Study of the mechanism behind the bacterial-load reducing activity of α-1-antitrypsin

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Approved by the advisor

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Research-Student's Affidavit when Submitting the Doctoral Thesis for Judgment

I Ziv Kaner, whose signature appears below, hereby declare that
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_✓_ I have written this Thesis by myself, except for the help and guidance offered by my Thesis Advisors.

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___ This Thesis incorporates research materials produced in cooperation with others, excluding the technical help commonly received during experimental work. Therefore, I am attaching another affidavit stating the contributions made by myself and the other participants in this research, which has been approved by them and submitted with their approval.

Date: _____ Student's name: Ziv Kaner
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Abstract

Background. Severe bacterial infection can cause sepsis, multiple organ dysfunction syndrome (MODS) and death. Human alpha-1-antitrypsin (hAAT) is an anti-inflammatory, immune-modulating and tissue-protective circulating serine-protease inhibitor that rises in the blood during acute phase responses. hAAT reduces the levels of inflammatory cytokines and chemokines while increasing those of anti-inflammatory proteins; nonetheless these outcomes are observed primarily during sterile immune responses. Plasma purified hAAT is indicated as an augmentation therapy for patients with genetic deficiency in hAAT, and it is presently evaluated in multiple clinical trials as a therapeutic for various inflammatory conditions in patients that are *not* hAAT-deficient. Unlike other anti-inflammatory molecules, hAAT augmentation displays reduction of infection frequency and bacterial burden through an unknown mechanism. While hAAT has no direct antibacterial properties, it has been shown to block bacterial growth upon *S*-nitrosylation (S-NO-hAAT) in the presence of excess local nitric oxide levels. Nonetheless, it has yet to be determined how the bacterial burden is reduced under anti-inflammatory conditions, and what is the effect of S-NO-hAAT on innate cell responses.
Aim. To investigate host immune cell responses during acute bacterial infections under conditions of elevated hAAT levels, and to analyze the properties of *S*-NO-hAAT with focus on immune cell responses during bacterial infection.
Methods. Peritonitis and sepsis animal models were performed using wild-type (WT) mice and mice transgenic for hAAT. Bacterial load, as well as MODS, cell activation markers, neutrophil infiltration, levels of circulating immune mediators and animal survival rates were determined. Formation of S-NO-hAAT *in-vivo* was assessed in lungs of transgenic mice 24 hours after intranasal LPS instillation (0.3 mg/kg) using the SNO-RAC method for pull-down of nitrosylated proteins. S-NO-hAAT was studied *in-vitro* using clinical-grade hAAT that underwent chemical *S*-nitrosylation in the presence of a nitric oxide donor. S-NO-hAAT was introduced to cultures of peritoneal macrophages, either alone or in the presence of LPS (10 ng/ml) and macrophage activation was evaluated. For control, equimolar concentrations of *S*-nitroso-glutathione (GSNO) were used. Signaling pathways were evaluated by addition of specific inhibitors and by

analysis of phosphorylation targets. The antibacterial effect of S-NO-hAAT was examined using live *Salmonella typhi* both directly in culture and indirectly in a cell-mediated manner using THP-1 cell line. To assess whether general physical properties are distinct between hAAT and S-NO-hAAT, thermal stability was determined.

Results. *In-vivo*, hAAT significantly reduced liver, pancreas and lung damage, as well as infection-induced leukopenia. Twenty-four-hour survival rates were significantly improved and peritoneal bacterial load was reduced. In examining the initial hours after bacterial inoculation, hAAT unexpectedly amplified the levels of pro-inflammatory mediators and neutrophil influx. However, 72 hours after infection, inflammatory markers were again markedly reduced. Formation of S-NO-hAAT was observed in inflamed lungs of hAAT-transgenic mice. *In-vitro*, in the absence of added LPS, hAAT reduced TNFα levels 1.9-fold and *S*-NO-hAAT increased TNFα levels 4.3-fold, compared to untreated cells. In the presence of LPS, hAAT and GSNO significantly reduced TNFα levels while S-NO-hAAT did not. After 6 hours of incubation, S-NO-hAAT induced transcript levels of IL-1β, TNFα, KC and IL-6, as well as iNOS and TLR2 (ranging 1.3- to 3.6-fold relative to untreated cells).

S-NO-hAAT was found to have some direct antibacterial properties, yet significantly lower concentrations of S-NO-hAAT were required in order to alter cells phenotype towards antibacterial and reduced CFU count. This change was achieved using S-NO-hAAT both as pre- and post-treatment. Structurally, S-NO-hAAT was found to be significantly more heat-labile compared to hAAT.

Conclusions. hAAT reduces bacterial burden after infection. The molecule displays a time-dependent dual-function activity profile that favors bacterial clearance and protection of organs from excessive inflammation. Nonetheless, the duality of hAAT appears to be context-specific, involving *S*-nitrosylation in a nitric oxide rich environment, which may play a role in enhancing bacterial clearance, in part by acting as a macrophage-stimulating agent. The established anti-inflammatory properties of hAAT are thus postulated to occur distal to the site of infection. The mechanism behind this duality involves P38 activation by added S-NO-hAAT. In this regard, it is possible that nitrosylation-induced structural changes in hAAT affect its interaction with various binding partners, thus modulating immune signaling to, ideally, minimize tissue

vulnerability and maximize the performance of innate cells towards a more effective host defense, as observed during physiological acute phase response.

These outcomes support the concept that prolonged hAAT treatment, although anti-inflammatory, is safe, and may render hAAT treatment an attractive preemptive approach for individuals at risk of bacterial infection.

Abbreviations

hAAT, human α1-antitrypsin;

S-NO-hAAT, S-nitroso-hAAT;

MODS, multiple organ dysfunction syndrome;

WHO, world health organization;

SERPIN, serine protease inhibitors;

RCL, reactive center loop;

GSNO, S-nitrosoglutathione;

TNFα, tumor necrosis factor α;

AST, serum aspartate aminotransferase;

ALT, alanine aminotransferase;

IL-, interleukin-;

KC, keratinocyte chemoattractant

MCP-1; monocyte Chemoattractant Protein-1

NO, nitric oxide;

GvHD, graft-versus-host disease

T1D, type 1 diabetes;

CF, cystic fibrosis;

WT, wild type;

CD, cluster of differentiation;

PBS, phosphate buffer solution;

CLP, cecal ligation and puncture;

MOI, Multiplicity of infection;

CFU, Colony-forming unit;

LB, luria broth

DTT, dithiothreitol;

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

Asc, ascorbate;

BCA, bicinchoninic acide;

NEM, n-ethylmaleimide;

MSA, multiple sequence alignment;

DTNB, ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid));

DSC, differential scanning calorimeter;

MAPK, mitogen-activated protein kinase;

JNK, c-jun n-terminal kinases;

P38, P38 mitogen-activated protein kinases;

ERK, extracellular signal-regulated kinase;

GAPDH, glyceraldehyde 3-phosphate dehydrogenase;

iNOS, inducible nitric oxide synthase;

TLR2, Toll-like receptor 2;

ThG, thioglycolate;

Cys, cysteine;

Lys, lysine;

Met, methionine;

CREB, cAMP response element binding;

RSK, ribosomal s6 kinase;

DMSO, dimethyl sulfoxide

Introduction

Bacterial infections are a major health concern, especially for individuals that are immune deficient or frequently hospitalized. Severe infections can lead to bacteremia, sepsis and septic shock, as well as to multiple organ dysfunction syndrome (MODS) and death [4, 5]. According to the world health organization (WHO), in 2008 more than 10 million people died from microorganism-related infections globally [6]. In the past few decades, this problem displayed an alarming rise as a result of the appearance of multiple treatment resistant bacterial strains [7, 8]. The swift evolving rate of resistant bacteria is further disturbing when compared to the slow rate of development of new antibacterial therapies [7, 9], although anti-infective drugs are still attractive commercially, representing the third largest therapeutic sector [10]. A major research goal is thus the development a novel approach, that is safe and efficient against bacterial infection, with minimal chance for emergence of bacterial resistance.

SERPIN superfamily and α1-antitrypsin

Serine protease inhibitors (SERPIN) are the largest and most widely distributed protease inhibitor superfamily; they include gene members in organisms as diverse as animals, plants, algae, viruses, bacteria and archaea [11, 12]. Although SERPINs are mostly characterized by their protease inhibition activity, some family members have gained new non-inhibitory-related roles [13].

This superfamily can be divided into 16 clades based on their evolutionary source, clades A-I exist in higher animal clusters [12]. Human α1-antitrypsin (hAAT or SERPINA1) is a 52kDa glycoprotein, which is considered the prototypical member of clade A, which all contain extracellular antitrypsin-like proteins.

hAAT is produced mainly by hepatocytes and circulates at steady-state levels of 0.9–1.75 mg/ml [14] with a biologic half-life of 3-5 days in humans [15]. As an acute phase protein [16], circulating levels of hAAT increase more than fourfold during acute phase responses, including infections acute phase responses.

As its name implies, hAAT was first discovered for its ability to inhibit trypsin [17]. However, it was later established that its greater affinity is towards neutrophil-related proteases, such as neutrophil elastase, proteinase 3 and cathepsin G [18, 19].

The serine-protease inhibition site within hAAT is located in the reactive center loop (RCL) that mimics amino acid sequence that fits the protease and serves as a trap; when a target protease is introduced to hAAT, it proteolytically cleaves the substrate-like site and releases a short peptide from hAAT [20]. However, unlike with the true substrate of the protease, it forms an irreversible covalent bond with hAAT, followed by a rapid conformation change in hAAT and a unique complex formation. Unlike the more conserved sequence inside the protein core [21], RCL is highly varied between SERPINs and directly dictates their affinity towards particular proteases; for example, in the rare hAAT Pittsburgh mutation, an Arg residue replaces Met^{358} in the RCL, dramatically increasing the affinity of hAAT towards thrombin [22] and other coagulant factors [23], and resulting in a fatal bleeding disorder.

hAAT appears to protect tissues from damage and proteolytic degradation. Since the 80's [24] hAAT augmentation therapy has been used to treat patients with genetic hAAT deficiency in order to slow lung emphysema progression [25-29]. Emphysema can also develop upon long exposure to tobacco smoke, without genetic deficiency; it has been established that hAAT is oxidized and locally neutralized in lungs of smokers [30-32]. However, hAAT augmentation therapy may decrease endothelial cell apoptosis [33, 34] and tissue breakdown [35], and therefore is proposed as a treatment to delay emphysema in smokers.

Until recently, hAAT deficiency-related emphysema was considered the outcome of protease/inhibitor imbalance. However, other protease inhibitors are not always sufficient in improving the pathology [36] and hAAT appears to further prevent pulmonary emphysema by inhibiting apoptosis [37]. Its anti-apoptotic effect is not restricted to lungs; it was shown to limit apoptosis of endothelial cells upon hypoxia [34, 38], preserve insulin secretion and increase survival of β-cells during exposure to cytokines and streptozotocin [39, 40] and protect hepatocytes from TNFα-induced apoptosis [41, 42]. Interestingly, hAAT was shown to inhibit the pro-apoptotic proteases, caspase-3 [43, 44]

and caspase-8 [44]. Similarly, hAAT inhibits MMPs [45], that, like the caspases, are not serine-proteases. These targets bind to hAAT at a significantly lower affinity compared to its native serine-protease targets.

Another important activity identified recently as part of the functions of hAAT is immune modulation, not unlike other SERPINs [12, 13]. Nonetheless, in the case of hAAT, this is not protease-inhibition related [46-48]. In as far as specific immunocytes, innate cells display intriguing behaviors when exposed to physiologically relevant concentrations of hAAT: dendritic cells become semi-mature and deliver a tolerogenic message to naïve T cells [49-51], and macrophages release IL-10 at the expense of IL-1β, IL-6 and TNFα; consistently, serum cytokines display an abrupt shift towards low IL-1β, low IL-6, low TNFα, low IL-8, high IL-10 and IL-1 receptor antagonist levels [50, 52-59]. All the while hAAT appears to exert no direct inhibitory effect on T cells [51, 60].

Immune modulation by hAAT is not fully understood, but it is presume to be more than a single cytokine or pathway specific effect; for example, hAAT can inhibit proteases like ADAM17 [44, 45] and caspase-1 [61], and therefore to reduce TNFα and mature IL-1β release, respectively. Another unique mechanism includes direct binding and neutralization of IL-8 [62] and TNFα receptors [54, 63, 64], and more mechanisms are expected to be discovered in the near future.

In accordance with its notable safety profile [14, 65] and its immune-modulatory attributes, various protocols using weekly hAAT infusions are currently evaluated for inflammatory pathologies in non-deficient individuals [66, 67]. Examples for immune conditions that were highly responsive to treatment with hAAT in preclinical studies include models of islet transplantation and type-1-diabetes [50, 51, 59, 65, 68-71], graft-versus-host-disease [72, 73] [74], ischemia-reperfusion injury [61, 75, 76], rheumatoid arthritis [77, 78] and multiple sclerosis [79]. According to the preclinical promising results, several phase II clinical trials presently infusion of hAAT to non-deficient patients, replicating the serum levels that would be present during physiological acute phase responses. These include treatment-resistant GvHD (NIH clinical trials registries NCT01523821, NCT01700036) and recently diagnosed type 1 diabetes (NCT02005848 and NCT01661192; completed: NCT01319331 [80], NCT01183455, NCT01304537 and

NCT01183468).

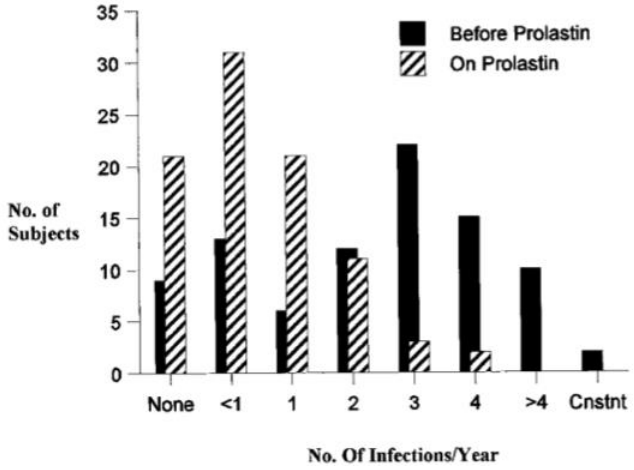

Figure 1. hAAT augmentation therapy reduces lung infections in patients. hAAT-deficient patients (ZZ phenotype), frequency of lung infections *before* (solid) and *during* (dashed) standard hAAT augmentation therapy[3].

While the anti-inflammatory profile of hAAT would seem to pose as a risk for opportunistic infections, individuals are *not* predisposed to infections during physiological 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-treated patients exhibit a significant reduction in bacterial colonization frequencies across several independent clinical studies (**Fig. 1** and [3, 14, 81]). Interestingly, a reduction in pulmonary *P. aeruginosa* bacterial burden has been established in patients with cystic fibrosis (CF) during hAAT inhalation therapy [82], a phenomenon that was corroborated in animal models [83, 84]. Moreover, this effect is not lung specific; cirrhosis patients with genetic hAAT deficiency are more susceptible to spontaneous bacterial peritonitis than in cirrhosis non-deficient individuals [85]. The mechanism behind hAAT's ability to provide an anti-inflammatory environment without interfering with bacterial clearance has yet to be determined.

S-nitrosylation of α1-antitrypsin

At infection sites, nitric oxide (NO) is produced in massive quantities, especially by innate immune cells, like macrophages [86, 87]. Nitric oxide has many activities, among them toxicity towards bacteria through several different mechanisms [88-91]. Nitric oxide is a reactive free radical and has a very short biological half-life [92]. Thus, importantly, its biological activity can be protected in a more stable form of S-nitrosothiols [93] or S-

nitrosylated albumin [94]. Therefore, most of the nitric oxide in the circulation is literally carried on proteins that serve as endogenous nitric oxide reservoirs [94-96].

