Acute Phase Protein α_1-Antitrypsin Reduces the Bacterial Burden in Mice by Selective Modulation of Innate Cell Responses

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Background. Severe bacterial infection can cause sepsis, multiple organ dysfunction syndrome (MODS), and death. Human α_1-antitrypsin (hAAT) is an antiinflammatory, immune-modulating, and tissue-protective circulating serine-protease inhibitor, with levels that increase during acute-phase responses. It is currently being evaluated as a therapeutic agent for individuals with diabetes and graft-versus-host disease. However, the concern of opportunistic bacterial infections has yet to be addressed. Therefore, we investigated host immune cell responses during acute bacterial infections under conditions of elevated hAAT levels.

Methods. Peritonitis and sepsis models were created using wild-type mice and hAAT-transgenic mice. Bacterial loads, MODS, leukopenia, neutrophil infiltration, immune cell activation, circulating cytokine levels, and survival rates were then assessed.

Results. hAAT significantly reduced infection-induced leukopenia and liver, pancreas, and lung injury, and it significantly improved 24-hour survival rates. Unexpectedly, bacterial load was reduced. Levels of early proinflammatory mediators and neutrophil influx were increased by hAAT soon after infection but not during sterile peritonitis.

Conclusions. hAAT reduces the bacterial burden after infection. Since hAAT does not block bacterial growth in culture, its effects might rely on host immune cell modulation. These outcomes suggest that prolonged hAAT treatment in patients without hAAT deficiency is safe. Additionally, hAAT treatment may be considered a preemptive therapeutic measure for individuals who are at risk for bacterial infections.

Keywords. inflammation; innate immunity; macrophages; neutrophils; cytokines; sepsis; cell infiltration.

Bacterial infections are a major cause of mortality, despite the availability of antibiotics; severe infections can lead to bacteremia, sepsis, and septic shock, as well as to multiple organ dysfunction syndrome (MODS) and death.

Human α_1-antitrypsin (hAAT) is a 52-kDa glycoprotein that is produced mainly by hepatocytes and circulates at steady-state levels of 0.9–1.75 mg/mL [1]. During acute phase responses, circulating levels of hAAT increase >4-fold for several days. hAAT inhibits a variety of important upstream inflammation-related proteases, such as neutrophil elastase, proteinase 3, and cathepsin G [2]. Indeed, hAAT decreases the levels and activity of interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), and interleukin 8 (IL-8) [3–5] and increases the levels of IL-1Ra and interleukin 10 (IL-10) under a multitude of inflammatory triggers [6].

In accordance with the notable safety profile [1] and antiinflammatory attributes of hAAT, various protocols using weekly hAAT infusions are currently being evaluated for the treatment of inflammatory pathologies in individuals without hAAT deficiency [7, 8]. These pathologies include treatment-resistant graft-versus-host-disease (clinical trials registrations NCT01523821 and NCT01700036) and recently diagnosed type 1 diabetes (NCT02005848, NCT01304537,

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and NCT01661192 [ongoing]; NCT01319331 [9], NCT01183455, and NCT01183468 [completed]).

While the antiinflammatory profile of hAAT would seem to pose a risk for opportunistic infections, individuals are not predisposed to infections during acute phase responses, and the duration and magnitude of elevated circulating hAAT levels are further increased during bacteremic acute phase responses. Administered in the context of its only current clinical indication as augmentation therapy for hAAT-deficient individuals, hAAT treatment has resulted in a significant reduction in bacterial colonization frequencies among recipients across several independent clinical studies [1, 10, 11]. Interestingly, a marked reduction in the pulmonary *Pseudomonas aeruginosa* bacterial burden has been established in patients with cystic fibrosis during hAAT inhalation therapy [12], a phenomenon that was corroborated by animal models [13, 14]. Yet the mechanism behind its ability to provide an antiinflammatory environment without interfering with bacterial clearance has yet to be determined.

In the present study, we examine the prognosis of extrapulmonary infections and study bacterial clearance functions of major innate immune cells, under conditions of chronically elevated levels of circulating hAAT.

MATERIALS AND METHODS

Animals

Mice transgenic to hAAT (background strain C57BL/6, kindly provided by A. Churg, University of British Columbia, Vancouver, Canada [15]) were bred in-house and genotyped routinely, as described elsewhere [16]. C57BL/6J mice were purchased from Harlan (Jerusalem, Israel). All animals were females 10–12 weeks old and housed in standard conditions. Experiments were approved by the Ben-Gurion University of the Negev Animal Care and Use Committee.

Fecal Suspension–Induced and Sterile Peritonitis Models

The fecal suspension injection protocol was performed as described previously [17], with minor modifications. Briefly, 4.0 ± 0.5 g of fresh feces from wild-type (WT) mice was suspended in 30 mL of phosphate-buffered saline (PBS; Sigma-Aldrich, Rehovot, Israel) by aggressive shaking at room temperature until the suspension reached uniform consistency. The suspension was filtered through double-layered surgical gauze, followed by filtration through a 70-μm cell strainer (BD Falcon, Becton Dickinson, New Jersey). Filtered fecal suspension stock was immediately aliquoted as is and stored at −80°C. Peritonitis was induced by directly introducing freshly thawed fecal stock solution (200 μL intraperitoneally per mouse); this dose is the result of a dilution calibration towards an optimal dose-dependent effect (not shown). Vital signs were assessed every 12 hours during the first 4 days and every 24 hours thereafter. Sterile peritonitis was induced by direct intraperitoneal introduction of thioglycolate (3% v/v, 1 mL per mouse; Sigma-Aldrich).

