72 h prior to intra-articular MSU/C16.0 injection. As shown in figure 3A, AAT-Fc given intraperitoneally 72 h before MSU/C16.0 was not effective. However, when 50 µg AAT-Fc was injected 48 h or 24 h prior to induction of gouty arthritis, the reduction in joint inflammation was comparable to that observed when AAT-Fc was injected 2 h prior to MSU/C16.0 (figure 3A). The reduction in joint inflammation was consistent with lower levels of IL-1β released from the synovial specimens when AAT-Fc was administered either 48 or 24 h before MSU/C16.0 (figure 3B). We also measured the concentrations of human AAT in the circulation in mice that received AAT-Fc at different time points. Figure 3C shows that the concentration of AAT-Fc declined after 72 h, which is consistent with the lack of efficacy of AAT-Fc at that time point.

Dose–response of AAT-Fc

C57Bl/6 mice were injected systemically with 50, 25 or 12.5 µg AAT-Fc 2 h prior to the induction of gouty arthritis. Figure 3D reveals a clear dose-dependent effect. A dose of 50 µg AAT-Fc provided optimal anti-inflammatory activity, which decreases progressively at 25 or 12.5 µg AAT-Fc. Again, the dose-dependent reduction in joint inflammation (figure 3D) was paralleled by a decrease in local IL-1β production (figure 3E). Histological analysis revealed that 50 µg AAT-Fc decreased the

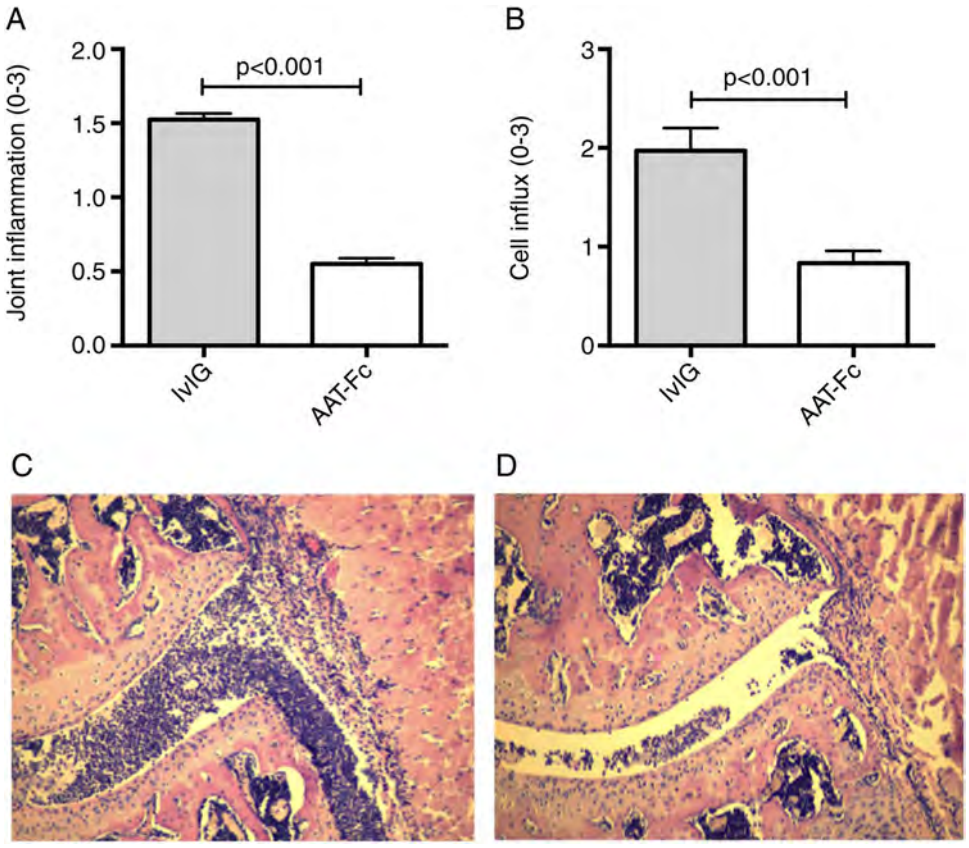

Figure 4 AAT-Fc suppresses joint inflammation. (A) Joint inflammation determined at 4 h after induction of gouty arthritis. N=5 mice per group, 10 knee joints were examined. Mice were treated with 50 μg of AAT-Fc or 50 μg IvIg, 1 h before induction of gouty arthritis. (B) Cell influx. The number of inflammatory cells in the joint cavity, scored on a scale ranging from 0 to 3. 10 joints per group were examined. Data are expressed as mean ± SD. Mann-Whitney U-test was used for statistical analysis. These experiments were repeated once with similar results. (C) Histopathology of an inflamed knee joint of an IvIg treated mouse, 4 h after induction of gouty arthritis. Note the severe infiltration of cells in the joint cavity. (D) AAT-Fc treated mouse. H&E staining, original magnification.

influx of inflammatory cells into the joint cavity of mice with gouty arthritis (figure 4B–D), whereas no reduction in cell influx was observed in mice injected with doses of 25 μg or 12.5 μg AAT-Fc (data not shown). Figure 4A shows the reduction in joint swelling with the 50 μg dose from figure 3D.