S-nitrosylation is a post-translational protein modification, that is defined as a covalent bond between nitric oxide and a sulfur atom on a cysteine residue. Unlike other post-translational protein modifications, we do not know whether the S-nitrosylation process is a spontaneous chemical event or rather enhanced by enzymatic activity. Some enzymes are known to remove NO from the nitrosylated amino acid and are presumed to be relevant to bound-NO homeostasis [97-100].

By now, protein S-nitrosylation is considered a modification that alters and regulates protein function and gene transcriptions, as well as cell activities such as inflammation [101-103] [104, 105] and survival [106-109]. In light of this, it is not surprising that S-nitrosylation is related to a plethora of pathologies [97, 110, 111].

hAAT has a single cysteine residue at position 232, which has an extremely low pKa [112] and is highly reactive [113], especially in a slightly acidic pH, such that is common at inflamed sites. In hAAT, this position is surrounded by three lysine residues (positions 233, 234 and 274), that can cause deprotonation of the thiol in cysteine. The combination of low pKa, local positive charge and an exposed surface cysteine are factors known for increasing the chance of cysteine S-nitrosylation [114-116].

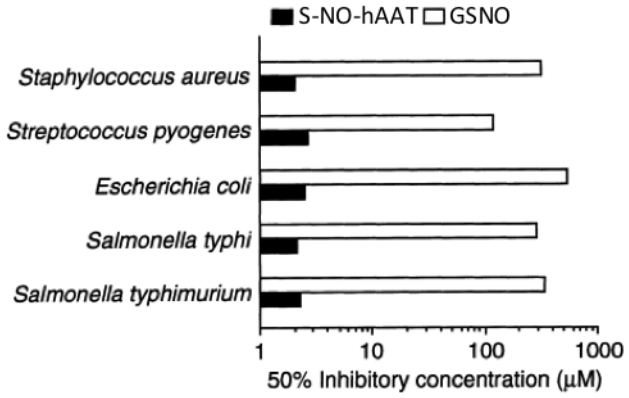

Figure 2. S-NO-hAAT inhibits growth of multiple bacterial strains in culture media. MIC50 of indicated bacterial strains in the presence of S-NO-hAAT compared to GSNO [1].

Although hAAT was shown to undergo S-nitrosylation (S-NO-hAAT) in previous studies, this phenomenon was relatively ignored compared to other hAAT modifications. One interesting function gained by S-nitrosylation of hAAT is growth arrest of bacteria of diverse strains (**Fig. 2**) [1, 117].

Research aims

In the present study, we investigate two main aspects of the bacterial burden reducing outcome of hAAT therapy; (1) the prognosis of extra-pulmonary infections under conditions of chronically elevated circulating hAAT and (2) investigate its direct effect on immune cells.

Materials and Methods

Animals

Mice transgenic to human AAT (background strain C57BL/6, kind gift from Churg A, University of British Columbia, Vancouver, Canada [118]), were bred in-house and genotyped routinely, as described [51]. 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 model

Fecal suspension injection protocol was performed as described [119] with minor modifications. Briefly, 4.0±0.5 grams freshly collected WT mouse feces were suspended in 30ml PBS (Sigma-Aldrich, Rehovot, Israel) by aggressive shaking at room temperature until reaching uniform consistency. The suspension was filtered through a double-layer surgical gauze, followed by filtration through a 70μm cell-strainer (BD Falcon^{TM}, Becton Dickinson, NJ). 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 (200μl per mouse, i.p.); this dose is the result of a dilution calibration, to 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 i.p. introduction of thioglycolate (3% v/v, 1ml per mouse; Sigma-Aldrich).

Cecal ligation and puncture (CLP) model

Minor modifications were made to the CLP procedure based on protocols described elsewhere [120]. Briefly, after anesthetizing the mice with ketamine and xylazine, the cecum was exposed through a 1cm abdominal midline incision. Ligation was performed 5mm from the cecal tip, and the cecal stump was punctured once with a 25G needle. A 1mm segment of stool was extruded into the peritoneal cavity, and the incision wound was stitched closed. Resuscitation of the animals was comprised of slow s.c. administration of 1ml, 37°C saline. Sham controls included mice subject to the same procedure, but with the cecum stump left intact.

Peritoneal lavage, whole blood and serum collection

Mice were sacrificed at indicated time points after induction of peritonitis. For peritoneal lavage production, 8ml ice-cold PBS were introduced i.p., then recovered using an 18G needle. In the peritonitis models, CFU count was determined in the fluid phase and cells were transferred to FACS analysis. Blood was collected by cardiac puncture to BD microtainer® (SST™ and K2E™ tubes, Becton Dickinson) 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 aspartate aminotransferase (AST), alanine aminotransferase (ALT) and pancreatic amylase were determined by clinical assay kits (all from Beckman Coulter, Switzerland). Serum, lavage or cell medium levels of TNFα and IL-6 were determined by ELISA (PeproTech, Israel), levels of IL-1β, IL-12, KC, MCP-1, IL-10 and IL-17 were determined by Q-Plex mouse cytokine chemiluminescence-based ELISA (Quansys Biosciences, Logan, UT).

Scoring of lung tissue damage

Lungs were removed from six randomly chosen mice from each group and fixed in 4% formalin. Paraffin sections were stained with H&E. Histologic examination was performed on blinded coded samples by two independent investigators, and scored as described elsewhere [121]. 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 four parameters.

Leukopenia evaluation

After whole blood collection, WBC count was determined using ADVIA® 2120 (Siemens). Bone marrow cells were isolated from single tibiae per mouse, washed in PBS and filtered through a 70μm cell-strainer. Samples were stained by trypan blue and cell number was determined by Countess® Automated Cell Counter (Life Technologies, NY).

FACS analysis

Cytomics FC 500 Beckman Coulter, BD Canto II was used for analysis. After washing with FACS buffer (PBS; 1% BSA, 0.1% sodium azide, and 2 mM EDTA at pH

7.4), 1×10^6 cells per sample were incubated with FcγRII/III blocker (BioLegend). *General peritoneal cell population* was stained with anti-CD11b pacific blue, anti-F4/80 APC, anti-CD86 PE, anti-MHCII APC/cy7 and anti-CD40 PE/cy7; *peritoneal neutrophils* were stained with anti-CD11b APC/cy7, anti-Ly6G FITS and anti-TNFα APC; R*AW 264.7 cells* (ATCC) were stained with anti-CD11b pacific blue. All antibodies were from BioLegend and diluted according to manufacturer's guidelines. Data were analyzed by FlowJo (Tree Star, Ashland, OR).

Bacterial count in-vivo

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

Bacterial growth assay

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

For S-NO-hAAT direct anti-bacterial assessment, logarithmic phase *Salmonella typhi* were washed with PBS, counted by OD_{600nm} using a spectrophotometer and seeded for final concentration of 1×10^5 per ml. The bacteria were introduced to several concentrations of S-NO-hAAT in comparison to hAAT and GSNO in the same molar ratio (diluted in PBS) for 2 hours at 37°C in the dark. Concentrated LB was added for its final optimal concentration for additional 8 hours. Relative bacterial population size was estimated by determining OD_{600nm} using an ELISA plate reader.

Bacterial uptake assay

RAW 264.7 cells were seeded in RPMI 1640 supplemented with 10% FCS in 60mm cell culture petri dishes, until reaching 80% confluence. After ×3 washes with PBS, cells were immersed in serum-free medium (RPMI 1640 supplemented with BIOGRO-2, Biological Industries, Ltd.) in the presence or absence of 0.5mg/ml hAAT, for 4 hours. *E. coli* (strain ER2566) expressing pSH21::his6-gfp plasmid (kind gift from Gur E, Ben-

Gurion University of the Negev, Israel) were grown on LB with ampicillin (100μg/ml, Sigma-Aldrich) at 37°C. Upon reaching logarithmic phase, IPTG (1mM, Sigma-Aldrich) was added and the temperature lowered to 30°C for 3 hours. Bacteria (MOI 100) were added onto RAW 264.7 cells for 20 minutes at 37°C then washed ×5 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

RAW 264.7 cells were plated in 48-well plates (0.5×10^5 per well in quadruplicates). Cells were allowed to adhere for 3 hours at 37°C in RPMI 1640 containing 10% FCS. The cells were then washed thoroughly with PBS and incubated for 24 hours with or without 0.5mg/ml hAAT in serum-free media. *E. coli* (strain ER2566) were added to the cells (MOI 100) and the plates were centrifuged at 2,500 rpm for 5 minutes; plates were then placed at 37°C for 15 min. Five washings steps with PBS were performed in order to remove free bacteria. Cells were then lysed with sterile double-distilled water in order to determine baseline readout (t-0). Serum-free media 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 out of t-0.

S-NO-hAAT mediated bacterial killing was carried out using human monocyte cell line, THP-1 cells. The THP-1 was maintained in RPMI 1640 containing 5 % (v/v) heat-inactivated fetal calf serum, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 % modified Eagle's medium with nonessential amino acids. For macrophage infection, the cells were plated in 24-well plates (0.5×106 per well in quadruplicates) and 40 ng/ml phorbol 12- myristate 13-acetate was added for 24 hours at 37°C for their differentiation, followed by RPMI wash. Macrophage infection was performed as described previously [122] with minor modifications. Briefly, *S. typhi* were grown on LB at 37°C until reaching logarithmic phase. The bacteria were counted by OD_{600nm} using spectrophotometer and opsonized by 10% human serum in RPMI, at 37°C in rotation for 30 minutes. The pretreated experiments were carried out by treating the cells with 27.5 μM S-NO-hAATin comparison to hAAT and GSNO in the same molar

ratio for 24 hours. The cells were washed and introduced to opsonized bacteria (MOI 1:10), followed by 5 minutes centrifugation at 800g and 30 minutes incubation at 37°C. To eliminate extracellular bacteria, the cells were washed 3 times and incubated for 2 h with medium containing 100 mg/ml gentamicin to kill extracellular bacteria, and then with 12 mg/ml gentamicin for additional 4 hours. Cells were washed 3 times and lysed by addition of sterile 0.5 ml 0.1 % (w/v) sodium deoxycholate in PBS per well, and the number of surviving bacteria was determined by ten-fold serial dilutions plated on LB agar plates. The post-treatment experiments were similar to the pre-treatment, with minor modifications; the opsonized bacteria were introduced to naïve cells (MOI 1:5) as mentioned. After the centrifugation, incubation and wash, medium with 100 mg/ml gentamicin and the treatments (S-NO-hAAT/ hAAT/ GSNO) was added for two hours, followed by replacing to 12 mg/ml gentamicin with another dose of treatments for 24 hours, and lysis as mentioned.

hAAT nitrosylation and S-NO-hAAT in-vitro measurements

hAAT (20 mg/ml or 450 µM) was reduced by incubation with 50 mM DTT (Sigma) for 10 min at 37 °C. Excess reducing agent was removed by gel filtration using Sephadex G-25 columns (GE Healthcare) equilibrated in the nitrosylation buffer (25 mM HEPES pH 7.4, 0.1 mM EDTA, 0.2 mM diethylenetriaminepentaacetate, 10 µM neocuproine, 100 mM NaCl). Reduced hAAT was then incubated for 30 minutes with 1000 µM Diethylamine NONOate (Cayman Chemical, Michigan, USA) followed by adding additional 500 µM Diethylamine NONOate for more 30 minutes at 37 °C. After excess NO donor was removed by Sephadex G-25 chromatography, the S-nitrosylation efficiency was calculated by measuring protein concentration and number of nitrosylated thiols. Protein concentration was determined using Bicinchoninic acide (BCA) protein assay kit (Santa Cruz) with hAAT in known concentrations as a calibration curve. hAAT's Nitrosylated thiols quantification was carried out by Saville-Griess assay [123]. Briefly, S-NO-hAAT was incubated in assay buffer (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dehydrochloride, 1% HCl) in the absence or presence of 1 mM $HgCl_2$ for 30 min, and absorbance readings were measured at 540 nM by ELISA plate reader. *S*-nitrosoglutathione (GSNO) treated identically served as a standard curve, and mercury-dependent absorbance was converted to SNO concentrations. hAAT's

nitrosylation efficiencies were more 50%. After production, S-NO-hAAT was aliquoted into dark tubes and stored at -80°C for a single thaw. Saville-Griess assay was also used for S-NO-hAAT transnitrosylation; after treating peritoneal macrophages with 100 mM N-Ethylmaleimide (NEM) or PBS for 15 minutes at R.T., they were introduced to S-NO-hAAT. Medium supernatant samples were taken in indicated time points, and the S-nitrosylated protein was measured by Saville-Griess assay.

hAAT sequence analysis.

Mammalian AAT-like protein sequences were collected using the NCBI BLASTp web interface with default parameters. The query sequence was hAAT variant (version ABG73380.1). For the database option, a non-redundant protein sequence (nr) was chosen and 19 more random sequences were chosen from diverse mammals (**Table 1**). For Multiple Sequence Alignment (MSA), all the amino acid sequences were inserted in FASTA format, and analyzed by Clustal Omega [124].

Table 1: Mammalian antitypsin-like sequences

Scientific name	Common name	order	Accession	Identity
Homo sapiens	Human	Primates	ABG73380	100%
Pan paniscus	Bonobo	Primates	XP_003832864.1	99%
Macaca mulatta	Rhesus macaque	Primates	NP_001252946.1	91%
Canis lupus familiaris	Domestic dog	Carnivora	NP_001073578.2	75%
Sus scrofa	Wild boar	Artiodactyla	NP_999560.1	74%
Rattus norvegicus	Brown rat	Rodentia	NP_071964.2	69%
Ovis aries	Domestic sheep	Artiodactyla	NP_001009799.1	69%
Bos taurus	Cattle	Cetartiodactyla	NP_776307.1	69%
Mus musculus	House mouse	Rodentia	NP_033270.3	64%
Tursiops truncatus	Common bottlenose dolphin	Cetacea	XP_004320745.1	73%
Oryctolagus cuniculus]	European rabbit	Lagomorpha	NP_001075666.1	64%
Odobenus rosmarus divergens	Walrus	Carnivora	XP_004394562.1	73%
Myotis davidii	David's myotis	Chiroptera	XP_006768021.1	72%
Pteropus alecto	Black flying fox	Chiroptera	XP_006925619.1	73%
Felis catus	Domestic cat	Carnivora	XP_006933138.1	72%
Lipotes vexillifer	Baiji	Cetacea	XP_007447379.1	68%
Physeter catodon	Sperm whale	Cetacea	XP_007118979.1	74%
Erinaceus europaeus	European hedgehog	Eulipotyphla	XP_007529139.1	64%
Mustela putorius furo	Ferret	Carnivora	XP_004754804.1	44%
Ceratotherium simum simum	Southern white rhinoceros	Perissodactyla	XP_004434291.1	56%

Table 1: Mammalian antitypsin-like sequences

Differential scanning calorimeter (DSC)

Differential scanning calorimeter (DSC). The thermal stability experiments were performed on a VP-DSC (Microcal, USA). The proteins were diluted to final concentration of 0.5 mg/ml and a volume of 0.6 ml was examined. Nitrosylation buffer served as a blank. In order to assess the pH influence on hAAT's thermal stability, nitrosylation buffer was adjusted, and the proteins underwent gel-filtrattion using Sephadex G-25 columns. Scan rates of 1°C/min and high gain were used. Each sample was scanned three times. Data analysis was performed by the Microcal Origin 7.0 software.

hAAT analysis on native and SDS PAGE

20 mg of unmodified and nitrosylated hAAT were analyzed on 12% native and SDS denaturative acrylamide gel, followed by Coomassie brilliant blue staining. For native PAGE, the proteins were first diluted with sample buffer (0.2 M Tris-HCl pH 6.8, 10% v/v Glycerol and 0.2% Bromophenol blue) and separated on ice using cold running buffer, without supplement of SDS or reducing reagents. The SDS-PAGE analysis was carried out after 5 minutes of boiling (95°C) the samples with Laemmli sample buffer.