Cecal Ligation and Puncture (CLP) Model

Minor modifications were made to the cecal ligation and puncture (CLP) procedure based on protocols described elsewhere [18]. Briefly, after anesthetizing the mice with ketamine and xylazine (80 and 12 mg/kg, respectively), the cecum was exposed through a 1-cm abdominal midline incision. Ligation was performed 5 mm from the cecal tip, and the cecal stump was punctured once with a 25-gauge needle. A 1-mm segment of stool was extruded into the peritoneal cavity, and the incision wound was stitched closed. Resuscitation of the animals was performed by slowly administering 1 mL of 37°C saline subcutaneously. Sham controls included mice subjected to the same procedure, but with the cecum stump left intact.

Peritoneal Lavage, Whole-Blood, and Serum Specimen Collection

Mice were euthanized at indicated time points after induction of peritonitis. For peritoneal lavage, 8 mL of cold PBS was introduced intraperitoneally and then recovered using an 18-gauge needle. The number of colony-forming units (CFU) was determined in the fluid phase, and cells were transferred to a flow cytometer for fluorescence-activated cell-sorting (FACS) analysis. Blood specimens were collected into a BD microtainer (SST and K2E tubes, Becton Dickinson) by cardiac puncture and either assayed or spun for serum separation. Lavage and serum aliquots were stored at −20°C.

Assessment of Organ Damage and Inflammatory Mediators

Serum levels of aspartate aminotransferase, alanine aminotransferase, and pancreatic amylase were determined by clinical assay kits (all from Beckman Coulter, Switzerland). Serum and lavage levels of TNF-α and IL-6 were determined by ELISA (PeproTech, Israel), levels of IL-1β, interleukin 12 (IL-12), keratinocyte chemoattractant (KC), monocyte chemotactic protein 1 (MCP-1), and interleukin 17 (IL-17) were determined by Q-Plex mouse cytokine chemiluminescence-based enzyme-linked immunosorbent assay (ELISA; Quansys Biosciences, Logan, Utah).

Scoring of Lung Tissue Damage

Lungs were removed from 6 randomly selected mice from each group and fixed in 4% formalin. Paraffin sections were stained with hematoxylin-eosin. Histologic examination was performed on blinded coded samples by 2 investigators, and findings were scored as described elsewhere [19]. Briefly, alveolar collapse, neutrophil infiltration, thickness of alveolar septae, and degree of alveolar fibrin deposition were graded on a scale of 0 (absent) to 4 (extensive); sample score is the sum of the 4 parameters.

Leukopenia Evaluation

After whole-blood specimen collection, the white blood cell (WBC) count was determined using ADVIA 2120 (Siemens). Bone marrow cells were isolated from a single tibia per

mouse, washed in PBS, and filtered through a 70-μm cell strainer. Samples were stained by trypan blue, and cell number was determined by the Countess Automated Cell Counter system (Life Technologies, New York).

FACS Analysis

The Cytomics FC 500 (Beckman Coulter) and BD FACSCanto II (BD) systems were used for analysis. After washing with FACS buffer (PBS, 1% bovine serum albumin, 0.1% sodium azide, and 2 mM ethylenediaminetetraacetic acid at pH 7.4), 1×10^6 cells per sample were incubated with FcγRII/III blocker (BioLegend). The general peritoneal cell population was stained with anti-CD11b Pacific Blue, anti-F4/80 allophycocyanin (APC), anti-CD86 phycoerythrin (PE), anti-MHCII APC/cyanine 7 (Cy7). and anti-CD40 PE/Cy7; peritoneal neutrophils were stained with anti-CD11b APC/cy7, anti-Ly6G fluorescein isothiocyanate, and anti–TNF-α APC; RAW 264.7 cells (ATCC) were stained with anti-CD11b Pacific Blue. All antibodies were from BioLegend and diluted according to the manufacturer's guidelines. Data were analyzed by FlowJo (Tree Star, Ashland, Oregon).

Bacterial Count

CFU counts were performed by 10-fold serial dilutions plated on blood agar plates (Hylab, Israel) for 24 hours at 37°C.

Bacterial Growth Assay

Logarithmic-phase *Escherichia coli* (ATCC, 25922) were allowed to multiply in cell culture medium (M-199, Biological Industries), in the absence or presence of 0.5 mg/mL hAAT (Glassia, Kamada, Israel) in quadruplicate. The relative bacterial population size was estimated by determining the $OD_{630\ nm}$, using an ELISA plate reader (EL800, BioTek Instruments).