AAT-Fc inhibits PR3-mediated processing of the IL-1β precursor into mature bioactive IL-1β

Serine proteinases, such as PR3, process the IL-1β precursor extracellularly into mature, active IL-1β.[20] Since plasma-derived AAT inhibits the enzymatic activity of PR3, we investigated whether recombinant AAT-Fc was able to inhibit PR3-mediated cleavage of the precursor into active IL-1β. A preparation of human IL-1β precursor[21] was incubated with PR3. After 20 min, the reaction mixture was added to the IL-1-responsive human A549 lung epithelial cell line to determine IL-1 bioactivity using the induction of IL-6 as a readout. As shown in figure 5A, the IL-1β precursor at 100 ng/mL did not induce IL-6 in the presence of either plasma-derived AAT or AAT-Fc. In contrast, the precursor incubated with PR3 induced a fivefold increase in IL-6 (figure 5A, right). Increasing concentrations of either plasma-derived AAT or AAT-Fc reduced the induction of IL-6. Either plasma-derived AAT or AAT-Fc alone without the IL-1β precursor had no effect on IL-6 production. The concentration of AAT-Fc that inhibit the generation of active IL-1β by PR3 was comparable to that of plasma-derived AAT (figure 5A, right).

AAT-Fc fusion protein inhibits IL-1β release from human CD14+ monocytes

To study the direct effect of AAT-Fc on the IL-1β release, we exposed freshly isolated $CD14^+$ human blood monocytes to AAT-Fc, followed by MSU/C16.0 stimulation. Figure 5B shows that $CD14^+$ monocytes release high levels (7000 pg/mL) of IL-1β when exposed to MSU/C16, which is dose-dependently reduced in the presence of AAT-Fc. At 25 μg/mL AAT-Fc, there was a 76% reduction ($p<0.01$) and at 12.5 μg/mL, the reduction was 60% ($p<0.01$).

AAT-Fc induces endogenous IL-1Ra

The balance of IL-1 and IL-1Ra, the natural inhibitor of IL-1, affects the severity of inflammation. Studies in a mouse model of pancreatic islet transplantation demonstrated that plasma-derived AAT induced IL-1Ra gene expression.[22] AAT also augmented IL-1Ra production in lipopolysaccharide (LPS)-stimulated human PBMCs.[6] We next investigated whether AAT-Fc induced the production of endogenous IL-1Ra in vitro and in vivo. Figure 5C shows that negatively selected primary human blood monocytes produce high levels (12-fold over control) of IL-1Ra during 24 h incubation in the presence of 25 μg/mL AAT-Fc. The same concentration of plasma-derived AAT did not induce IL-1Ra (figure 5C). We next measured the levels of circulating IL-1Ra in mice injected with AAT-Fc. Indeed, levels of resting state mouse IL-1Ra rose approximately fourfold at 6 h after intraperitoneal injection with 50 μg AAT-Fc (figure 5D). In addition, we