Peritoneal macrophage isolation and activation

Mice were sacrificed 4 days after 2 ml thioglycolate i.p. injection. For peritoneal lavage production, 8ml ice-cold PBS were introduced i.p., then recovered using an 18G needle. The cells were counted and left to adhere for at least 2 hours in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine and 1% Penicillin-Streptomycin (all from Biological Industries, Ltd.). All the *in-vitro* activation experiments were carried out using RPMI with 5% FCS medium and 27.5 μM of S-NO-hAAT and GSNO. hAAT amount was adjusted to the same final protein concentration of S-NO-hAAT. At indicated time points, the medium was used for cytokines analysis while the cells were lysed for mRNA and protein analysis as will be further detailed.

Similar macrophage activation was also used in two other experiments, after cysteine blocking and with signaling inhibition. Measuring the importance of free membranal cysteins was done by blocking them with 3mM 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) for 30 minutes at 37°C. For P38, JNK and ERK inhibition, the cells were

pretreated 30 minutes at 37°C with 30mM SB203580, SP600125 and PD98059, respectively. In both cases, after cysteine block or signaling inhibition, the cells were washed and introduced to S-NO-hAAT for 1 hour, followed by mRNA purification. DMSO was used as a vehicle in the same volume as the pretreatments in both cases.

Real-Time PCR assays

Total RNA was extracted and purified from activated peritoneal macrophages with a total RNA purification kit (Norgen Biotek Corp. Thorold, ON, Canada). After quantification of RNA with a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE) reverse transcription of RNA to cDNA was performed with the qScriptTM cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD) using 1μg of template RNA and a blend of random hexamers and oligo (dT) primers. Quantification of gene transcription was performed with the StepOnePlus Real-Time PCR system and the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The levels of gene expression were normalized to GAPDH levels, and the primers sequences are detailed in **Table 2,** from '5 to '3**.**

Table 2: Real-Time PCR primers sequences

Gene	Forward	Reverse
GAPDH	TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA
TNFα	GACCCTCACACTCAGATCATCTTC	CGCTGGCTCAGCCACTCC
IL-1β	AAAGCCTCGTGCTGTCGGACC	TTGAGGCCCAAGGCCACAGGTA
KC	AGACCATGGCTGGGATTCAC	AGTGTGGCTATGACTTCGGT
IL-6	CCAGTTGCCTTCTTGGGACT	GGTCTGTTGGGAGTGGTATCC
iNOS	TTCACTCCACGGAGTAGCCT	CCAACGTTCTCCGTTCTCTTG
TLR2	GCATCCGAATTGCATCACCG	CCTCTGAGATTTGACGCTTTGTC
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG

Table 2: Real-Time PCR primers sequences

Kinase array and western blot

After activation as previously described, peritoneal macrophages were lysed and the phosphor-proteins quantities were compered using human phosphor-kinase antibody array (R&D Systems), according to the manufacturer guidelines. The array dots were digitally analyzed using the open-source software ImageJ version 1.49 (NIH, Bethesda, MD). The lysate proteins were also denatured and separated on SDS-PAGE as previously described and transferred to PVDF membrane followed by Western blot using rabbit anti-pP38 (sc-17852-R, Santa Cruz biotech), rabbit anti-P38 (#9212, Cell signaling

technology), rabbit anti-pSAPK/JNK (#9251, Cell signaling technology), rabbit anti-SAPK/JNK (#9252, Cell signaling technology), rabbit anti-pErk1/2 (#9101, Cell signaling technology), rabbit anti-Erk1/2 (#9102, Cell signaling technology) and mouse anti-actin (MAB1501, Merk Millipore, Darmstadt, Germany). To detect primary antibody binding, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibody. The immobilized antibodies were detected with an ECL reagent (Advansta, Menlo Park, CA, USA).

SNO-RAC analysis

hAAT transgenic mice were lightly anesthetized using isoflurane and inoculated intranasally (I.N.) with 20 µl LPS (0.3 mg/kg). After 24 hours, the mice were sacrificed, and the lungs were harvested and homogenated in 2 ml lysis buffer (50 mM HEPES pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.1 mM diethylenetriamine pentaacetate, 50 mM NEM with protease inhibitors) using the gentleMACS Dissociator and M tubes (Miltenyi Biotec, Cologne, Germany) followed by supernatant collection after 15 minutes of 10,000 g at 4 °C centrifuge. The protein concentration was measured by BCA and SNO-RAC (resin- assisted capture) method was carried out [125] for isolation of the nitrosylated proteins. Briefly, a total of 6 mg protein was diluted in HEN buffer (100 mM HEPES pH 7.5, 1 mM EDTA and 0.1 mM Neocuproine) supplemented with 20 mM NEM and 2.5% SDS and blocked at 50 °C with frequent vortexing. To remove excess NEM, proteins were precipitated with 3 volumes of acetone at -20 °C for 30 min. The proteins were recovered by centrifugation at 10,000 g for 5 min, and the pellets were resuspended in HENS buffer (HEN buffer with 1% SDS). This material was added to 150 µl of Thiopropyl sepharose 6B (GE Healthcare, Little Chalfont, UK) resin in the presence or absence of 40 mM sodium ascorbate. After overnight rotation in the dark at 4 °C, the resin was washed with 4 × 1 ml of HENS buffer, then 2 × 1 ml of HENS/10 buffer (HENS diluted 1:10). Captured proteins were eluted with 30 µl of HENS/10 containing 100 mM 2-mercaptoethanol for 20 min at room temperature and analyzed in parallel to whole lung lysate by Western blotting using a goat anti-hAAT (A80-122A, Bethyl laboratories, Montgomery, TX, USA) antibody.

Statistical analysis

Analyses were performed using GraphPad Prism 5 software (GraphPad Prism 5, Pugh computers, UK). Results are expressed as the mean ± standard error of the mean. Significance of differences between groups was determined by two-tail student Mann-Whitney test. If more than two groups were compared, a nonparametric Kruska- Wallis test was used to analyze the variance. If the variance was significant, the difference between each two groups was determined by two-tail student Mann-Whitney test for statistical significance. Results are considered significant at $p \leq 0.05$.

Results

hAAT protects from polymicrobial peritonitis–induced organ failure and leukopenia

In order to assess whether hAAT protects from polymicrobial peritonitis–induced multiple organ failure, organ damage was assessed in WT mice and in mice transgenic for hAAT, 24 hours after peritoneal cavity bacterial contamination by CLP. Sham-operated mice were used as controls.

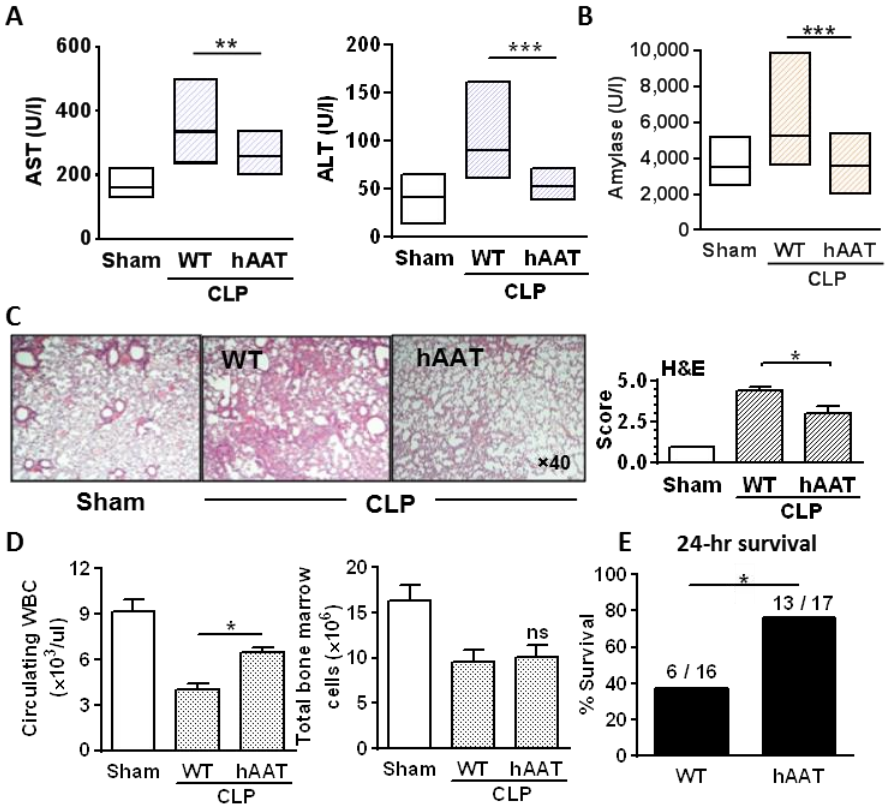

Figure 3. ***In-vivo*** **hAAT protects from polymicrobial peritonitis–induced organ failure and leukopenia.** Serum samples obtained 24 hours after CLP were examined for **(A)** AST, ALT and **(B)** amylase (n = 13). **(C)** Representative lung tissue H&E staining and lung scoring 24 hours after CLP (n = 6). **(D)** Circulating WBC and bone marrow cell count 72 hours after CLP (n = 5). **(E)** 24-hr Survival. Uniform preparations of fecal suspension introduced i.p. to WT mice and to hAAT mice. A and B; floating bars min to max, line at mean. C, D and E; mean ± SEM. For all panels * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to WT mice.

As shown in **Figure 3**, the levels of circulating markers for liver damage **(Fig. 3A)** and pancreatic damage **(Fig. 3B)** displayed a marked elevation in WT mice that underwent CLP. However, hAAT transgenic mice exhibited a limited rise in injury markers. Parameters for lung injury were assessed by histological scoring **(Fig. 3C)**. As shown, 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 leukopenia **(Fig. 3D)** was assessed alongside total bone marrow cell count 72 hours after CLP. As shown, while WBC population size was lower in WT mice that underwent CLP, it was markedly improved in the hAAT group. However, the reduction of bone marrow cells was similar between WT and hAAT mice. Twenty-four-hour survival was determined in the fecal injection model so as to minimize variance between outcomes **(Fig. 3E)**; as shown, WT mice exhibited <40% survival rate in comparison to hAAT mice (>75% survival rate). Not shown, 48-hour and 72-hour survival rates were similar in both groups.

Long-term systemic hAAT decreases polymicrobial-peritonitis–induced serum inflammatory mediators and cell activation markers.

Since systemic inflammation is the major cause of organ failure during peritonitis, we evaluated circulating cytokine levels 72 hours after CLP. As shown in **Fig. 4**, hAAT group displayed reduced circulating levels of IL-1β, TNFα, IL-6 and MCP-1.

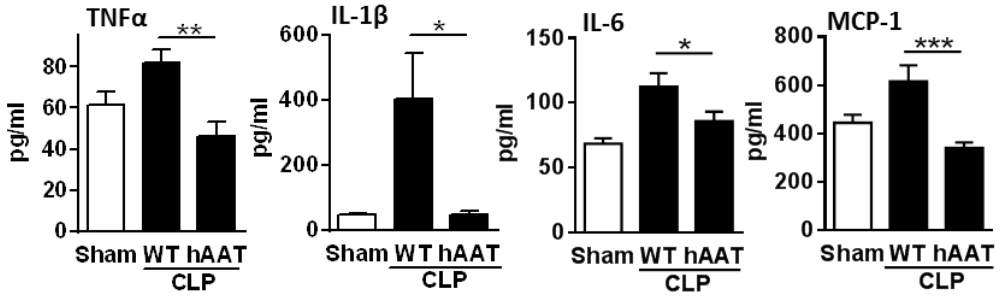

Figure 4. ***In-vivo*** **systemic hAAT decreases peritonitis-evoked circulating inflammatory mediators.** Sham-operated mice, WT and hAAT mice, 72 hours after CLP (n = 8). Serum inflammatory mediators were analysed by ELISA multiplex. Data is presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the WT mice. Results are representative of three independent experiments.

Inflammatory markers at infection site were also examined. Peritoneal cells were collected from the site of infection 72 hours after CLP. As shown in **Fig. 5**, inducible surface CD40, CD86 and MHC class II were significantly reduced in the hAAT group.

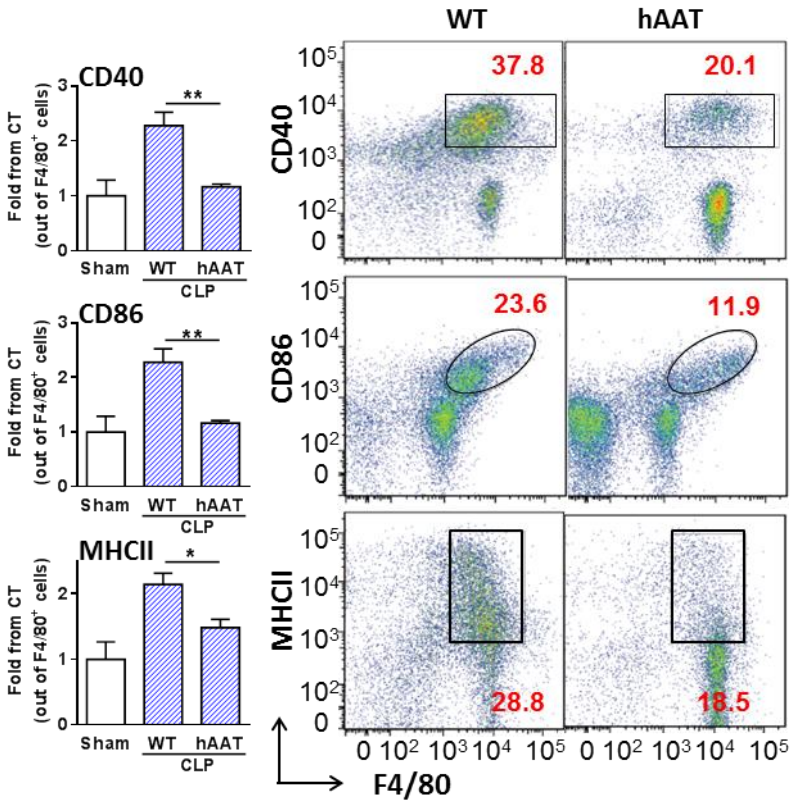

Figure 5. hAAT reduces macrophage cell-surface activation markers at infection site. Sham-operated mice, WT and hAAT mice, 72 hours after CLP (n = 8). Surface CD40, CD86 and MHCII levels on peritoneal macrophages. Results gated to F4/80^{+} cells. *Right*, representative FACS histograms. *Left*, pooled data, presented as mean ± SEM, * $p < 0.05$ and ** $p < 0.01$ compared to WT. Results are representative of three independent experiments.

While not antibacterial, hAAT significantly reduces bacterial burden in-vivo

Based on the multi-level outcomes observed thus far, the possibility that *bacterial load* might be affected by hAAT was raised. Thus, 24 hours after CLP peritoneal lavage was performed and bacterial CFU were determined. As shown in **Fig. 6A**, a significant reduction in the bacterial load was observed in hAAT-expressing mice compared to WT mice. A consistent outcome was found at 72 hours (*not shown*). However, *E. coli* (**Fig. 6B**) and peritonitis-isolated bacteria (*not shown*) that were allowed to grow in medium displayed intact growth rates when added hAAT.