Bacterial Uptake Assay

RAW 264.7 cells were seeded in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS; both from Biological Industries) in 60-mm cell culture Petri dishes until reaching 80% confluence. After washing 3 times with PBS, cells were immersed in serum-free medium (RPMI 1640 supplemented with BIOGRO-2, Biological Industries) in the presence or absence of 0.5 mg/mL hAAT, for 4 hours. *E. coli* (strain ER2566) expressing pSH21::his6-gfp plasmid (kindly provided by E. Gur, Ben-Gurion University of the Negev, Israel) were grown on LB with ampicillin (100 μg/mL, Sigma-Aldrich) at 37°C. Upon reaching logarithmic phase, IPTG (1 mM, Sigma-Aldrich) was added and the temperature lowered to 30°C for 3 hours. Bacteria (multiplicity of infection [MOI], 100) were added onto RAW 264.7 cells for 20 minutes at 37°C, washed 5 times in PBS, and gently collected by scraper for FACS analysis. For negative control, bacteria were added to RAW 264.7 cells on ice.

Bacterial Killing Assay

Bacteria killing assay was performed as previously described [20], with minor modifications. Briefly, RAW 264.7 cells were plated in 48-well plates (0.5×10^5 cells per well in quadruplicates). Cells were allowed to adhere for 3 hours at 37°C in RPMI 1640 medium containing 10% FCS. The cells were then washed thoroughly with PBS and incubated for 24 hours with or without 0.5 mg/mL hAAT in serum-free medium. *E. coli* (strain ER2566) were added to the cells (MOI, 100), and the plates were centrifuged at 1083*g* for 5 minutes; plates were then incubated at 37°C for 15 minutes. Five washings with PBS were performed to remove free bacteria. Cells were then lysed with sterile double-distilled water to determine baseline readout (t-0). Serum-free medium was added to the remaining cells at 37°C for 30 and 60 minutes. Cell lysates were plated in serial-dilutions on ampicillin LB agar plates, and bacterial counts were determined after 24 hours. Bacterial killing was calculated as the proportion of killed bacteria among those present at t-0.

Statistical Analysis

Analyses were performed using GraphPad Prism 5 software (GraphPad Prism 5, Pugh computers, United Kingdom). Results are expressed as the mean or median ± standard error of the mean. The statistical significance of differences between groups was determined by a 2-tailed Student Mann–Whitney test. Results are considered significant at a P value of $\leq .05$.

RESULTS

hAAT Protects Against Polymicrobial Peritonitis–Induced Organ Failure and Leukopenia

To assess whether hAAT protects against polymicrobial peritonitis–induced multiple organ failure, organ damage was assessed 24 hours after peritoneal cavity bacterial contamination by CLP in WT mice and in mice transgenic for hAAT. Sham-operated mice were used as controls. As shown in Figure 1, the levels of circulating markers for liver damage (Figure 1*A*) and pancreatic damage (Figure 1*B*) displayed a marked elevation in WT mice that underwent CLP. However, hAAT transgenic mice exhibited a limited rise in levels of injury markers. Parameters for lung injury were assessed by histologic scoring (Figure 1*C*). As shown in the figure, WT mice that underwent CLP displayed a massive neutrophil infiltrate and alveolar collapse; in contrast, hAAT transgenic mice displayed an almost intact lung tissue. Alveolar thickness and fibrin accumulation were not observed in either group. The severity of leukocytopenia (Figure 1*D*) was assessed alongside total bone marrow cell count 72 hours after CLP. As shown in the figure, while the WBC population size was lower in WT mice that underwent CLP, it was markedly improved in the hAAT group. However, the reduction in the number of bone marrow cells was similar