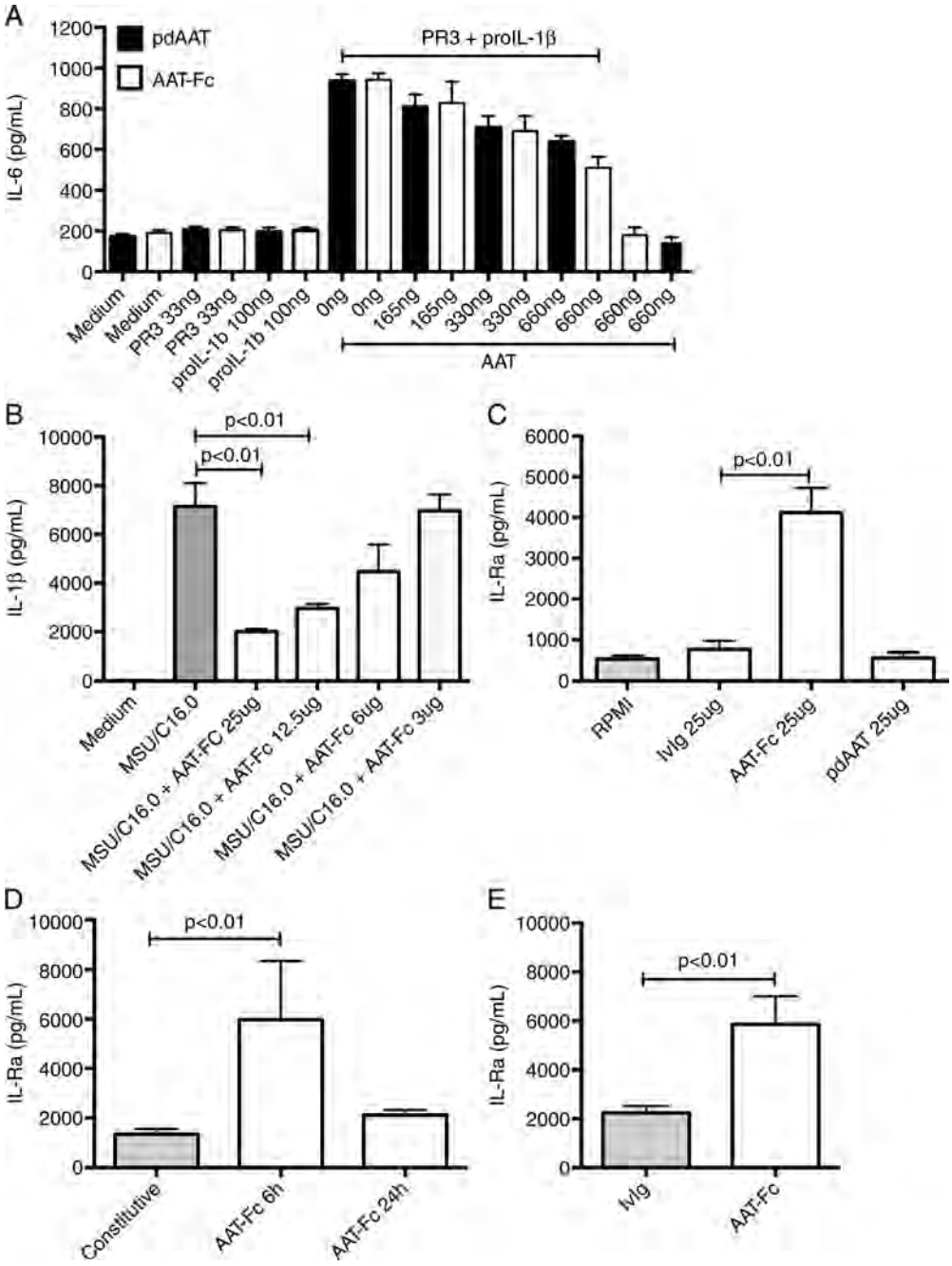

Figure 5 AAT-Fc inhibits PR3-mediated IL-1β processing, suppressed MSU/C16.0-induced IL-1β- and induces IL-1Ra- production. (A) Proteinase 3 and proIL-1β were incubated with or without either pdAAT or AAT-Fc in different concentrations. PR3 was pre-incubated for 30 min with pdAAT or AAT-Fc. IL-1β bioactivity was determined using the A549 cell line. (B) Human PBMCs were incubated for 24 h with MSU/C16.0 (300 μg/ml; 200 μm) with or without AAT-Fc. IL-1β was determined by ELISA. N=6 donors. (C) Negative selected human primary monocytes were incubated for 24 h with IvIg, AAT-Fc and pdAAT. N=4 donors. IL-1Ra was measured with ELISA. (D) Wild-type mice (n=5) were injected i.p. with 50 μg AAT-Fc. Before AAT-Fc injection, after 6 h and 24 h blood was collected for IL-1Ra determination. (E) Serum IL-1Ra levels in mice with gouty arthritis treated with AAT-Fc. N=10 mice per group. Data are expressed as mean ± SEM. Mann-Whitney U-test was used for statistical analysis.

observed that prior to intra-articular MSU/C16.0 injection, mice treated with AAT-Fc exhibited elevated levels of serum IL-1Ra 6 h later (figure 5E).