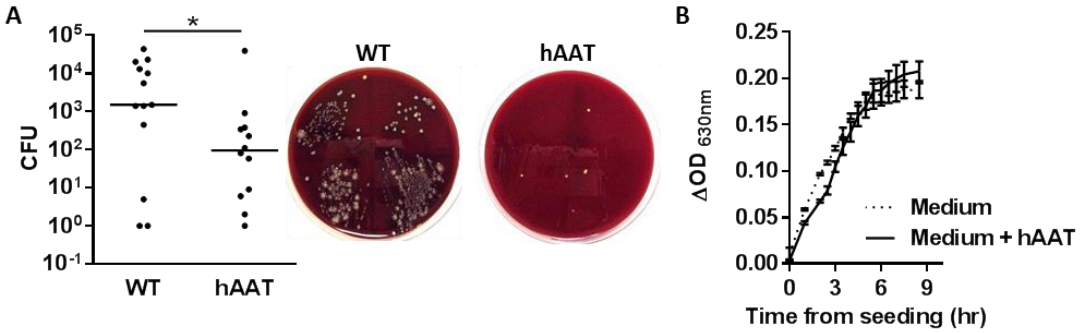

Figure 6. hAAT reduces bacterial burden *in-vivo*, but not *in-vitro*. (A) 24-hr peritoneal CFU counts from WT and hAAT mice (n = 13). *Right*, representative blood agar plates. *Left*, pooled data presented as values and median on a logarithmic scale. Representative of three independent experiments. * $p < 0.05$. **(B)** Logarithmic-phase *E. coli* grown in culture medium, in the absence or presence of hAAT in quadruplicate. Mean ± SEM.

hAAT does not interfere with macrophages bacterial uptake and killing

Major macrophage antibacterial activities, including bacterial uptake and killing, were examined *in-vitro*. Macrophages were preincubated with or without hAAT for four hours, and then washed and added GFP-expressing bacteria. Cells were then analyzed by FACS for fluorescent readout (**Fig. 7A**).

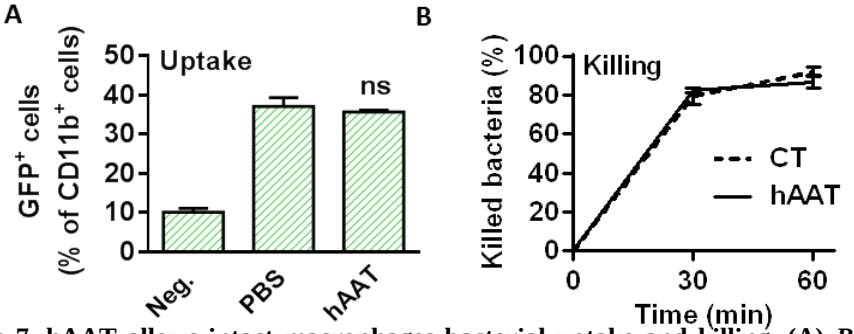

Figure 7. hAAT allows intact macrophages bacterial uptake and killing. (A) *Bacterial uptake.* hAAT pretreated macrophages co-cultured with GFP-expressing BL21 *E. coli* at 37°C. *Neg.*, negative control (cells placed on ice). Pooled data presented as mean ± SEM. **(B) *Bacterial killing.*** hAAT pretreated macrophages co-cultured with logarithmic phase BL21 *E. coli*. Post-uptake CFU converted to calculated killing capacity. Shown, percent killed *E. coli* compared to t = 0; 100% set at CFU = 0. Data presented as mean ± SEM.

As shown, bacterial uptake was undisturbed in the presence of hAAT. Based on CFU counts after bacterial uptake by macrophages (**Fig. 7B**), hAAT readily allowed macrophages to execute bacterial killing.

hAAT increases the size of the early neutrophil population during bacterial peritonitis

Neutrophil infiltration was examined shortly after bacterial-evoked peritonitis in mice. Two hours after i.p. injection of fecal suspension, peritoneal lavage was collected and examined by FACS analysis.

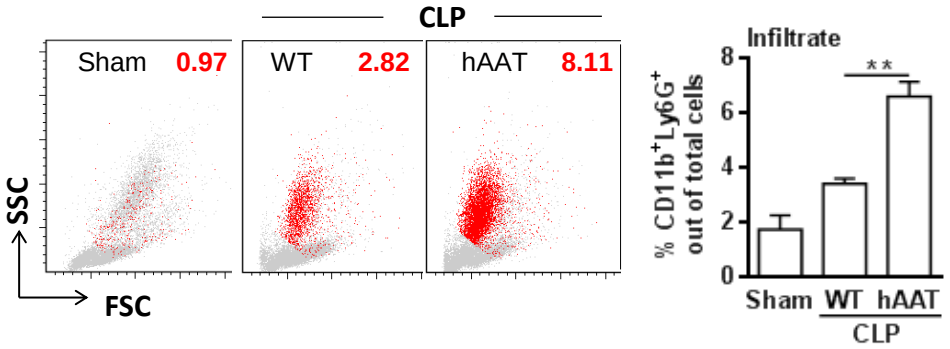

Figure 8. Early neutrophil population size is increased by hAAT during bacterial peritonitis. WT, hAAT and sham mice, 2 hours after fecal injection (n = 6). *Red*, Neutrophils ($Ly6G^+/CD11b^+$). *Left*, representative FACS images. *Right*, pooled data, presented as mean ± SEM, ** $p < 0.01$ compared to the WT mice.

As shown in **Figure 8**, a massive neutrophil infiltration was observed 2 hours after induction of peritonitis. Importantly, this population of cells was over 2-fold greater in the hAAT group.

hAAT increases local and systemic inflammatory mediators at initial hours after initiation of peritonitis in-vivo

In investigating the duality of the outcomes of elevated hAAT, i.e., reduced bacterial load with a concomitant increase in the anti-inflammatory profile, the initial hours after peritoneal stimulation were examined. Fecal injection–induced peritonitis was compared to sterile (ThG–elicited) peritonitis (**Fig. 9A**). Six hours after the inflammatory trigger, TNFα levels were measured in the peritoneal lavage fluid and in the serum. As shown, after stimulation, TNFα levels displayed a rise both in the infection model and in the sterile model; however, the outcomes in the hAAT group compared to the WT group were significantly profound only in the infective model. TNFα was also assessed 2 hours after fecal injection by measuring the proportion of peritoneal cells carrying high levels of membrane-associated TNFα (**Fig. 9B**). Accordingly, the hAAT group displayed more membrane-$TNF\alpha^{HI}$ peritoneal cells, a time point that precedes measurable TNFα release into the peritoneal compartment. Selected circulating cytokines and chemokines were

determined 24 hours after CLP (**Fig. 9C**); unlike the previously observed 72-hour anti-inflammatory profile in the infected hAAT group, here at 24 hours post-infection, the group displayed an early inflammatory spike.

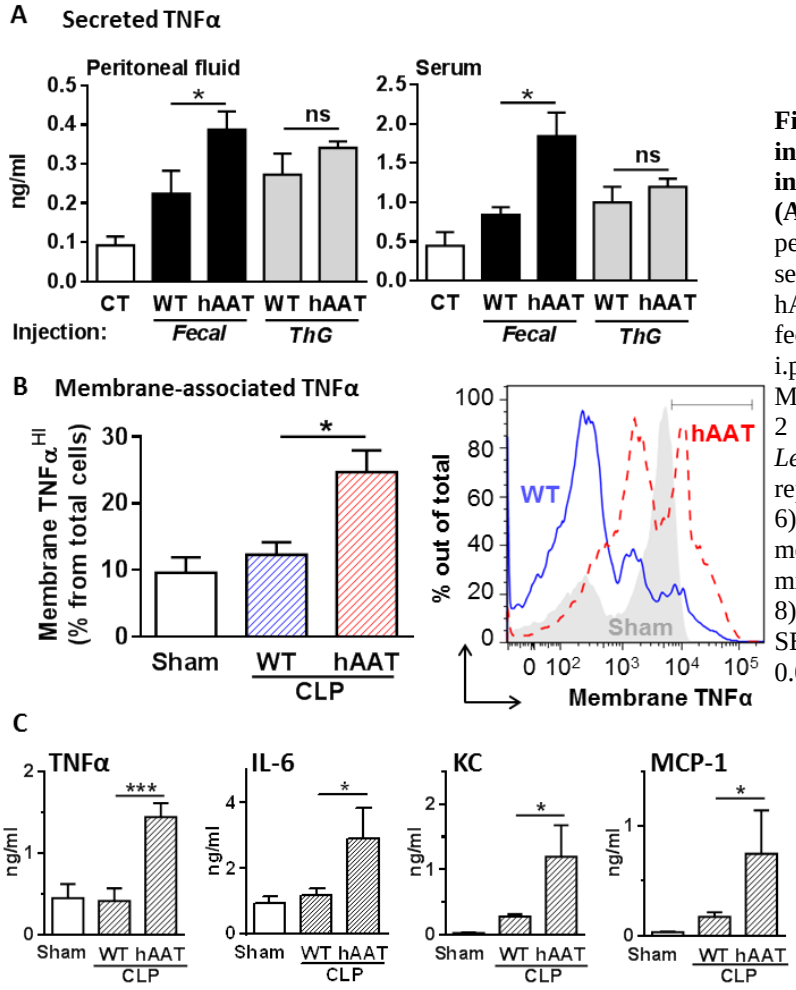

Figure 9. hAAT increases inflammatory mediators at initial hours post-infection. **(A)** TNFα levels in peritoneal fluid (*left*) and serum (*right*) of WT and hAAT mice, 6 hours after fecal or thioglycolate (ThG) i.p. injection (n = 8). **(B)** Membrane-associated TNFα, 2 hours after fecal injection. *Left*, pooled results; *right*, representative overlay (n = 6). **(C)** Serum inflammatory mediators. WT and hAAT mice 24 hours after CLP (n = 8). Data presented as mean ± SEM, * $p < 0.05$ and *** $p < 0.001$ compared to WT.

Structure and conservation of cysteine and lysine residues

hAAT crystal structure (**Fig. 10A**) reveals that the surface cysteine is surrounded by three positively-charged lysine residues (Lys^{233}, Lys^{234} and Lys^{274}), without nearby negatively-charged amino acids (e.g., glutamate or aspartate). This configuration is consistent across a variety of 20 hAAT-like sequences from diverse mammalian species, as determined by Multiple Sequence Alignment (MSA, **Fig. 10B**). Interestingly, Lys^{274} is highly conserved with 95% lysine or other positive amino acids (e.g., arginine or histidine) and no negative amino acids in any of the sequences at the same position. Lys^{234} is moderately conserved with 60% positive amino acids and no negative amino

acids in any of the sequences. However, Lys[233] is not conserved; only 15% of the sequences have positively charged amino acids at that position, and 60% have a negative charge.

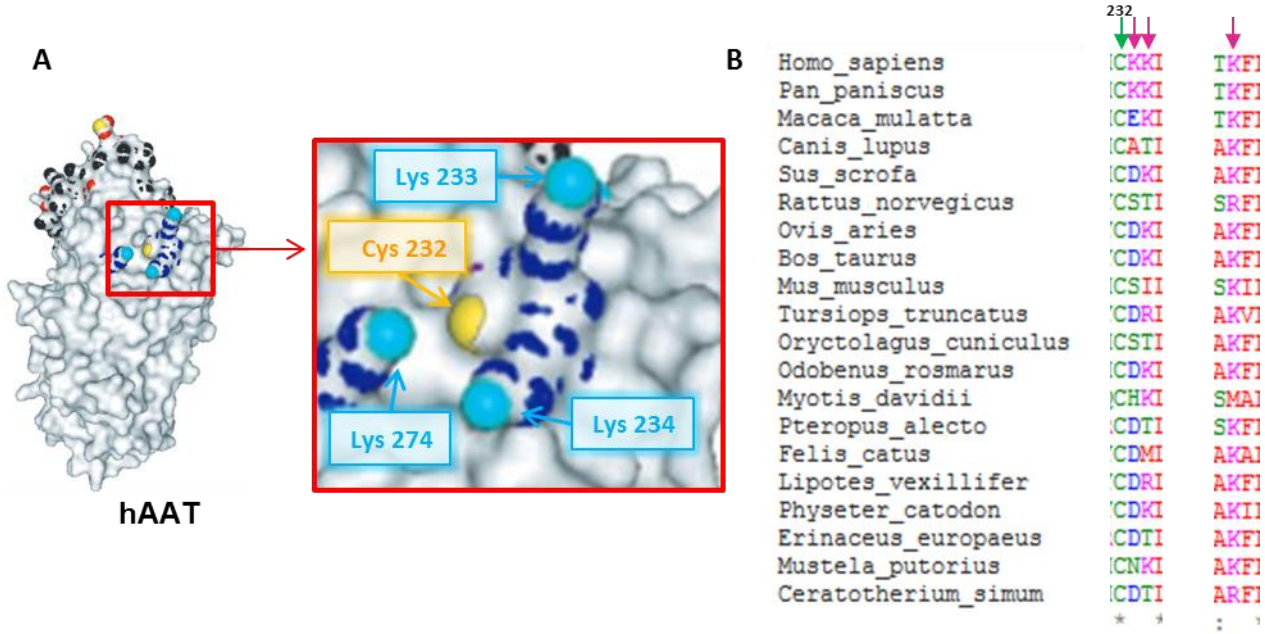

Figure 10. hAAT contains a single surface cysteine residue surrounded by lysine residues in a conserved manner. (A) hAAT crystal structure. *Yellow*, Cys[232]; *blue*, Lys[233, 234, 274]; *black*, RCL (modified from [2]). **(B)** Multiple sequence alignment (MSA) and Evolutionary Conservation in mammals. Cys[232] (*green arrow*); Lys (three *pink arrows*).

Biochemical properties of S-NO-hAAT

S-NO-hAAT was chemically produced and compared to unmodified hAAT. Analyzed S-NO-hAAT on SDS-PAGE (**Fig. 11A** *top*), S-NO-hAAT appeared as a 52 kDa protein, much like unmodified hAAT, suggesting that S-nitrosylation did not cause protein cleavage or aggregate formation. However, in examining a non-denatured form of S-NO-hAAT using native gel analysis (**Fig. 11A** *bottom*), S-NO-hAAT migrated slightly less than unmodified hAAT, supporting the notion that a structural change had occurred. Such conformational change would most probably result in an altered stability profile; thus, thermal stability was deterined using Differential Scanning Calorimetry (DSC). As shown (**Fig. 11B** *left*), S-NO-hAAT appears to be more temperature sensitive than unmodified hAAT. Moreover, the denaturation plot is asymmetrical, and requires division into two denaturation plots (**Fig. 11B** *right*): the first represents a partial unfolding of the protein, while the second represents the final denaturation towards the

primary structure. Shown in *red*, S-NO-hAAT demonstrates a lower first and second Tm values. Consistent results were obtained at three different pH conditions (**Fig. 11C**); S-NO-hAAT appears to be a molecule with an inferior stability compared to unmodified hAAT.