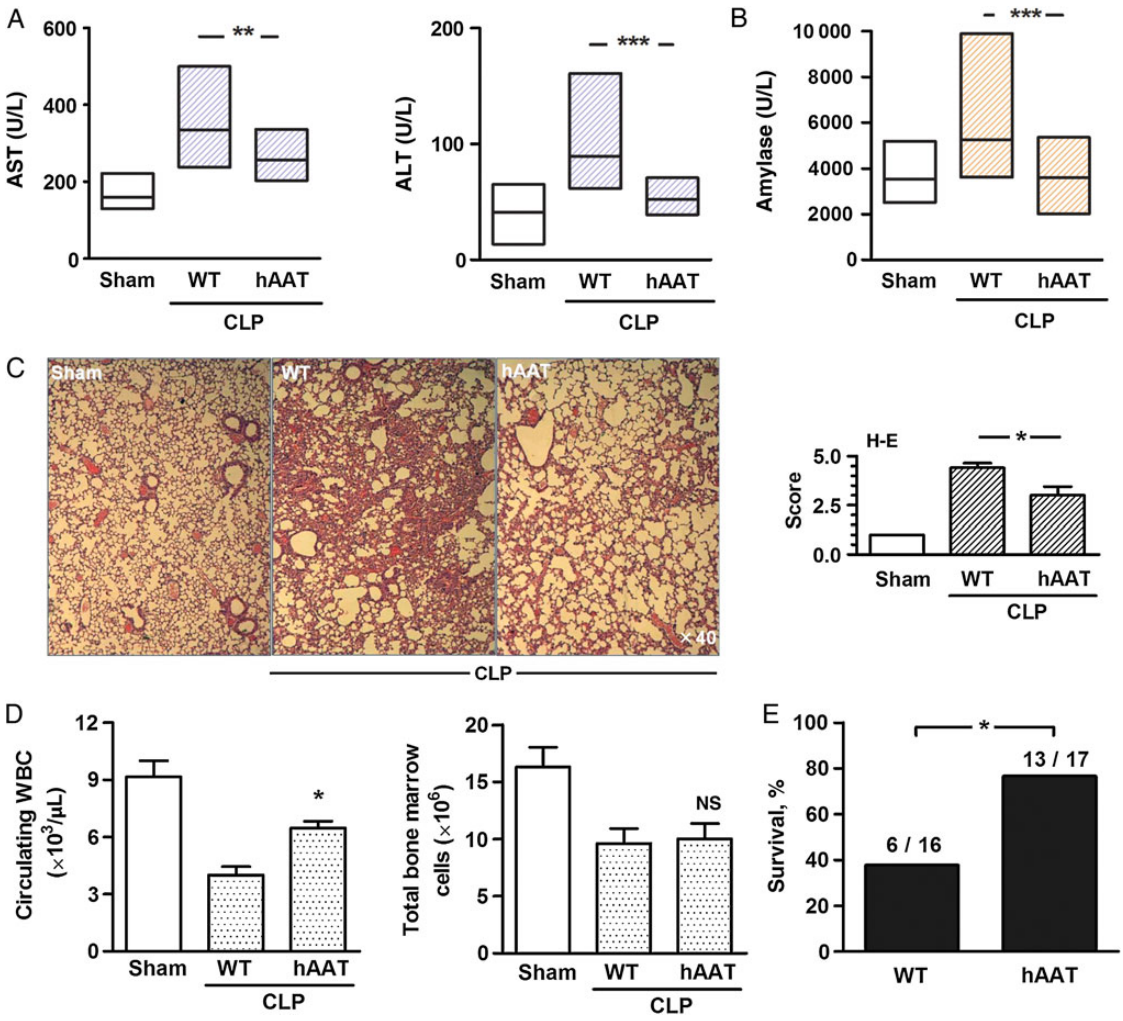

Figure 1. Human α_1-antitrypsin (hAAT) protects from polymicrobial peritonitis–induced organ failure and leukopenia. Serum samples obtained 24 hours after cecal ligation and puncture (CLP) were examined for aminotransferase (AST) and alanine aminotransferase (ALT; *A*) and amylase (*B*; n = 13). *C*, Representative lung tissue hematoxylin–eosin (H–E) staining and lung scoring 24 hours after CLP (n = 6). *D*, White blood cell (WBC) and bone marrow cell count 72 hours after CLP (n = 5). *E*, Survival at 24 hours. Uniform preparations of fecal suspension introduced intraperitoneally to wild-type (WT) mice and to hAAT-expressing mice. **P* < .05, ***P* < .01, and ****P* < .001, for comparisons to WT mice. Abbreviation: NS, not significant.

between WT and hAAT-expressing mice. Twenty-four-hour survival was determined in the fecal injection model, to minimize variance between outcomes (Figure 1*E* and Supplementary Figure 1); as shown in the figures, WT mice exhibited a survival rate of <40%, compared with >75% in hAAT-expressing mice. Survival rates at 48 and 72 hours were similar in both groups (Supplementary Figure 1).

Long-term Systemic hAAT Decreases Levels of Polymicrobial Peritonitis–Induced Serum Inflammatory Mediators

Since systemic inflammation is the major cause of organ failure during peritonitis, we evaluated circulating cytokine levels 72 hours after CLP. As shown in Figure 2*A* and Supplementary Figure 2, the hAAT group displayed reduced circulating levels of IL-1β, TNF-α, IL-6, MCP-1, IL-12, and IL-17. Inflammatory markers at the infection site were also examined. Peritoneal cells were collected from the site of infection 72 hours after CLP. As shown in Figure 2*B* and Supplementary Figure 3, inducible surface CD40, CD86, and major histocompatibility complex class II were significantly reduced in the presence of hAAT.

hAAT Reduces the Bacterial Burden In Vivo

Based on the multilevel outcomes observed thus far, the possibility that bacterial load might be affected by hAAT was raised. Twenty-four hours after CLP, peritoneal lavage was performed, and bacterial CFU was determined. As shown in Figure 3*A*, a significant reduction in the bacterial load was observed in hAAT-expressing mice, compared with WT mice. A consistent outcome was found at 72 hours (not shown). Nonetheless, when *E. coli* (Figure 3*B*) or peritoneal-isolated bacteria (not shown)