Human AAT transgenic mice are protected from gouty arthritis

Mice transgenic for human AAT (AAT-tg), which is driven by the surfactin C promoter for selective expression in type-2 lung epithelium,[16] have low circulating levels of human AAT but are nevertheless protected in models of multiple sclerosis[23] and *Pseudomonas* pneumonia.[24] Here we demonstrated that human AAT-tg mice were also protected against joint inflammation induced by local MSU/C16.0 injection. Figure 6A shows that 4 h after intra-articular injection of MSU/C16.0, there was moderate joint swelling in wild-type C57Bl/6 mice, whereas AAT-tg mice showed sixfold ($p<0.001$) less swelling. We could confirm these findings in a subsequent study (figure 6B). Histological analysis revealed that the number of inflammatory cells was

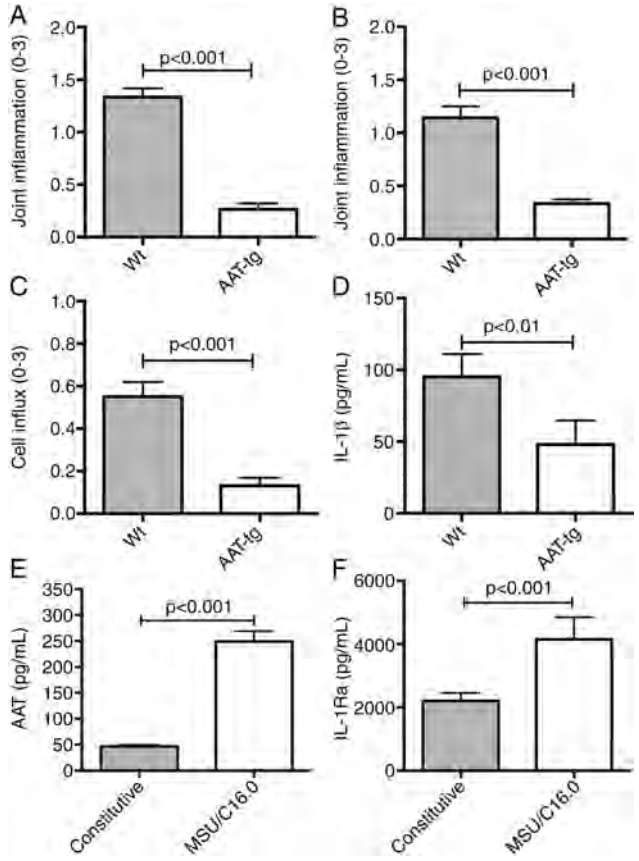

Figure 6 Suppression of gouty arthritis in human AAT-tg mice. (A, B) Gouty arthritis was induced in wild-type and human AAT-tg mice. N=5 mice per group, 10 joints were injected. Joint inflammation was determined after 4 h. (C) Influx of inflammatory cells in the joint cavity. N=10 joints per group. (D) IL-1β levels in synovial tissue explants. N=10 per group. (E) Human AAT levels in hAAT-tg mice. Constitutive and induced serum hAAT levels. MSU/C16.0 (3 mg/2 mM) was injected i.p., serum was collected after 6 h. N=10 AAT-tg mice. (F) Serum IL-1Ra levels in AAT-tg mice before and after injection i.p. with MSU/C16.0. Data are expressed as mean ± SEM. Mann-Whitney U-test was used for statistical analysis.

significantly reduced in AAT-tg mice compared with the wild-type mice (figure 6C). The IL-1β production in synovial tissue explants was reduced by 50% compared with wild-type mice (figure 6D). In line with these results, IL-6 and KC concentrations were also reduced (see online supplementary figure S2). Figure 6D reveals the constitutive level of circulating endogenous human AAT in AAT-tg mice. Following intraperitoneal injection of MSU/C16.0, circulating AAT levels increased remarkably (figure 6D) and serum IL-1Ra concentrations were increased further in the AAT-tg mice (figure 6E).

DISCUSSION

We demonstrate that the novel AAT-Fc fusion protein markedly suppresses joint inflammation in murine acute gouty arthritis. The study also reveals that AAT-Fc inhibits the release of IL-1β by human blood monocytes elicited by the combination of MSU crystals plus the C16.0 fatty acid. In addition, the AAT-Fc fusion protein induced IL-1Ra, the natural inhibitor of IL-1, in vitro and in the circulation of mice. Related to the administration of AAT-Fc to mice, transgenic mice expressing human AAT are protected from MSU crystal-induced arthritis and also exhibit elevated endogenous levels of mouse IL-1Ra compared with wild-type mice. Together, the data provide the basis for AAT-Fc as a possible treatment option for acute gout attacks in humans. Furthermore, infusions of plasma-derived AAT have a 20-year history of safety in deficient patients.[25 26]

IL-1β is clearly the causal cytokine in the pathogenesis of acute gout attacks. Clinical trials using anakinra,[17–19] the IL-1 trap (rilonacept)[27] or a neutralising anti-IL-1β antibody (canakinumab)[14 28] reveal a rapid and sustained reduction in the clinical manifestations as well as the number of acute attacks. Treatment with canakinumab was superior to the standard corticosteroid therapy in patients with refractory gout.[14] The present findings support the concept that AAT-Fc would be effective in humans since the fusion protein targets the production as well as the activity of IL-1β.