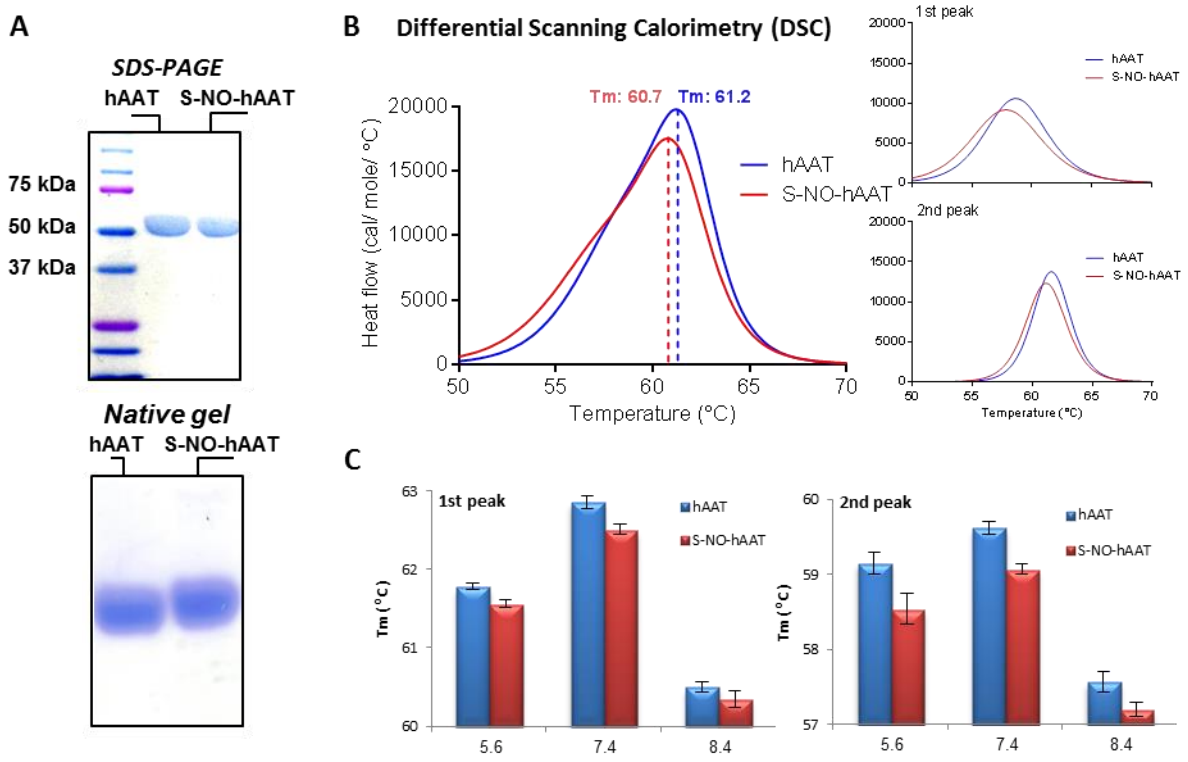

Figure 11. Biochemical properties of S-NO-hAAT are distinct from unmodified hAAT. **(A)** Denaturating (*top*) and native (*bottom*) electrophoretic gel analysis. **(B)** Thermal stability analysis one peak (*left*) and two peaks (*right*). **(C)** Denaturation temperature (Tm) per pH.

S-NO-hAAT is an inflammatory agent that increases cytokine production by macrophages in a concentration-dependent manner

The initial observation that provided evidence for the inflammatory profile of S-NO-hAAT aimed at determining whether S-NO-hAAT maintains the anti-inflammatory profile of unmodified hAAT; thus, stimulated macrophages were examined (**Fig. 12**). As shown, while unmodified hAAT significantly reduced LPS-induced release of TNFα, S-NO-hAAT did not. An apparent loss-of-function with regards to the well-established anti-inflammatory attributes of unmodified hAAT. However, the possibility of gain-of-

function required specific investigation.

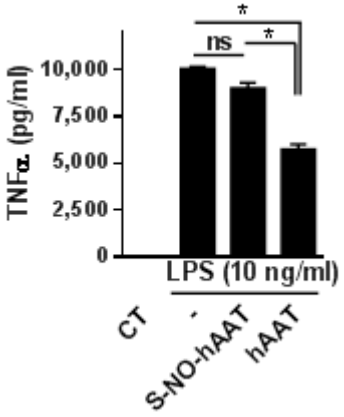

Figure 12. *In-vitro* effect of S-NO-hAAT on LPS-induced TNFα release. Peritoneal macrophages (0.5×10^6 per well). Pretreatment with equimolar S-NO-hAAT and hAAT followed by LPS stimulation. 48-hour supernatant. The variance was calculated using a nonparametric Kruska- Wallis test followed by two-tail student Mann-Whitney test. Data presented as mean ± SEM, * $p < 0.05$.

Primary peritoneal macrophages were introduced either S-NO-hAAT or unmodified hAAT without added stimulation. To control for the presence of another nitric oxide donor, GSNO was introduced at equimolar concentrations. As shown in **Figure 13A**, unlike unmodified hAAT or GSNO, S-NO-hAAT significantly increased the levels of the inflammatory mediators TNFα, IL-1β and KC. Moreover, TNFα levels were altered by S-NO-hAAT in a concentration-dependent manner (**Fig. 13B**), depicting a range of hAAT concentrations that exists in the circulation during acute infection (2.5 and 5 mg/ml); interestingly, at 1 mg/ml (a value typical of a non-inflamed individual) S-NO-hAAT did *not* evoke the release of TNFα.

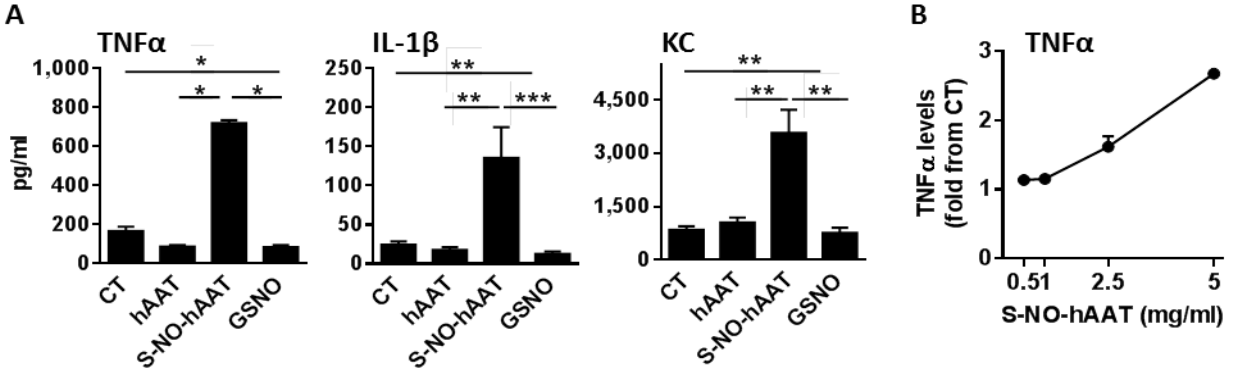

Figure 13. S-NO-hAAT induces an inflammatory response in a concentration-dependent manner. **(A)** Peritoneal macrophages (0.5×10^6 per well). S-NO-hAAT, hAAT and GSNO at equimolar concentration (27.5 µM). 48-hour supernatant, ELISA multiplex (n = 4). **(B)** Peritoneal macrophages (0.5×10^6 per well). S-NO-hAAT concentrations indicated. 48-hour supernatant TNFα levels. CT, cells treated with nitrosylation buffer (n = 2). The variance was calculated using a nonparametric Kruska- Wallis test followed by two-tail student Mann-Whitney test. mean ± SEM, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In examining whether S-NO-hAAT affects the inflammatory profile by altering early gene expression profiles, macrophages were incubated with S-NO-hAAT, hAAT or

GSNO and then mRNA levels were determined by qRT-PCR. Similar to the previous pbservations, S-NO-hAAT appeared to have induced an inflammatory cassette of genes (**Fig. 14A**), including iNOS and TLR2. The inflammatory profile was accompanied by evidence for reduced expression of IL-10, most pronounced at 72 hours upon examination of intracellular IL-10 protein levels by FACS (**Fig. 14B**). The kinetics of this phenomenon is extremely rapid (**Fig. 14C**), but also well tempered as the peak observed in the first two hours of exposure to S-NO-hAAT is short-lived and is followed by reduction in inflammatory gene expression after 2-3 hours.

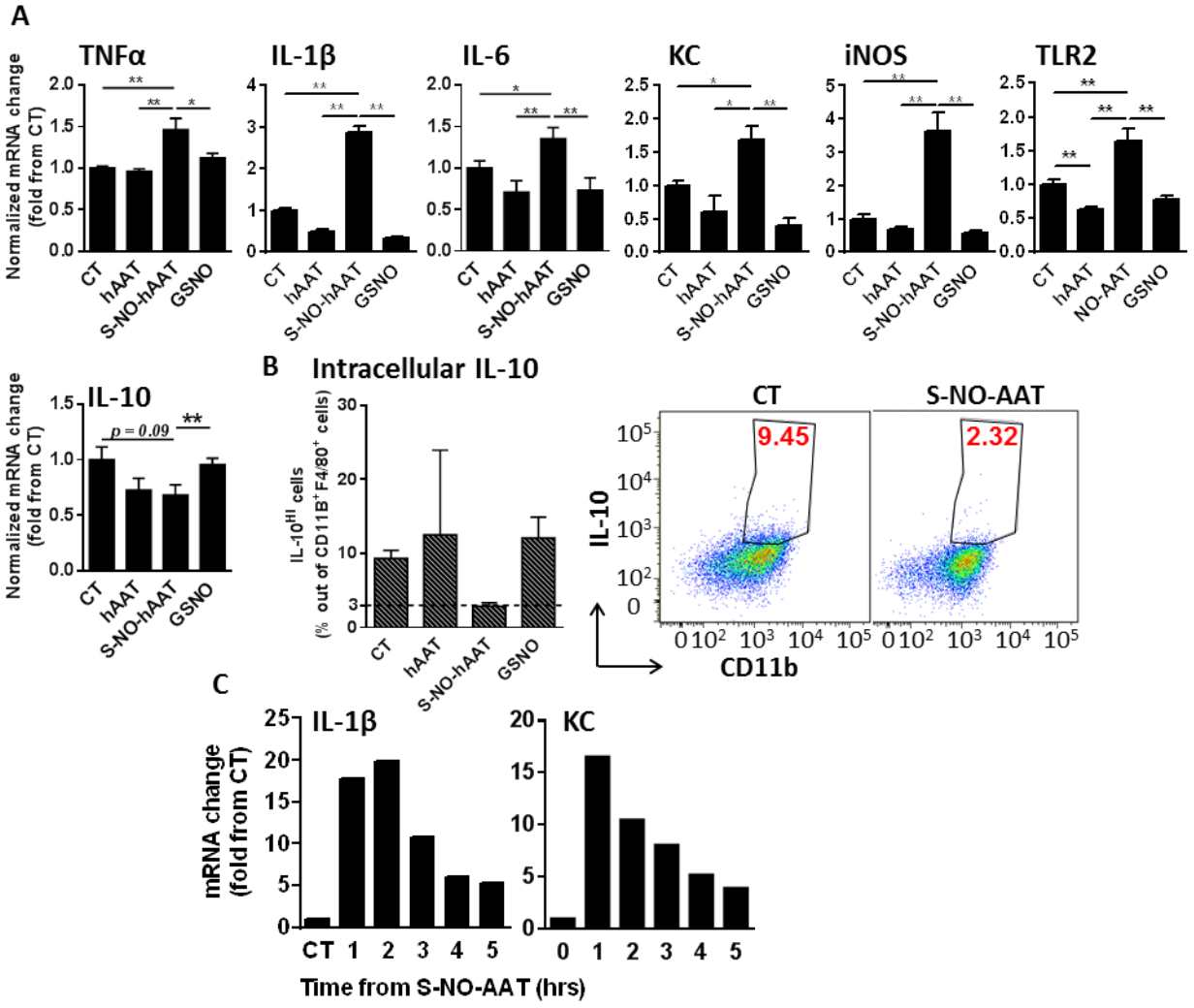

Figure 14. S-NO-hAAT induces a rapid inflammatory phenotype. **(A)** Peritoneal macrophages (0.25×10^6 cells per well) S-NO-hAAT, hAAT and GSNO at equimolar concentration (27.5 µM). 6-hour relative mRNA levels (n = 6). **(B)** BMDM (1.5×10^5 cells per well). S-NO-hAAT, hAAT and GSNO at equimolar concentration (27.5 µM) for 72 hours. FACS analysis. *Left*, pooled data. *Right*, representative FACS images. **(C)** Kinetics. Relative mRNA levels. The variance was calculated using a nonparametric Kruska- Wallis test followed by two-tail student Mann-Whitney test. Data presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Transnitrosylation-activity dependent of S-NO-hAAT

In order to examine whether S-NO-hAAT interacts directly with cells, peritoneal macrophages were incubated with unmodified or nitrosylated hAAT for 5, 15 and 30 minutes, followed by WB analysis for hAAT in cell lysates.

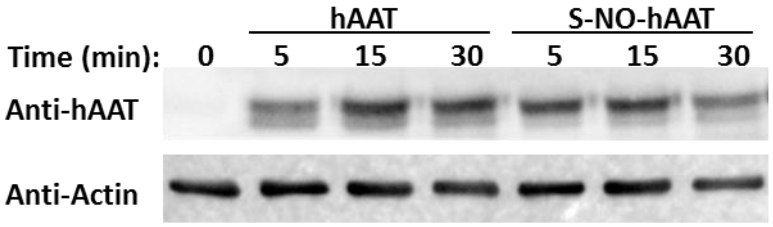

Figure 15. Recovery of S-NO-hAAT and unmodified hAAT from macrophage lysates. Peritoneal macrophages (1×10^7 per well). Unmodified or S-NO-hAAT (27.5 μM) for indicated durations. Representative Western blot analysis.

As shown (**Fig. 15**), both unmodified and nitrosylated hAAT are readily demonstrated in cell lysates in all examined time points. Whether this activity enables the transferring of the nitric oxide from S-NO-hAAT to another cell-associated molecule, and whether such a process is required for cell activation is the topic of the following experimental setup.

Transnitrosylation was allowed to occur in cultured cells that were pretreated with NEM, a method that pre-blocks free cysteine residues in treated cells. After washing, NEM-treated and non-treated cells were incubated with S-NO-hAAT, and the supernatant was sampled at several time points; nitrosylated entities were then determined in supernatants (**Fig. 16A**).

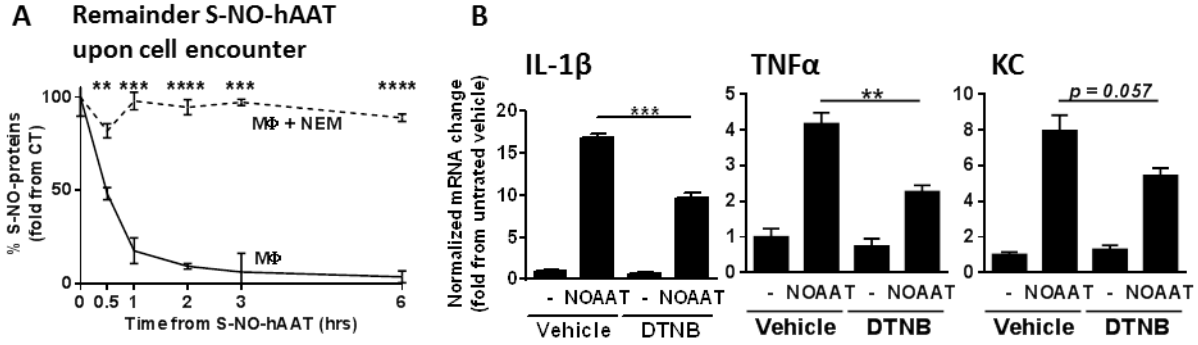

Figure 16. Transnitrosylation-dependent activity of S-NO-hAAT. (A) ***Time dependent distribution of nitrosylated entities.*** Naïve and NEM-pretreated peritoneal macrophages (1×10^6 per well). Amount of remaining nitrosylated proteins in supernatant. **(B)** ***Rapid surface transnitrosylation-dependent inflammatory response.*** DTNB or DMSO (vehicle)–pretreated cells (0.25×10^6 per well). S-NO-hAAT for one hour. mRNA levels. Data presented as mean ± SEM, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (t-test analysis).