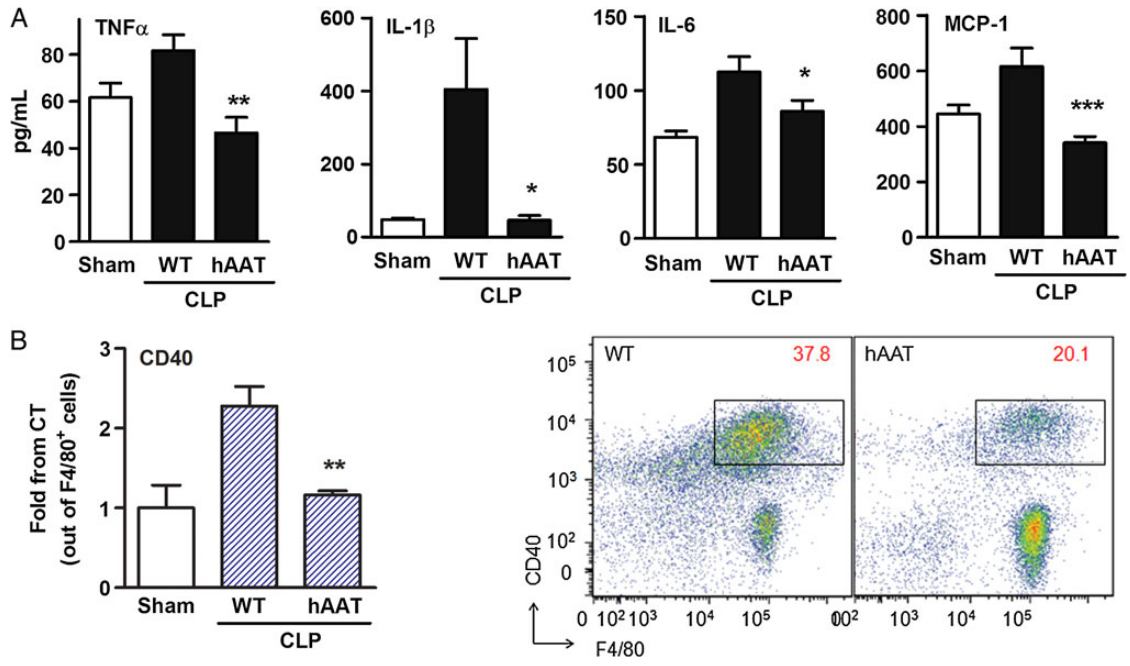

Figure 2. Long-term systemic human α_1-antitrypsin (hAAT) decreases peritonitis-evoked serum inflammatory mediators, as well as cell-surface activation markers at the infection site. Sham-operated mice, wild-type (WT) mice, and hAAT-expressing mice 72 hours after cecal ligation and puncture (CLP) (n = 8). *A*, Serum inflammatory mediators. Representative results out of 3 independent experiments. *B*, Surface CD40, peritoneal cells. Results are gated to F4/80$^+$ cells. *Right,* Representative histograms of data from fluorescence-activated cell sorter analysis. *Left,* Pooled data obtained from 3 independent experiments, presented as mean ± standard error of the mean. **P* < .05, ***P* < .01 and ****P* < .001, for comparisons to WT mice. Abbreviations: IL-1β, interleukin 1β; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein 1; TNF-α, tumor necrosis factor α.

were allowed to grow in medium, added hAAT allowed intact bacterial growth.

hAAT Increases Levels of Inflammatory Mediators in the Initial Hours After Initiation of Peritonitis

In investigating the duality of the outcomes of elevated hAAT (ie, reduced bacterial load with concurrent increased antiinflammatory profile), the initial hours after peritoneal stimulation were examined. Fecal injection–induced peritonitis was compared to sterile peritonitis (Figure 4*A*). Six hours after the inflammatory triggers, TNF-α levels were measured in the peritoneal lavage and in the serum. As shown in the figure, after infection, higher TNF-α levels were observed in the hAAT group, compared with the WT group, but sterile inflammation resulted in no statistically significant differences between the groups. TNF-α levels were also assessed 2 hours after fecal injection by measuring the proportion of peritoneal cells carrying high levels of membrane TNF-α (Figure 4*B*). Accordingly, hAAT-expressing mice displayed more membrane TNF-α^{HI} peritoneal cells, before measurable TNF-α release into the peritoneal compartment. Levels of circulating cytokines and chemokines were determined 24 hours after CLP (Figure 4*C*); unlike the 72-hour profile (Figure 2), a proinflammatory profile emerged in the hAAT-expressing mice.

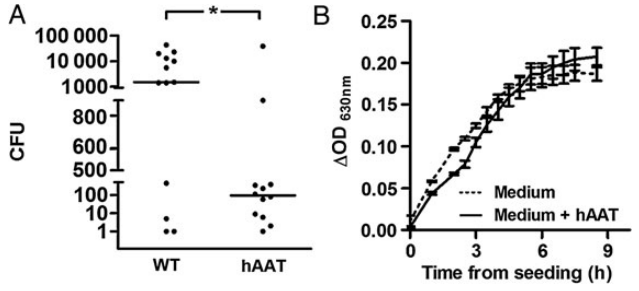

Figure 3. Human α_1-antitrypsin (hAAT) reduces the bacterial burden in vivo. *A*, Peritoneal colony-forming unit (CFU) counts at 24 hours from wild-type (WT) and hAAT-expressing mice (n = 13). Data represent values and medians from 3 independent experiments. **P* < .05. *B*, Logarithmic-phase *Escherichia coli* were allowed to proliferate in culture cell medium, in the absence or presence of hAAT in quadruplicate. $OD_{630\ nm}$ was determined, and background levels were subtracted. Data are mean ± standard error of the mean.