Not merely one mechanism accounts for the efficacy of AAT-Fc in this model of gouty arthritis. Gene expression, synthesis, processing and release of active IL-1β from mononuclear phagocytes are tightly regulated.[29 30] Despite active caspase-1 in primary human blood monocytes,[30] MSU crystals alone do not induce the synthesis nor processing of IL-1β,[13] but require an additional signal, for example, LPS or TLR2 ligands. Palmitic acid (C16.0) also provides a second signal.[31] Nevertheless, non-caspase-1-mediated cleavage of the IL-1β precursor into active IL-1β is known to occur.[32] Fantuzzi and coworkers reported that the systemic inflammatory response elicited by subcutaneous turpentine was nearly the same in caspase-1-deficient mice as in wild-type mice.[3] Therefore, caspase-1-mediated cleavage of the IL-1β precursor bypassed particularly during neutrophil-dominated inflammation. Once the IL-1β precursor is released from dying cells, neutrophil-mediated processing takes place in the extracellular space. In an attempt to phagocytose urate crystals, cells release their contents, which would include the IL-1β precursor.[33 34] Several neutrophil proteases such as elastase, proteinase 3[20] and Granzyme B[35] can cleave the IL-1β precursor at sites close to the caspase-1 site as reviewed in ref.36.

AAT is the primary serum serine proteinase inhibitor,[4 5 37–39] and in AAT-deficient persons, the uncontrolled activity of serine proteinases, mostly neutrophil elastase, is regarded as causal in the pathogenesis of lung, liver and pancreatic chronic inflammation in these patients.[25 26 40] Since neutrophils are the dominant inflammatory cells in the joint space of acute gout, it is likely that neutrophil proteases bypass the inflammasome-caspase-1 pathway and cleave the IL-1β precursor extracellularly. Another possible proteinase that can process the inactive IL-1β precursor is mast cell chymase.[41] However, PR3 is highly effective in cleaving the IL-1β precursor into bioactive IL-1β.[20] In addition, AAT treatment results in remarkable downregulation of PR3 expression in mouse PBMCs.[42] Here we demonstrated that both plasma-derived AAT and AAT-Fc fusion protein inhibit PR3 conversion of the IL-1β precursor to an active cytokine (figure 5A).

In addition to inhibition of PR3, the reduction in synovial IL-1β production by AAT-Fc may be due to inhibition of caspase-1, as reported for plasma-derived AAT in a model of acute myocardial infarction in the mouse[8] and renal ischaemia.[43] However, another study indicates that plasma-derived AAT did not reduce caspase-1 activity in the THP-1 macrophage cell line or in cell free extracts, and had no effect on whole human blood production of IL-1β.[44] Because IL-1 stimulates caspase-1 gene expression in human blood monocytes[45] and caspase-1 cleavage of the IL-18 precursor is IL-1β dependent,[46] a reduction in IL-1β will also reduce the level of caspase-1 itself without directly affecting the enzymatic function of caspase-1.

Another mechanism by which AAT-Fc affects IL-1β production and activity is via a reduction in the expression of TLR. In humans with recent onset diabetes treated with eight weekly infusion of AAT, IL-1β synthesis by circulating monocytes was reduced significantly upon stimulation with TLR agonists ex vivo.[47] AAT-Fc added directly to mouse insulin-producing islets suppresses the surface expression of TLR2 and TLR4[6]

The ability of AAT-Fc to increase circulating levels of endogenous IL-1Ra also contributes to the efficacy of AAT-Fc in suppressing MSU/C16.0-mediated inflammation. In addition to the present study, elevated endogenous IL-1Ra by AAT was reported in a model of graft versus host disease in mice.[42] Acute gout in humans responds to a relatively low dose of anakinra of 100 mg, which results in a peak plasma level of <1 μg/mL. AAT-tg mice have a low level of serum IL-1Ra, which may account for the resistance of these mice to acute MSU/C16.0.