As shown, nitrosylation state in the supernatant rapidly declined in the presence of naïve cells within 0.5-1 hours, but remained mostly unchanged in cultures of cells pretreated

with NEM. While this method depicts total nitrosylation of cell proteins, DTNB affords a blockade of cysteine residues restricted to cell membrane; using this approach (**Fig. 16B**) we observed that blockade of free cell surface cysteine residues by DTNB had no significant effect in and of itself, it did reduce the inflammatory flare triggered by S-NO-hAAT, as determined by transcript levels of IL-1β, TNFα and KC.

S-NO-hAAT activates MAPK pathways

Evaluation of the signaling pathways that are involved in S-NO-hAAT activities was carried out in three parallel approaches: kinase array, western blot analysis and specific signaling inhibitors. For kinase array and western blot analysis, peritoneal macrophages were introduced S-NO-hAAT for 5, 15 and 30 minutes, then lysed and examined for phosphorylated signaling mediators.

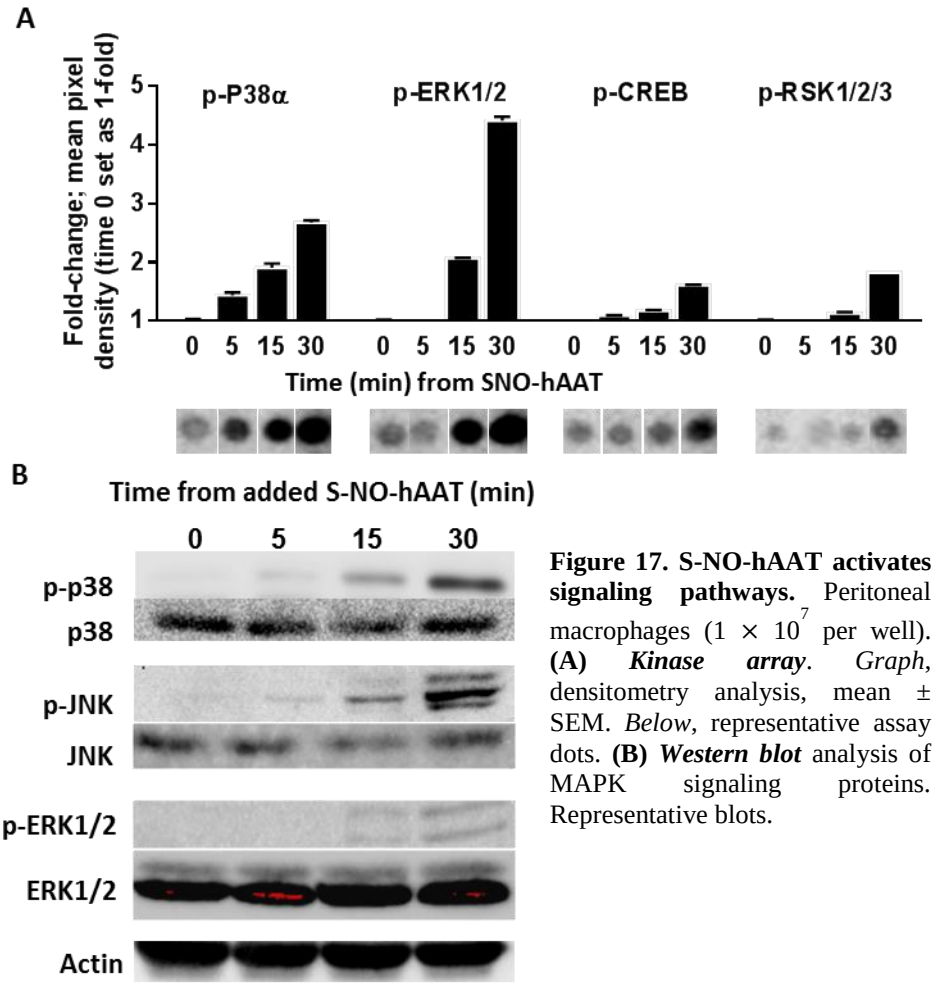

Figure 17. S-NO-hAAT activates signaling pathways. Peritoneal macrophages (1×10^7 per well). **(A)** ***Kinase array***. *Graph*, densitometry analysis, mean ± SEM. *Below*, representative assay dots. **(B)** ***Western blot*** analysis of MAPK signaling proteins. Representative blots.

As shown in **Figure 17**, the earliest phosphorylated mediators appear to be P38 (panel A for kinase array; panel B for WB) and JNK (WB) that are phosphorylated within 5 minutes from introduction of S-NO-hAAT. ERK is found phosphorylated at the 15-minute mark in both methods, and the transcription factors CREB and RSK are phosphorylated by 30 minutes.

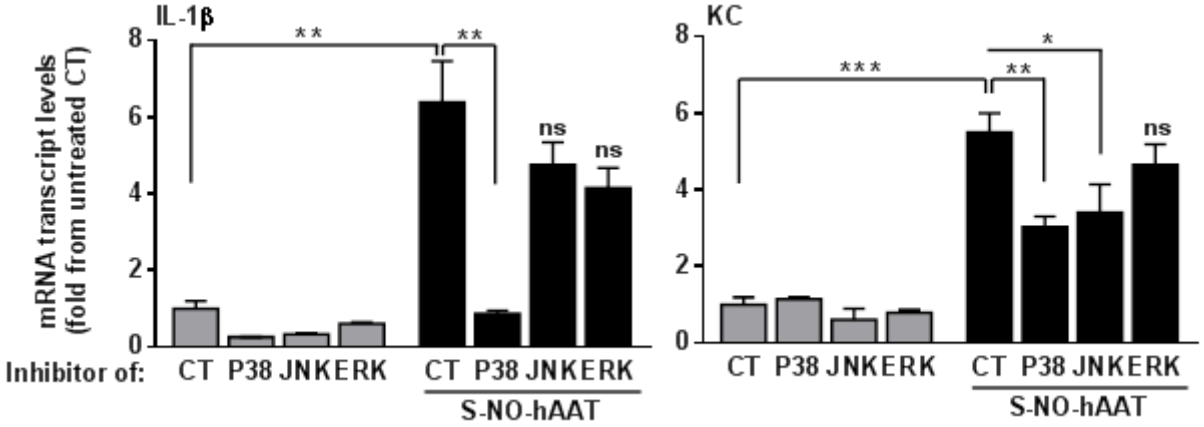

Figure 18. Inflammatory response to S-NO-hAAT requires intact P38 and JNK. Peritoneal macrophages (0.25×10^6 per well). 30-minute-preincubation with indicated signaling inhibitors or DMSO (CT), 1-hr treatment with S-NO-hAAT. mRNA transcript levels normalized to 18s. Data presented as mean ± SEM. ns, non-significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (t-test analysis).

Inhibition of signaling molecules was conducted by incubation of peritoneal cells with specific inhibitors for P38, JNK and ERK (or DMSO for control), followed by S-NO-hAAT treatment (**Fig. 18**). The relative transcript level of two pivotal inflammatory molecules was chosen as an early end-point, i.e., one hour after exposure to S-NO-hAAT. As shown, both IL-1β and KC transcript levels increased several-fold in the presence of S-NO-hAAT; however, this inducible profile was significantly diminished in the presence of the P38 inhibitor. In addition, the presence of a JNK inhibitor resulted in reduced induction of KC by S-NO-hAAT, albeit inducible IL-1β transcript levels did not change in a marked manner. These results strengthen our previous observations by which MAPK pathways are required for the rapid inflammatory effects of S-NO-hAAT.

S-NO-hAAT improves macrophage bacterial killing

Macrophage bacterial killing activity was examined using THP-1 cells. The cells were pretreated with unmodified hAAT, S-NO-hAAT or GSNO followed by introduction to live opsonized *S. Typhi* (**Fig. 19A**). In a parallel post-treatment experiment, the cells

were co-cultured with *S. Typhi* and *then* treated with unmodified hAAT, S-NO-hAAT or GSNO (**Fig. 19B**). In both settings, after 30 minutes of co-culture the extracellular bacteria were displaced by washing and Gentamycin supplement. Based on CFU counts of lysed macrophages, the antibacterial activity of the macrophages was enhanced by S-NO-hAAT in both the pretreated cells and post-treated cells, the former exhibiting a log reduction in live bacteria. Unmodified hAAT and GSNO had no significant effect on bacterial killing.

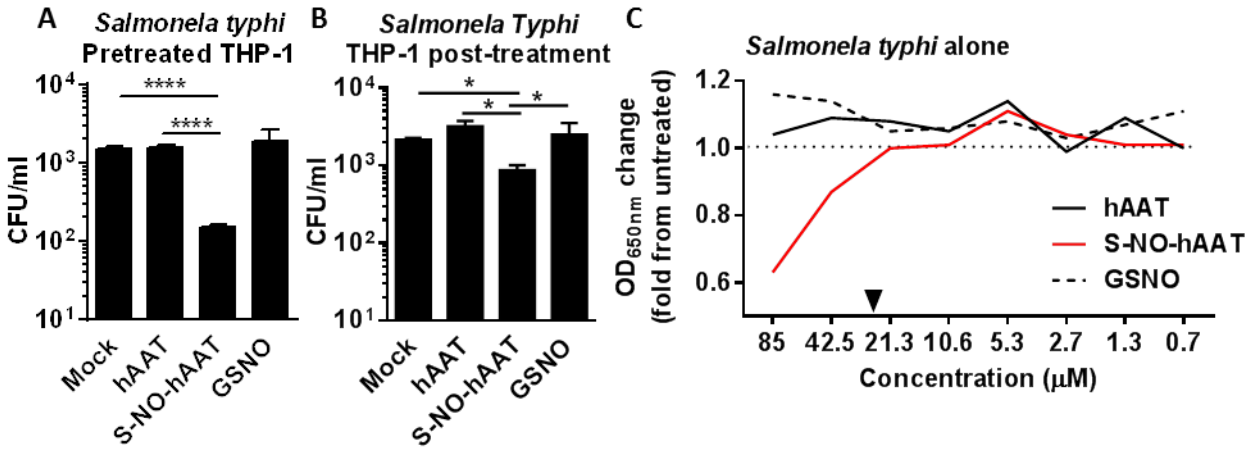

Figure 19. S-NO-hAAT–activated macrophages kill intracellular *S. typhi*. Bacterial infected THP-1 cells (0.5×10^6 per well). **(A)** Bacterial killing by *pretreated* macrophages. **(B)** Bacterial killing by *post-infection*–treated macrophages. **(C)** Bacterial killing in the absence of macrophages; *S. typhi* (10^5 CFU/ml LB medium). Mean ± SEM, * $p < 0.05$ and **** $p < 0.0001$ (t-test analysis).

To test whether the bacterial reduction is indeed *cell*-mediated and not a direct antibacterial effect of S-NO-hAAT, live *S. Typhi* were directly treated with S-NO-hAAT and allowed to grow for 8 hours. As shown in **Figure 19C**, S-NO-hAAT reduced bacterial growth at high concentrations (42.5 - 85 µM), but not at the lower concentrations used in cell culture experiments (≤27.5 µM). Considering that the lower physiological concentrations of hAAT resulted in enhanced bacterial killing in the presence of macrophages and that the pro-inflammatory effects were observed at the same concentrations, one can conclude that S-NO-hAAT may reduces bacterial burden by macrophage activation.

S-nitrosylation of hAAT occurs under similar conditions in-vivo

Thus far, the results obtained were based on using S-NO-hAAT formed in the laboratory by exposing hAAT to a nitric oxide donor. In order to ascertain that S-NO-

hAAT occurs physiologically under similar conditions to those tested here, hAAT transgenic mice were inoculated with intranasal LPS or PBS (Mock). After 24 hours mice were sacrificed and lungs were homogenized and lysed. To investigate whether hAAT was nitrosylated, SNO-RAC method for nitrosylated proteins pull-down was applied, followed by elution and western blot analysis. Ascorbate supplementation is essential for this method; lack of ascorbate represents background readout of non-specific pull-down entities.

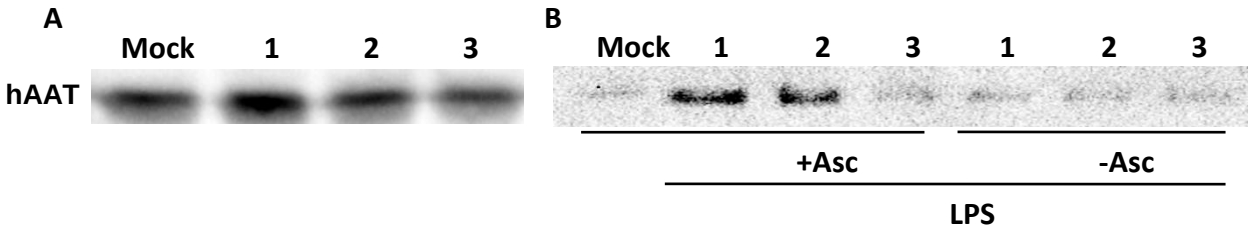

Figure 20. S-nitrosylation of hAAT *in-vivo*. hAAT-transgenic mice (n = 3; individual numbering). LPS (0.3 mg/kg) i.n. inoculation, 24-hour lungs lysates. **(A)** WB analysis of whole lung lysate. **(B)** WB analysis of nitrosylated proteins (SNO-RAC method) with and without ascorbate (Asc).

As depicted in **Figure 20A**, using species-specific ELISA for hAAT, all mice expressed transgenic lung hAAT in a comparable manner. However, upon intranasal LPS stimulation (**Fig. 20B**), 2 out of 3 mice displayed a sizeable rise in the amount of lung S-NO-hAAT upon LPS inoculation.

Discussion

Treatment with repeated infusions of hAAT is currently considered for non-deficient individuals with the rationale that hAAT is a modulator of immune-related pathologies [66]. Being an anti-inflammatory protein, the concern was raised as to an increase in the 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.

Bacterial peritonitis is a life threatening condition, frequently related to peritoneal dialysis complication, as well as to gut perforation as a result of trauma and post-operative complications. Up to 4% of peritoneal dialysis-related peritonitis cases are lethal [126, 127]. Here, we employed two bacterial peritonitis models: (a) CLP, whereby bacteria are extruded from the gut into the peritoneal cavity. In this model, the entry of bacteria to the cavity is continuous and the injured cecum introduces necrotic tissue elements. (b) Fecal injection, in which 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 scenario [119, 128, 129].

Multiple organ dysfunction syndrome (MODS) is a major life threatening complication of peritonitis and sepsis. This study demonstrates some organ protective effects of hAAT during the pathogenesis of bacterial peritonitis, agreeing with its function as a tissue protective protein. Indeed, the hallmark outcome of genetic hAAT deficiency involves lung tissue degradation. Transgenic hAAT mice express the human protein in lung alveolar epithelial cells under the surfactant promoter, and therefore its intracellular presence may directly minimize cell death [37, 130]. However, the present study demonstrates improved *extra*-pulmonary organ function as well. The positive outcomes, in as far as MODS is concerned, during hAAT therapy in the present study may, thus, relate to other recently described activities of hAAT. The hyper-inflammatory phase of early sepsis is characterized by a cytokine-storm, a major cause of MODS. Massive and systemically production of IL-1β, IL-6 and TNFα, as well as danger

associated molecular pattern (DAMP) molecules [131, 132] promotes tissue injury. Here, the tissue protective effect of hAAT was observed 24 hours after bacterial inoculation, at a time that inflammation was, unexpectedly, *more pronounced* in the hAAT group.