Early Infiltration, Bacterial Uptake, and Killing in the Presence of hAAT

Neutrophil infiltration and antibacterial innate cell activity were examined (Figure 5*A*). As shown in the figure, a massive neutrophil infiltration was observed 2 hours after induction of peritonitis, and it was greater in the hAAT-expressing mice. Bacterial uptake and killing were examined in vitro; macrophages were introduced to green fluorescent protein–expressing bacteria after 4

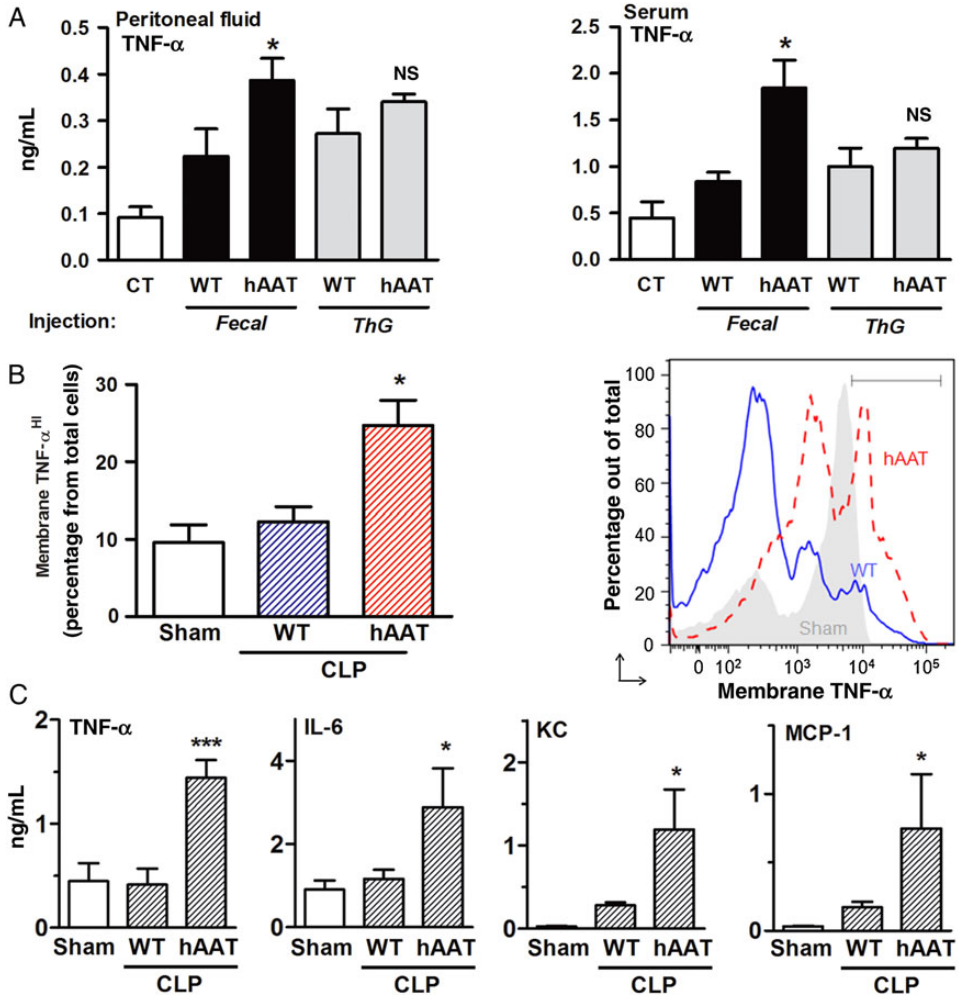

Figure 4. Human α_1-antitrypsin (hAAT) increases inflammatory mediators in the initial hours after evoked peritonitis. *A*, Secreted tumor necrosis factor α (TNF-α) levels in peritoneal fluid (left) and serum (right) from wild-type (WT) and hAAT-expressing mice 6 hours after fecal or thioglycolate injection (n = 8). *B*, Membrane TNF-α level 2 hours after fecal injection in WT mice (blue), hAAT (dashed red), and sham mice (gray; n = 6). *C*, Serum analysis of inflammatory mediators was conducted on WT and hAAT-expressing mice 24 hours after cecal ligation and puncture (CLP) (n = 8). Data are presented as mean ± standard error of the mean. **P* < .05 and ****P* < .001, for comparisons to WT mice. Abbreviations: IL-6, interleukin 6; KC, keratinocyte chemoattractant; MCP-1, monocyte chemotactic protein 1; NS, not significant.

hours of incubation with or without hAAT and then analyzed by FACS for fluorescent content (Figure 5*B*). As shown, bacterial uptake was intact in the presence of hAAT, and its addition to cells allowed intact bacterial killing (Figure 5*C*).

DISCUSSION

Treatment with repeated infusions of hAAT is currently considered for non–hAAT-deficient individuals with the rationale that hAAT is a modulator of immune-related pathologies [7, 8]. Because hAAT is an antiinflammatory protein, there is concern about an increased risk for opportunistic bacterial infections. The present study directly evaluates the impact of chronically elevated circulating levels of hAAT on the immune response to bacterial infections at extrapulmonary sites.