Although the fusion of AAT to the Fc domain of IgG1 created a new molecule,[48] fusion molecules of a naturally occurring serum protein such as tumour necrosis factor receptors to the Fc domain IgG1 (etanercept) are used commonly as therapeutics in humans to increase plasma half-life.[49] One advantage of AAT-Fc over plasma-derived AAT is that injection of only 50 μg prevented MSU/C16.0-induced joint inflammation comparable to 2000 μg of plasma-derived AAT (40-fold less). In humans, AAT-Fc could be injected subcutaneously compared with the need of an intravenous infusion of plasma-derived AAT. The greater potency of AAT-Fc over plasma-derived AAT may relate to the method of purification as discussed in ref.6. The purification of plasma-derived AAT begins with cold ethanol precipitation (Cohn fractionation), which can oxidise the molecule followed by subsequent steps intended to maintain the elastase inhibition properties of AAT. In contrast, AAT-Fc is purified by a signal affinity step using Protein A chromatography.[48]

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Contributors LABJ: initiated the study, perform experiments, analysed the data and wrote the manuscript. TOC, TA, MCPC: performed experiments. MIK and SK: performed experiments and analysed data. FLvdV, MGN: analysed data, wrote manuscript. CAD: initiated the study, analysed data and wrote manuscript.

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Competing interests CAD is Chief Scientific Officer of Omni Bio Pharmaceuticals and holds shares in this company. The AAT-Fc is patented by the University of Colorado and licensed by OMNI Bio Pharmaceutical. The US patent number is 8,633,305.

Ethics approval The animal experiments were approved by the ethical committees of the University of Colorado (Denver, USA) and the Radboud University Medical Center (Nijmegen, The Netherlands).

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Alpha-1-anti-trypsin-Fc fusion protein ameliorates gouty arthritis by reducing release and extracellular processing of IL-1β and by the induction of endogenous IL-1Ra

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Supplemental Information.

Materials and Methods

Preparation of MSU crystals. MSU crystals were prepared according to Seegmiller *et al.* [1]. Briefly, a 0.03M solution of MSU was prepared after diluting 1.0 g of uric acid (Sigma-Aldrich, St. Louis, MO) in 200 mL of sterile water containing 24g of NaOH. The pH was adjusted to 7.2 with HCl and subjected to 120 ºC for 6h to inactivate endotoxins. LPS contamination was controlled by LAL assay. The solution was left to cool at room temperature and stored at 4°C. The crystals were 5–25μm in length. To prepare MSU for in vitro and in vivo experiments, the fluid was removed; the crystals were weighed under sterile conditions and combined with palmitic acid (C16.0). A solution of 800μM C16.0, BSA 0.4% and ethanol 0.4% in RPMI was filter-sterilized (0.2μm) and thereafter mixed with uric acid crystals 1200μg/mL. This preparation was kept at 4ºC.

Generation of AAT-Fc fusion protein. Human pCAGGs-IgG1/AAT plasmids were co-transfected with the pSV-dihydrolfolate reductase (DHFR) vector (ATCC, Manassas, VA, USA) into the DHFR-deficient CHO cell line [2]. Stable clones were selected in medium containing G418 (500 μ/mL) and subsequently subjected to methotrexate selection for gene amplification. AAT-Fc was purified using protein A-conjugated beads that had been equilibrated with phosphate-buffered saline (PBS) containing 0.5 mol/L NaCl. AAT-Fc was examined for purity, LPS contamination and stored at -80 ºC until use. The preparation (second generation) used in the present study inhibited elastase using the method described in the Supplement of Jonigk, et al [3].

***Monocytes isolation*s.** Purified monocyte suspensions were obtained from PBMCs using MACS beads according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ cells were isolated using CD14 microbeads followed by magnetic separation over selection columns. Negative selection of monocytes was performed after an initial monocyte-lymphocyte separation of PBMCs using high-density hyperosmotic Percoll solution, followed by MACS. Thereafter, monocytes were isolated by using the pan monocyte isolation kit (Miltenyi Biotec)

according to the manufacturer's instructions. Monocyte suspensions were adjusted to $1x10^6$ cells/mL and experiments were performed using $1x10^5$ cells/well.

Joint inflammation measurement. Joint inflammation was measured by macroscopic scoring method [4, 5]. Macroscopic joint swelling was scored on a scale ranging from 0-3. After the skin was removed the knee joint was scored, 0= no inflammation, 1= mild, 2= moderated and 3= severe inflammation. The scores start with 0.25, 0.50, 0.75, 1,0, up to 3.0. 0.25 is giving when the first signs of swelling and redness is seen. All values exceeding 0.25 are assigned as joint swelling. Joint swelling scoring was performed without knowledge of the experimental groups.

Cytokine determinations. Protein levels of murine IL-1β or IL-6 were measured in patellae washouts or synovial tissue extracts. Four hours after injection of MSU/C16.0, patellae were isolated from inflamed knee joints and cultured 2h at RT in RPMI 1640 medium containing 0.1% bovine serum albumin (200µL/patella). For total IL-1β levels, patellae with surrounding tissue were directly transferred to 200µL 0.05% Triton-X 100 in PBS. After repeated freeze-thawing, IL-1β was determined. Mouse cytokines, including mouse IL-1Ra were determined by ELISA from R&D Systems, Minneapolis, MN, USA. Human IL-1β and IL-6 was measured by ELISA (R&D Systems).