How can hAAT protect cells from injury in an excessive inflammatory environment? hAAT was shown in several models to inhibit caspase-3, and as a result reduce hepatocyte death in acute liver failure [44], pancreatic islet β-cell death [39] and lung endothelial cell cigarette smoke–induced injury [43]. In addition, hAAT modulation of response to TNFα was demonstrated to be protective of cell injury in lung endothelial cells [37, 43, 54, 63, 130]. Excessive cell death causes a rise in DAMP molecules, which can further facilitate organ damage, as occurs, e.g., when excessive levels of heat shock protein gp96 inflict multi-organ damage [133]. hAAT was recently found to directly neutralize gp96, reducing inflammation in various models including the cecal ligation model [134]. Cell and organ protection by hAAT can also relate to bacterial toxin neutralization; indeed, hAAT was shown to reduce LPS-induced lethality *in-vivo* [135].

Lymphocytes and dendritic cells are extremely sensitive to sepsis-induced cell death (reviewed in [136]), rendering a septic host further vulnerable to opportunistic infections. According to our results, hAAT significantly minimizes the degree of sepsis-induced leukopenia. However, hAAT did not significantly affect the decline in bone marrow cell count, suggesting that the benefit of hAAT under these particular conditions is most-probably restricted to peripheral WBC homeostasis.

It is also evident according to our results that unmodified hAAT does not act as an antibiotic; bacteria multiply freely in its presence, as others have previously shown [83]. However, hAAT does appear to enhance bacterial clearance *in-vivo.* Unlike *in-vitro* bacterial models, after inoculation of live bacteria into a host, they must penetrate through tissues in order to establish a vital colony. Indeed, *P. aeruginosa* pneumonia has been shown to be blocked by hAAT in part by protecting the epithelial barrier from becoming compromised [84].

Several studies indicate that inhibition of proteolytic activity by hAAT treatment in CF patients can protect essential host proteins from excessive cleavage and inactivation, and therefore provide further support against bacterial colonization. For example, in

examining CF patients, one learns that such targets of cleavage include membrane complement receptor 1 (CR1) on the surface of neutrophils and C3bi on opsonized bacteria [137]; recent reports establish that cleavage of membrane CR1 is directly inhibited by hAAT [138]. IL-8 receptor is also important for bacterial clearance by directing neutrophil migration, and was shown to be cleaved on the surface of neutrophils in CF patients; proteolysis of neutrophil IL-8 receptor is also blocked by hAAT [139]. However, CF patients typically suffer from chronic inflammation with repeated opportunistic infections, and not so much from acute infection as in our model, thus the mechanism behind the benefit of hAAT for CF patients may count as an outcome of hAAT therapy.

One possibility for a lower CFU may be related to *24 hours* increased levels of KC and MCP-1 release, associated with massive neutrophil infiltration, in the presence of hAAT. As the main producers of hAAT targeted proteases, neutrophils are perhaps the most investigated immune cell in the context of hAAT thus far, and their mutual complex effects. This interesting result is therefore not obvious *inhibition*. Indeed, hAAT is known for its ability to reduce neutrophil chemotaxis under a series of stimuli, including LPS [47], FMLP [140], thioglycolate [50] and allogeneic cell transplants [50]. This apparent contradiction with the present findings may be resolved by considering that the present study addresses a response to *live* bacteria, rather than to sterile inflammation. Support for this hypothesis can be found in the observed differential effect of hAAT on TNFα release. TNFα is one of the most responsive cytokines to hAAT, and its release from membranes is dependent on a recently identified direct target of AAT, ADAM17 [44, 62]. However, here its enhancing capacity appears to be restricted to the context of live bacterial inoculation (e.g., fecal injection and CLP), and absent in sterile inflammation.

The outcomes of hAAT treatment in the present study exceed its function as an anti-inflammatory agent. Blocking IL-1β and TNFα in animal sepsis models has failed to diminish lethality or reduce lung neutrophil sequestration in other reports [141]. While IL-1 pathway blockade in septic mice reduces inflammation, it increases mortality rates and bacterial burden [142]. While inflammation appears to be essential for bacterial clearance [143], excess inflammation is associated with negative consequences, particularly in the form of multiple organ damage and high risk of mortality [143].

Indeed, hAAT is not a classic anti-inflammatory protein; hAAT apparently has context-dependent pro- and anti-inflammatory properties. In the present study, hAAT reduced immune cell activation at the infection site and pro-inflammatory cytokine levels in the circulation after *72 hours*, but achieved the opposite effect before the end of the first *24 hours* after infection. Previous publications support this duality in the activity of hAAT; short exposure to LPS followed by hAAT treatment in cells results in a pro-inflammatory profile, and is then followed by an anti-inflammatory profile [144]. This phenomenon was suggested by Nita et al. to be CD14-related: hAAT increases the levels of soluble CD14 and anti-CD14 antibodies reduce LPS-induced hAAT-related cytokine release only in the first few hours after treatment [145].

One may ask, how can a single protein act as a pro- and anti-inflammatory mediator in a time-dependent manner? Also, how is it possible that according to our *in-vitro* results, hAAT only causes CFU reduction *in-vivo*? One possible answer to both questions is that hAAT is chemically modified at the inflamed site and that as a result its activity is altered. To further investigate this aspect, we aligned hAAT's sequence and several AAT-like sequences from different organisms. We focused on cys^{232} and the lysine residues that surround it, that may be involved in the S-nitrosylation process and the direct anti-bacterial activity of S-NO-hAAT *in-vitro* [1, 117]. Baised on this *in-silico* exploration, this single cysteine is completely evolutionary conserved (100%) and there is a 95% conservation rate for its nearest lysine (position 274). This observation joins further unique molecular details such as that hAAT's cysteine is highly reactive, is not engaged in a disulfide bond and is not located within the RCL. Moreover, hAAT's cysteine single mutation to the relatively similar amino acid serine did not affect protein structure or function [146, 147]. We can carefully assume, therefore, that considering its evolutionary conservation, Cys^{232} hold some significance for the proper function of hAAT, particularly in its capacity to undergo local S-nitrosylation.

hAAT point mutations may lead to formation of aggregates [148, 149]. However, interestingly nitrosylated hAAT does not form aggregate, while some other nitrosylated proteins [150]. Taking together the outcome of the native gel comparison and thermal stability results, we propose that S-nitrosylation of hAAT does cause a conformation change, as demonstrated in other nitrosylated circulating proteins, such as human serum

albumin [113] and hemoglobin [151, 152]. S-NO-hAAT may be somewhat less condensed than unmodified hAAT, thus appearing as a higher band in the native gel and as having a lower Tm, unlike other S-nitrosylated proteins [153].

It is becoming increasingly acknowledged that the activities of hAAT rely on its structure; point mutations, not necessarily within the RCL, can prevent protease inhibition (e.g. the Z mutant). Yet, Interestingly, S-NO-hAAT can still inhibit serine proteases, just as unmodified hAAT does. Nonetheless, S-NO-hAAT gains new activities that unmodified hAAT lacks, such as inhibiting cysteine proteases, including human Cathapsin B and *S. pyogenes* virulence factor SPE-B [117].

A well-studied activity of hAAT involves its anti-inflammatory effects. Here, we display for the first time that upon S-nitrosylation, hAAT alters its activity towards enhancing inflammation. This surprising short term activity is demonstrated by both elevated released cytokines, as well as up-regulation of their mRNA levels. Moreover, the level of the anti-inflammatory cytokine IL-10, that is normally elevated by hAAT, is decreased in the presence of S-NO-hAAT. It is interesting to note that the effect on TNFα levels is concentration-dependent, and can be observed only at higher concentrations of S-NO-hAAT that most probably are not found in the normal steady state, but may exist during acute phase responses.

The kinetics of the cytokines expression after S-NO-hAAT induction is not obvious. Most inflammatory triggers, such as bacterial molecules and cytokines, increase the production and level of inflammatory cytokines, reaching a plateau and then later decline, at a relatively long time frame. S-NO-hAAT however, activates cytokine expression to the peak within 1-2 hours, followed by a rapid reduction. We speculate that the augmentation of the pro-inflammatory cytokines, including IL-1β and TNFα, may cause a positive feedback loop. Also, this short and temporary effect may represent the rapid degradation of S-NO-hAAT. Indeed, our results show that unlike in the case of cysteine-blocked cells, S-NO-hAAT levels rapidly decline upon meeting naïve cells. The rapid removal of nitric oxide from S-NO-hAAT, also termed trasnitrosylation, may thus result in concomitant elevated anti-inflammatory hAAT, explaining the short nature of S-NO-hAAT kinetics. However, the swift nitric oxide removal raises another question: is S-NO-

hAAT affected by transnitrosylation, or does the trasnitrosylation inhibit S-NO-hAAT activity? By blocking the membranal cysteines with DTNB, we noticed that cell activation by S-NO-hAAT treatment was significantly reduced, indicating that free membrane associated cysteins are essential for the S-NO-hAAT effect. Another support for this hypothesis is S-NO-hAAT's presence in the cells' lysate 5 minutes after introduction, and therefore it is more likely that S-NO-hAAT affects directly.

Most known relationships between S-nitrosylation and inflammation are towards anti-inflammation, including the NFκB pathway [154-158]. However, MAPK pathways seem to play a major role in the activity of S-NO-hAAT, but unlike NFκB, this effect is less obvious. The activity of RAS, the upstream mediator of ERK, is enhanced after S-nitrosylation [159]. This is followed by ERK activation, as observed using macrophage coltures [160], activated T lymphocytes [161, 162] and neurons [163]. Our results similarly, indicate that S-NO-hAAT affects ERK phosphorylation, but it is mostly JNK and P38-related. JNK and P38 were phosphorylated within 5 minutes and their inhibition by selective inhibitors, especially P38, had a high impact on gene expression. This agrees with the fact that most of their downstream pathways are joint. JNK is negatively regulated by its direct S-nitrosylation, as observed using cytokine-stimulated macrophages [164, 165] as well as neurons subjected to ischemia–reperfusion injury [166]. On the other hand, S-nitrosylation of neuronal P38 alters its activity according to the source of nitric oxide; enhanced by endogenous NO, but inhibited by exogenous NO [167]. Apoptosis signal-regulating kinase 1 (ASK1) is an upstream signaling protein shared by JNK and P38, and is also inhibited by its direct S-nitrosylation [168-170].

Another potential mechanism may be interactions between S-NO-hAAT and novel protein targets, as demonstrated by the circulating S-nitrosylated serum albumin [113, 171-173], similar to other S-nitrosylated proteins [174-176].

hAAT has several hydrophobic domains and direct interacts with cholesterol [177, 178], it has recently found to exert a synergistic cytoprotective effect together with HDL [179, 180]. On cell membranes, hAAT has been shown to dock onto membrane lipid rafts [181], which usually contain multiple inflammatory receptors. Interestingly, hAAT was shown to enter the cytosol [130, 182, 183], but according to the S-NO-hAAT rapid effect,

it is more likely that S-NO-hAAT has membrane-associated targets, while unmodified hAAT may not necessarily interact with.

Similar to hAAT, surfactant D (SP-D), another extracellular protein that becomes inflammatory instead of anti-inflammatory upon S-nitrosylation, is abundant in the lungs [184-187]. The basic structure of SP-D is a trimer held by disulfide bonds [188-190]. However, SP-D contains a hydrophilic "head" and hydrophobic "tail", and therefore self-assembles to form a multimer of 12 subunits (4 trimers) or more, resulting in a globular complex. In its globular form, SP-D acts as an anti-inflammatory complex in two different pathways: by interacting with SIRP-1α followed by phosphatase activation that reduction in inflammatory signaling [191], and by interacting with TLR4/MD-2 thus preventing LPS binding [192-196]. However, during lung inflammation, SP-D is modified through S-nitrosylation (SNO-SP-D) and *induces* inflammation [197-199], but not through augmentation of iNOS inhibitor *in-vivo* [199] or ascorbate treatment *in-vitro* [197]. After being S-nitrosylated in two cysteine residues, SNO-SP-D complex disintegrates to the trimeric subunits, and its hydrophobic tail is exposed, then binds and activates the scavenger receptor, CD91 [176, 186, 200]. CD91 activates pro-inflammatory signaling pathways that are P38-dependent. Although S-NO-hAAT was not found to form complexes, its mechanism may be similar to SNO-SP-D in other aspects. For example, CD91 binds LDL and DAMPs, such as GP96 [201-203], similar to hAAT. CD91 is also known as a hAAT receptor [203, 204] that internalizes hAAT [183] . In addition, hAAT seems to undergo conformation change after S-nitrosylation that may alter its affinity towards target proteins, and appears to act in a P38-dependent manner.

In the present study, S-NO-hAAT increased iNOS expression, forming an apparent positive feedback loop for reaching greater bacterial killing capacity at sites of infection. S-NO-hAAT also increased expression of bacterial sensing proteins, such as TLR2/4. Therefore, it is not surprising that S-NO-hAAT enhanced macrophage antibacterial activity, both pre- and post-treatment.

This attribute of hAAT is not exempt from a bacterial counter-response: a group of pathogenic bacteria have developed proteolytic enzymes that directly degrade hAAT, including *S. aureus*, *S. marcescens*, *S. erythraeus* and *P. aeruginosa* [205-208]. One may

speculate that the major antibacterial effect of S-NO-hAAT is direct and does not involve macrophages, yet our results indicate that a direct antibacterial effect can only be achieved using super-physiological concentrations, unlike previously published [1, 117]. This difference may be the result of disparity between bacterial strains in both studies, as well as the source of hAAT and the details of S-nitrosylation procedure.

We and others [1] have shown that unmodified hAAT can undergo local S-nitrosylation in biologic systems. In our study, hAAT was S-nitrosylated in the lungs of two out of three transgenic mice after inflammation was provoked, and was absent in the non-stimulated mouse. The variance within the treated group may be the result of insufficient LPS inoculation or to differences within the animals; yet we do have evidence that hAAT transgenic mice display a significantly lower bacterial load after lung infection with *S. pneumonia* (not shown). This can be explained by both effects (antibacterial S-NO-hAAT and its production *in-vivo*). However, additional experiments are needed to prove this association, and should also be extended to include *in-vivo* iNOS inhibitors.

In light of our results, we suggest a mechanism of action for hAAT and its S-nitrosylation during bacterial infections (**Fig. 21**). We propose that hAAT exists as an abundant protein in the circulation, with distinct context-specific activities. According to this concept, in the periphery, hAAT remains in its unmodified form and acts as an anti-inflammatory and tissue protective agent. However, during infection, immune cells infiltrate and become activated, iNOS expression is induced locally and nitric oxide levels rise; hAAT that reaches an infection site is thus S-nitrosylated and can assist in the reduction of the bacterial burden by further activating immune cells. Since S-NO-hAAT increases iNOS expression, more S-NO-hAAT is formed upon entry of unmodified hAAT to the site of infection. Nitric oxide may then be transferred from S-NO-hAAT to immune cell–associated proteins, thus reducing the probability of residual inflammatory S-NO-hAAT in the periphery. After losing its nitric oxide molecule, hAAT acquires again its unmodified anti-inflammatory form and acts to reduce inflammation distal to the

infected site.