We used 2 bacterial peritonitis models: CLP and fecal injection. In the CLP model, bacteria are extruded from the gut into the peritoneal cavity. The entry of bacteria into the cavity is continuous, and the injured cecum introduces necrotic tissue elements. In the fecal injection model, uniformly aliquoted fecal bacterial preparations are introduced into the peritoneal cavity in a single injection; no necrotic tissue is formed in the process, and the inoculated mass is introduced in a single instillation. In addition, fecal injection is superior in outcome uniformity between mice. Each of these models alone would be insufficient in representing the complete clinical condition [21, 22].

The present study demonstrates some organ-protective effects of hAAT during the pathogenesis of bacterial peritonitis, which accords with its function as a tissue-protective protein. Indeed, the hallmark outcome of genetic hAAT deficiency

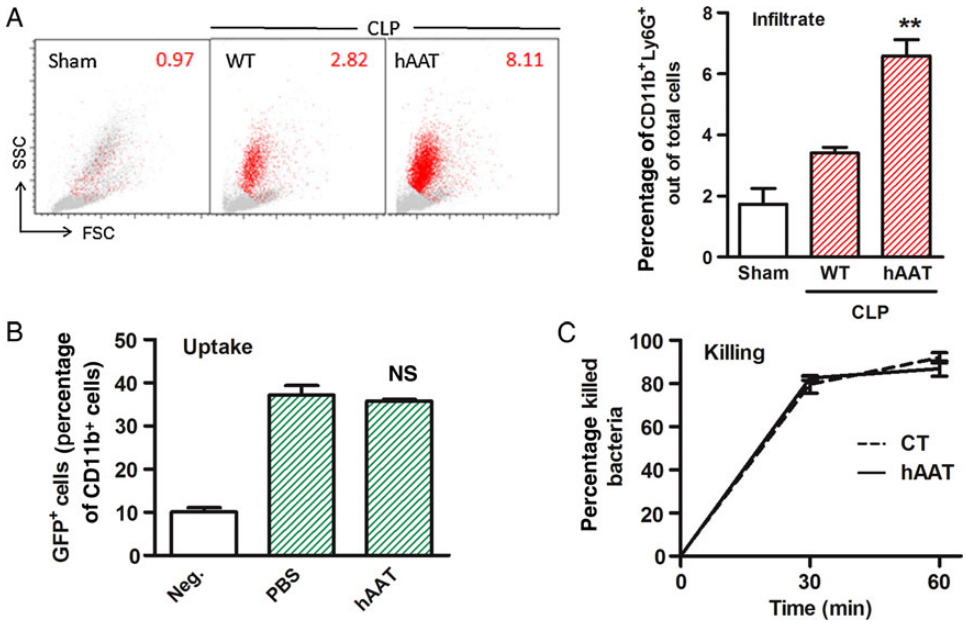

Figure 5. Infiltration, bacterial uptake, and killing. *A*, Neutrophil infiltration (red) in wild-type (WT) mice, human α_1-antitrypsin (hAAT)–expressing mice, and sham mice 2 hours after fecal injection (n = 6). Neutrophils were distinguished by $Ly6G^+/CD11b^+$ staining and their positioning on a forward scatter (FSC)/side scatter (SSC) graph. *Top*, Representative fluorescence-activated cell-sorting (FACS) images. *Bottom*, Pooled data, presented as mean ± standard error of the mean (SEM). *B*, hAAT-preincubated macrophages cocultured with green fluorescent protein (GFP)–expressing BL21 *Escherichia coli* at 37°C. Data are pooled and presented as mean ± SEM. *C*, hAAT-preincubated macrophages cocultured with logarithmic-phase BL21 *E. coli* at 37°C. Killing capacity is shown as the percentage of killed *E. coli*, compared with the population at t = 0; 100% killing was set as a CFU of 0. Data are presented as mean ± SEM. Abbreviations: CFU, colony-forming units; CLP, cecal ligation and puncture; Neg, negative control (cells placed on ice); NS, not significant; PBS, phosphate-buffered saline.

involves lung tissue degradation. Transgenic hAAT-expressing mice express the human protein in lung alveolar epithelial cells under the surfactant promoter, and therefore its intracellular presence may directly minimize cell death [23]. However, the present study demonstrates improved extrapulmonary organ function, as well. The positive outcomes with regard to MODS may be related to other recently described nonpulmonary activities of hAAT. The hyperinflammatory phase of early sepsis is characterized by a cytokine storm, a major cause of MODS, characterized by massive production of IL-1β, IL-6, and TNF-α, as well as danger-associated molecular pattern (DAMP) molecules [24, 25]. Here, the tissue protective effect of hAAT was observed 24 hours after bacterial inoculation, a time when inflammation was, unexpectedly, more pronounced in the hAAT group.

How can hAAT protect cells from injury? hAAT was shown in several models to inhibit caspase 3 and, as a result, to reduce hepatocyte death in acute liver failure [26], pancreatic islet β-cell death [27], and lung endothelial cell cigarette smoke–induced injury [28]. In addition, improved cell viability can be found in association with inhibition of MMP-2, MMP-9, and NO production by hAAT treatment of liver cells [29], and modulation of response to TNF-α was demonstrated to be protective of lung endothelial cells [5, 23, 30]. Excessive cell death causes an increase in the number DAMP molecules, which can further facilitate organ damage, as occurs, for example, with extracellular heat shock protein gp96 [31]. hAAT was recently found to directly neutralize gp96 and to reduce inflammation in various models, including the cecal ligation model [32].