AAT determination. Levels of human AAT in mouse sera were determined using an AAT ELISA kit (Immunology Consultant Laboratory, Inc., Newberg, OR, USA). Blood was collected from mice and serum was diluted 20 times in ELISA diluent.

Histological analysis. Mice were sacrificed by cervical dislocation. Whole knee joints were removed and fixed in 4% formaldehyde for 7 days before decalcification in 5% formic acid and processing for paraffin embedding. Tissue sections (7µm) were stained with Haematoxylin/Eosin. Histopathological changes in the knee joints were scored in the patella/femur region on 5 semi-serial sections. Scoring was performed on decoded slides by two separate observers, using the following parameters: the amount of cells infiltrating the synovial lining and the joint cavity was scored from 0-3 [6].

Cleavage of the IL-1β precursor by PR3. 100ng of recombinant human IL-1β precursor [7] was incubated for 2h with 33ng of PR3 at pH 7.2 (Athens Research & Technology, Athens, Georgia, USA). Thereafter, the bioactivity of the cleaved product was assessed by stimulation of IL-6 from human A549 cells. After 24h, IL-6 in the supernatants were determined by ELISA (R&D Systems). To inhibit PR3, the enzyme was pre-incubated for 30 minutes with AAT-Fc or plasma-derived AAT before mixed with the IL-1β precursor.

Statistical analysis. Differences between experimental groups were tested using the Mann-Whitney U-test. Data are expressed as mean ±SEM, unless stated elsewhere. Graphpad Prism 6.0 was used for statistical analysis.

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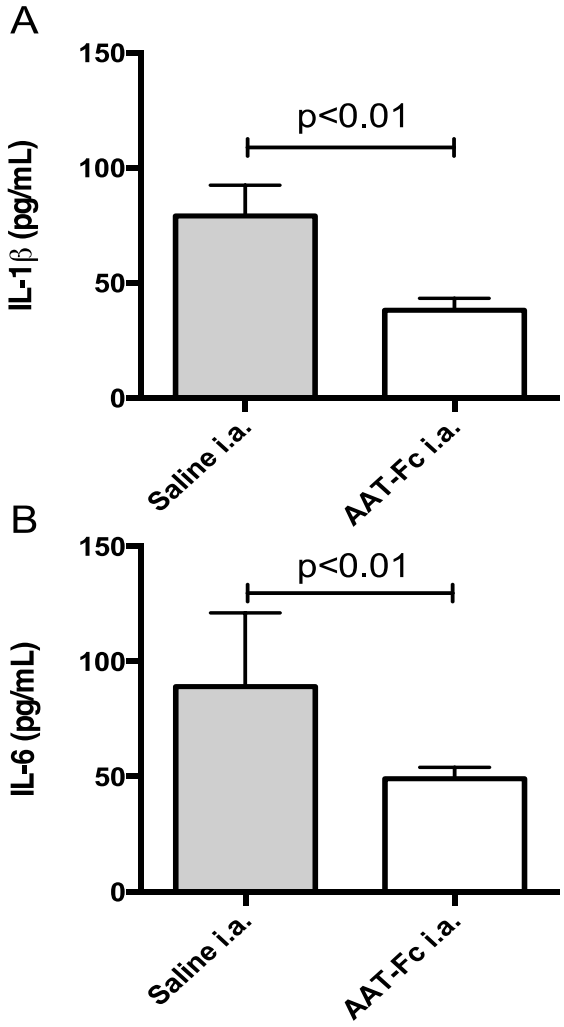
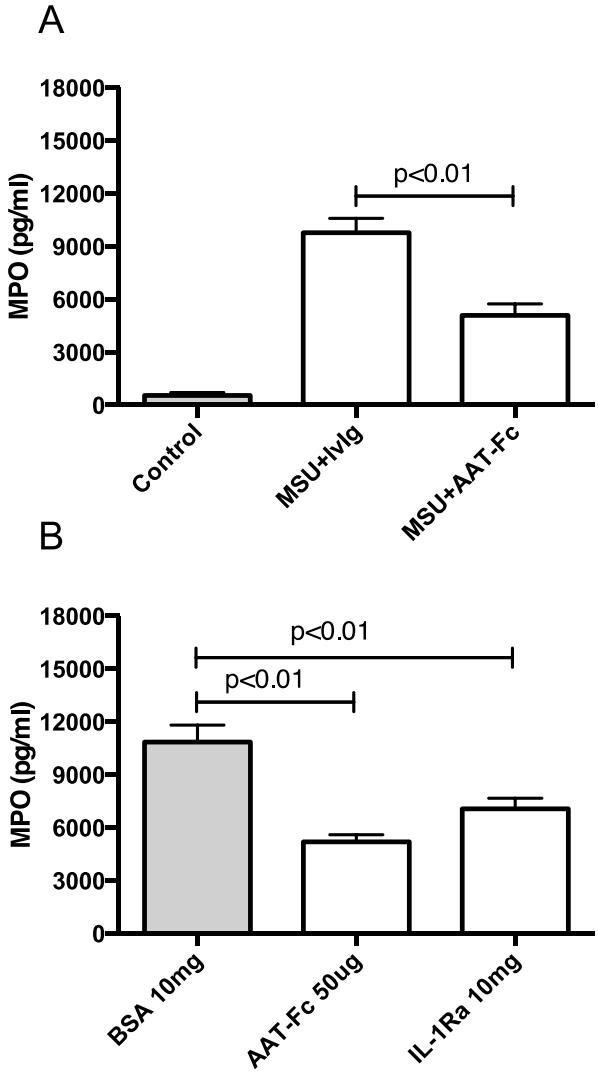
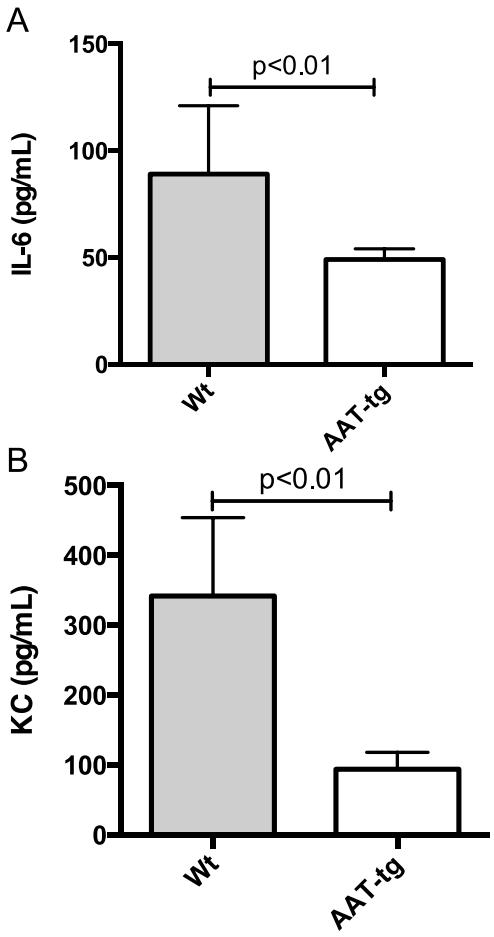
Supplemental Figures.



Supplementary Figure 1. Intraarticularly (i.a.) application of AAT-Fc. C56/Bl6 mice were i.a. injected with 6ul of MSU/C16.0 (300µg/200µM) or MSU/C16.0 (300µg/200µM) plus AAT-Fc (2µg). After 4h, synovial tissue specimens were isolated and the cytokine release was determined. **A.** IL-1β production. **B.** IL-6 production. N= 5 specimens per group. Data are expressed as mean+/-SEM. Mann-Whitney U-test was used for statistical analysis.



Supplemental Figure 2. A. C57/Bl6 mice (n=5 per group) were i.p injected with 50μg IvIg, 50μg AAT-Fc or saline (control). After 2h, 3mg (0.5ml of a solution of 6 mg/ml) MSU crystals were i.p. injected, only in the mice that received IvIg or AAT-Fc previously. Four hours later, mice were sacrificed and 10ml of cold PBS was i.p. injected. Thereafter peritoneal fluid was isolated. Cell influx was determined by cell counting. MPO levels in the peritoneal fluid by Elisa. Mann Whitney U-test. Data are expressed as mean+/-SEM. **B.** Comparison AAT-Fc with IL-1Ra. BSA (10mg per mouse), AAT-Fc (50μg per mouse) or IL-1Ra (10mg per mouse) were injected i.p., 2h before MSU (3mg)crystals were injected. Thereafter peritoneal fluid was isolated. Cell influx was determined by cell counting. MPO levels in the peritoneal fluid by Elisa. Mann Whitney U-test. Data are expressed as mean+/-SEM.



Supplementary Figure 3. Wild-type (C56/Bl6) and AAT-tg mice were i.a. injected with 6ul of MSU/C16.0 (300µg/200µM). After 4h, synovial tissue specimens were isolated and the cytokine release was determined. **A.** IL-6 production. **B.** KC production. N= 5 specimens per group. Data are expressed as mean+/-SEM. Mann-Whitney U-test was used for statistical analysis.