Lymphocytes and dendritic cells are extremely sensitive to sepsis-induced cell death [33], rendering the host further vulnerable to opportunistic infections. According to our results, hAAT significantly minimized the degree of leukopenia. However, hAAT did not affect the decline in bone marrow cell count, suggesting that the benefit of hAAT under these particular conditions is most probably regulated at the level of peripheral homeostasis.

The outcomes of hAAT treatment in the present study exceed its function as an antiinflammatory agent. Blocking IL-1β and TNF-α in animal sepsis models fails to diminish lethality or reduce lung neutrophil sequestration [34]. The IL-1 pathway blockade in septic mice reduces inflammation but increases mortality rates and bacterial burden [35]. hAAT is not a classic antiinflammatory protein. Similar to other acute phase proteins, such as C-reactive protein [36] and α_1-acid glycoprotein [37], hAAT has proinflammatory and antiinflammatory properties. In the present study, hAAT reduced immune cell activation at the infection site and levels of circulating inflammatory cytokines after 72 hours but achieved the opposite effect before the end of the initial 24 hours after infection. Previous publications

support this duality in the activity of hAAT; short exposure to lipopolysaccharide (LPS) followed by hAAT exhibits a proinflammatory profile [38], a phenomenon suggested by Nita et al to be CD14 related [39]. In addition, hAAT is neutralized by excessive free radicals, which is precisely the environment it meets when an initial local oxidative burst is formed.

hAAT does not act as an antibiotic; bacteria multiply in its presence. However, in vivo, bacteria must penetrate host tissues to thrive. Indeed, *P. aeruginosa* pneumonia is blocked by hAAT, in part by protecting the epithelial barrier [14]. In addition, tissues contribute NO, which then binds to hAAT, generating *S*-nitrosylated hAAT with potent bacteriostatic activity [40].

In addition to immune cell activation by proinflammatory cytokines during the first 24 hours, hAAT increased KC and MCP-1 release, associated with massive neutrophil infiltration. This interesting result is not obvious as the relation between hAAT and neutrophils predominantly involves neutrophil inhibition. Indeed, hAAT is known for his ability to reduce neutrophil chemotaxis under a series of stimuli, including LPS [41]. This apparent contradiction with our findings may be resolved by considering that the present study addresses a response to live bacteria, rather than to sterile inflammation. Evidence for this hypothesis can be found in the observed differential effect of hAAT on TNF-α release; its enhancing capacity in the present study is restricted to the context of live bacterial inoculation (eg, fecal injection and CLP) but absent in the context of sterile inflammation.

In addition to greater neutrophil infiltration and a more inflammatory environment shortly after bacterial infection, as shown here, hAAT also allowed uninterrupted antibacterial activity, such as uptake and intracellular killing. Indeed, several studies indicate that inhibition of proteolytic activity can protect essential host proteins from cleavage and inactivation. One such target of cleavage includes membrane complement receptor 1 (CR1); cleavage of membrane CR1 is directly inhibited by hAAT [42]. In patients with cystic fibrosis, IL-8 receptor is found cleaved on the surface of neutrophils, a process blocked by hAAT [43].

The fact that hAAT is naturally occurring and that it rises in the circulation during acute phase responses suggest that it may be a naturally occurring and safe immunomodulator. During bacteremic acute phase responses, the profile of circulating hAAT is particularly prolonged, as recently reviewed by Vandevyver et al [44]. Yet this attribute of hAAT is not exempt from a bacterial counterresponse: a group of pathogenic bacteria developed enzymes that degrade hAAT, including *Staphylococcus aureus, Serratia marcescens, Saccharopolyspora erythraea* (formerly known as "*Streptomyces erythraeus*"), and *P. aeruginosa* [45–48].

Bacterial proteases are considered important therapeutic targets [49]. According to Suda et al, protease inhibition activity alone may improve animal survival in sepsis models, as established using a synthetic neutrophil elastase inhibitor [50]. The mechanism of action of hAAT in relation to reducing bacterial burden may thus include its antiprotease activities.

In a related preliminary study (data not shown), exogenous hAAT pretreatment of infected WT mice reduced bacterial load but was less protective of organs. One explanation relates to the compartment in which hAAT is deposited, as bacterial load reduction in the peritoneum may involve the activity of locally injected hAAT, while changes in organ damage and lethality may require more-sustained systemic hAAT. Another explanation can be that hAAT transgenic mice are exposed to hAAT beginning at parturition and are conditioned with sustained levels of ectopically produced unregulated levels of hAAT, possibly representing superior distribution.

The ability to facilitate bacterial clearance by hAAT is a nonobvious added function to its ability to modulate inflammation. Since clinical use of hAAT is safe and readily available, pretreatment of patients with hAAT may be beneficial as a preventive measure before either hospitalization, an immunosuppressive course, or elective surgical procedures, yet such avenue deserves further investigation. The optimal dose and timing, and the complete mechanism of action behind these positive phenomena, necessitate further studies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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