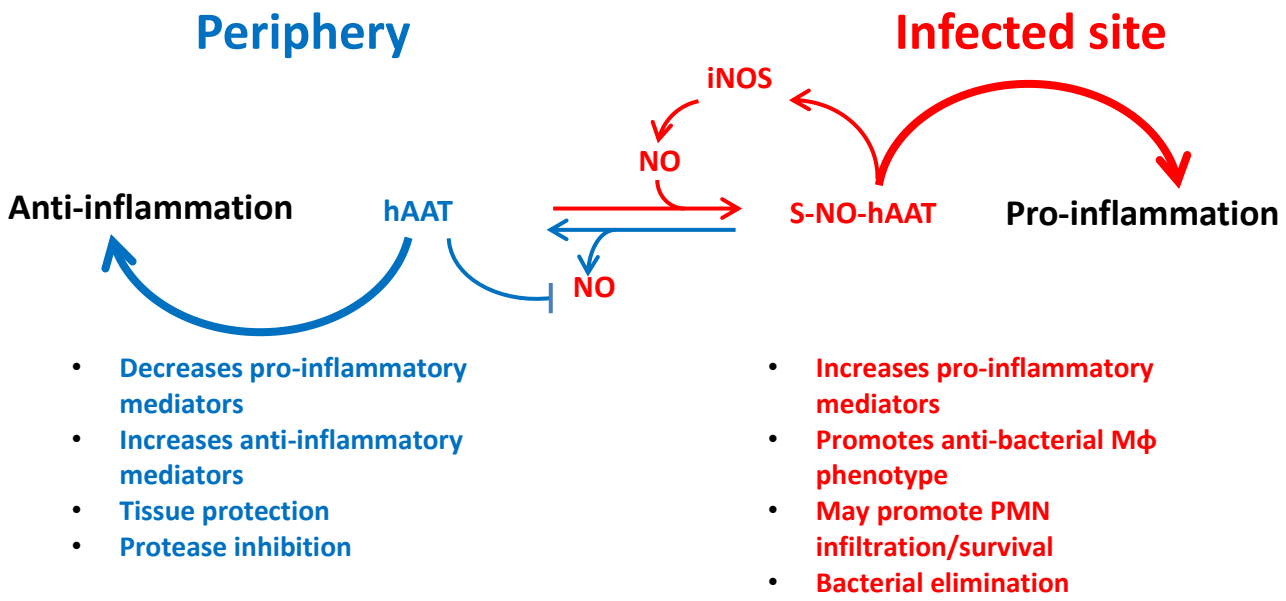

Figure 21. hAAT duality: proposed physiology. It is suggested that hAAT acts in two different manners according to its S-nitrosylation state. In an infected inflamed, nitric oxide-rich site, hAAT is nitrosylated and can reduce the bacterial load directly, by transnitrosylation of bacterial proteins, and indirectly, by acting as an inflammatory trigger for immune cells. However, in the periphery, nitric oxide levels are low and hAAT is presumed to maintain its unmodified anti-inflammatory and tissue-protective activities.

In addition to S-nitrosylation, hAAT is known to undergo two more post-translational modifications at inflamed sites: proteolytic cleavage, and oxidation (**Fig. 22**). hAAT oxidation, similar to S-nitrosylation, is a reversible modification mediated by free radicals (reactive oxygen species) that are typically abundant at an inflamed tissue. Although Cys^{232} is highly reactive, it is *not* oxidized *in-vivo* [2]); the oxidation of hAAT occurs on methionine residues, particularly Met^{351} and Met^{358} [209]. hAAT oxidation has been found in many oxidative stress–related pathologies [210-215], and in a few, interestingly, in complex with LDL [216, 217]. Oxidized hAAT lacks protease inhibitory ability [19, 209], yet it becomes an inflammatory molecule to monocytes [218] and epithelial cells [219]. The proteolytic cleavage of hAAT results in a 36-amino acid–long peptide (C-36) which is released from the carboxyl terminus of hAAT. C-36 was demonstrated in bile, spleen and lungs of inflamed patients [220-222] , as well as in urine samples from patients with IgA nephropathy [223]. C-36 is hydrophobic [221] and typically hidden inside unmodified hAAT (**Fig.22**). Upon release, it causes human monocytes to release inflammatory cytokines [222, 224] and facilitates human neutrophil

chemotaxis and proteases release [225]. Indeed, S-nitrosylation, oxidation and proteolytic-cleavage occur primarily in inflammatory conditions, such that contain excessive levels of free radicals and proteases.

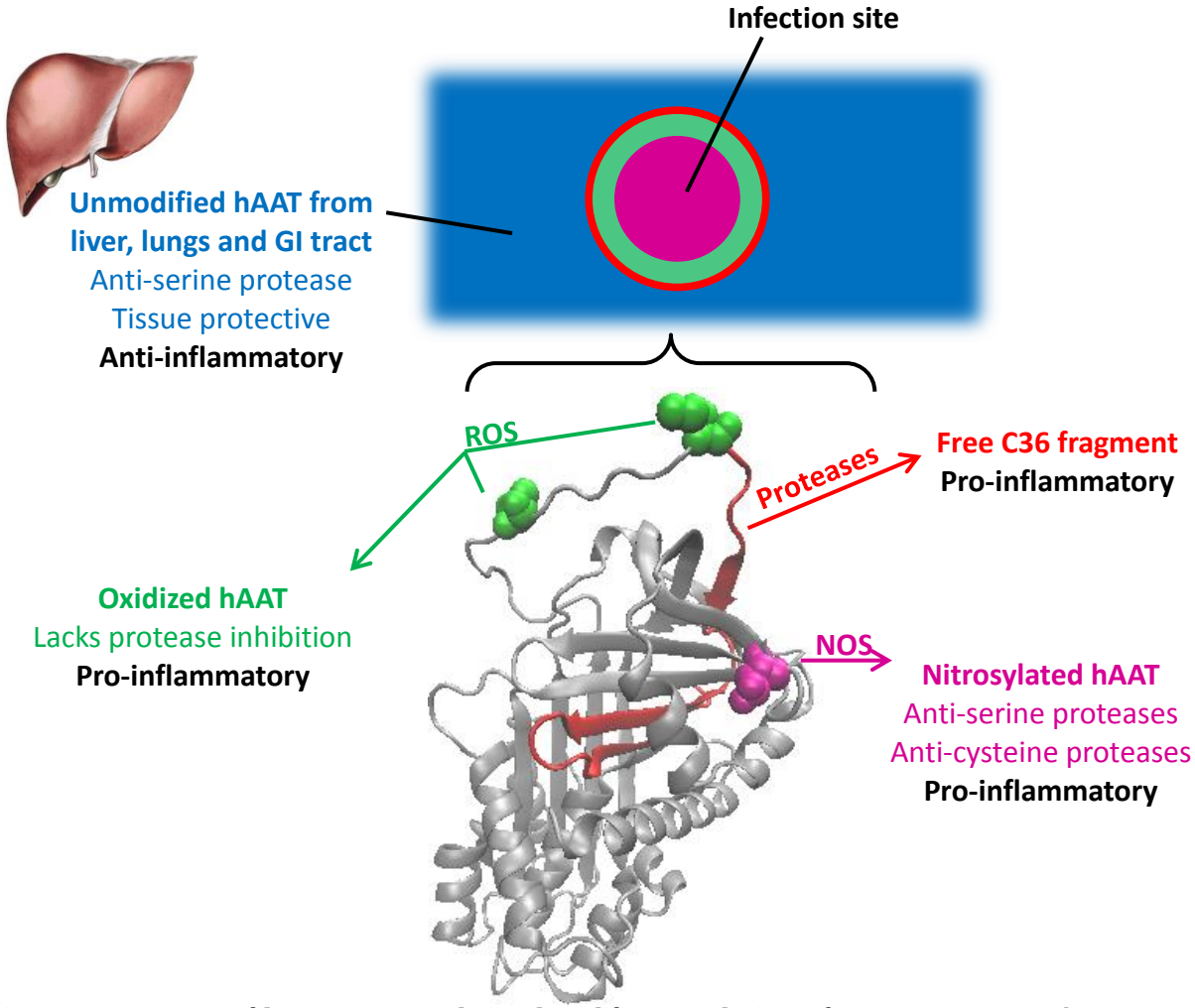

Figure 22. Summary of hAAT post-translational modifications during infection. As an acute phase protein that rises during inflammation, hAAT serves as a potent anti-inflammatory protein in its unmodified form. In an infected and inflamed site, hAAT may be modified in all or some known manners: oxidation (Met^{351} and Met^{358}, *green*), S-nitrosylation (Cys^{232}, *pink*) and proteolytic cleavage followed by release of a C36 peptide (*red*) . This model suggests that circulating hAAT elicits two opposing effects based on its location. Immune activation towards enhanced bacterial killing at the infection site, and anti-inflammatory and tissue-protective effects in the periphery. In addition, its anti-protease repertoire is different for each modification, a possible mechanistic element in its multiple context-dependent functions.

Why would hAAT undergo these modifications? As we know, inflammation is a two-edged sword; on the one hand, it is essential for pathogen elimination, but on the other hand, when uncontrolled, it will cause tissue damage or even sepsis, as establishged in infected IL-10 KO mice [143]. At infected sites, augmentation of an anti-inflammatory

molecule, such as IL-1Ra [142], may lead to bacteremia and death, yet hAAT, being a systemically abundant protein particularly during the acute phase response, must be locally regulated in a manner that is tightly linked to the presence of the pathogen. Thus, S-nitrosylation/oxidation/cleavage of hAAT ensure that inflammation is enhanced at the infection site, and at the same time, high levels of unmodified hAAT may reduce inflammation and protect tissues from unwanted cell injury in the periphery.

S-NO-hAAT has previously displayed a protective effect in the context of ischemia-reperfusion injury, more than unmodified hAAT did and more than other nitric oxide carriers did [226]. This beneficial effect was attributed to superior blood flow relating to the presence of vasodilation-levels of nitric oxide; indeed, NOS inhibitors exacerbate ischemia-reperfusion injuries. However, in other studies, unmodified hAAT improved the outcome of ischemia-reperfusion injuries by reducing inflammation [61, 75, 76]. We do not know how S-NO-hAAT will affect inflammation in that scenario. It may donate its nitric oxide to endothelial cells and then serve as a unmodified anti-inflammatory hAAT. Another option may be that due to high levels of nitric oxide during ischemia-reperfusion, unmodified hAAT may be nitrosylated. Supporting evidence relate to caspase-1 inhibition by hAAT [61]. As previously discussed, S-NO-hAAT, unlike unmodified hAAT, has a higher affinity to some cysteine-proteases, and caspase-1 may be such one. Nonetheless, the mechanism behind the protective effects of S-NO-hAAT is yet unknown, and requires further investigation into other sterile inflammatory pathologies.

Considering hAAT's influence on pathogenic bacteria, it is interesting to investigate its effect on non-pathogenic microbiome especially in the lungs and intestine, as currently evaluated in a clinical trial (NIH clinical trial registry NCT01832220). Although healthy lower respiratory tract lung segments have primarily been considered a sterile environment, they are in fact populated by microbiome. Chronic obstructive pulmonary disease (COPD) is characterized with changing of the lung microbiome [227, 228] and, similarly, intestinal microbiome is also altered during inflammatory bowel disease (IBD) [229-231]. During chronic inflammation, such as in both COPD and IBD, local microbiome diversity is typically decreased [232], suggesting an involvement of bacteria-regulating processes. While COPD has a strong and known correlation with hAAT, as

previously described, the relation of hAAT to IBD is mainly diagnostic per its presence in patient stool. However, hAAT deficient patients have been reported to be more likely to develop severe ulcerative colitis [233, 234], and animal models for IBD show benefit by treatment with hAAT [235]. In light of the results presented in the present study, it will be interesting to investigate whether hAAT improves COPD and IBD prognosis by regulating microbiome-immune cell interactions.

To conclude, hAAT augmentation therapy is safe, and unlike immunosuppression therapies or anti-inflammatory agents, spares treated individuals from opportunistic infections. In addition, S-nitrosylation of hAAT is a natural post-translational modification, which alters the function of hAAT towards inflammation through, at least in part, MAPK signal transduction. S-NO-AAT may thus possess beneficial properties in the context of bacterial infections and other pathologies. Further investigations are needed in order to understand the complete mechanism of action of S-NO-hAAT, and the full spectrum of possible clinical applications.

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תקציר

מבוא. זיהום חיידקי חמור עלול לגרום לאלח דם (ספסיס),תסמונת כשל רב מערכתי (MODS) ומוות. אלפא-1-אנטיטריפסין הומאני (hAAT) הינו מעכב סרין-פרוטאזות הנפוץ בזרם הדם וריכוזו אף עולה במצבי עקה. hAAT הינו נוגד דלקת המבקר את מערכת החיסון ומגן מפני נזק רקמתי. השפעת hAAT הינה על ידי הפחתה של חלבונים מתווכים גורמי דלקת והגברה של נוגדי דלקת; אולם, תוצאות אלו התקבלו במצבי תגובה חיסונית כלפי דלקת סטרילית. hAAT מופק מפלסמה הומאנית ומשמש משנות השמונים כטיפול לחולים בעלי חסר גנטי בו. כיום טיפול זה נבדק כנגד מחלות כגון סכרת מסוג 1 ("נעורים", T1D) ומחלת שתל כנגד מאכסן (GvHD). בשונה מטיפולים נוגדי דלקת אחרים, תוספת hAAT לחולים מראה הפחתה בתדירות הזיהומים במנגנון שאינו ברור לחלוטין. בעוד ש- hAAT חסר פעילות אנטי-חיידקית, נצפה כי לאחר חשיפה לריכוזים גבוהים של חנקן חד-חמצני (nitric oxide) hAAT עובר ניטרוזילציה (S-NO-hAAT) ולאחר מכן מסוגל לעכב ישירות גדילת חיידקים. עם זאת, אין כיום ידע לגבי השפעת S-NO-hAAT על תגובת תאי מערכת החיסון המולדת כלפי זיהום חיידקי.

מטרות. המטרה הראשונה הינה לבחון את תגובת מערכת החיסון לזיהום חיידקי אקוטי במצב של רמות hAAT גבוהות כרונית. המטרה השנייה הינה להשוות בין S-NO-hAAT לבין hAAT נאיבי תוך התמקדות בתאים חיסוניים בהקשר של זיהומים חיידקיים.

שיטות. מודלים של צפקת (פריטוניטיס) וספסיס יושמו על עכברים טרנסגנים ל hAAT ועל עכברי זן הבר (wild-type). הפרמטרים שנבחנו הם העומס החיידקי, הנזק המערכתי, סמני אקטיבציה תאיים, הגעת ניוטרופילים, רמות סרום של מתווכי דלקת ושרידות. היווצרות S-NO-hAAT בחיה השלמה (in-vivo) נבחנה בעזרת SNO-RAC על ליזאט ריאות של עכברים טרנסגנים 24 שעות לאחר הזלפת ליפופוליסכריד (0.3 mg/kg LPS) לנחיר. S-NO-hAAT נוצר בצורה כימית תוך שימוש ב hAAT הומאני בדרגת ניקיון קלינית והוסף לתרבית מקרופגים פריטוניאלים עם או ללא תוספת של 10 ננוגרם למ"ל של ליפופוליסכריד ורמת האקטיבצייה של התאים נבדקה; כביקורת על רמות החנקן החד-חמצני, הוסף GSNO בכמות מולארית זהה. בהמשך, השפעת S-NO-hAAT על מסלולי סיגנלינג נבחנה תוך שימוש במעכבים ספציפיים כלפי חלבוני מפתח במסלולים השונים ובדיקת רמת זירחונם. הפעילות האנטי חיידקית של S-NO-hAAT נבחנה על חיידקי סלמונלה (*Salmonella typhi*) בצורה ישירה כמו שפורסם בעבר וכן בצורה עקיפה על ידי איקטוב תאי THP1. השוואה בין המאפיינים הפיזיקאליים של S-NO-hAAT לבין hAAT נערכה על ידי בדיקת היציבות התרמית שלהם.

תוצאות. hAAT הפחית בצורה ניכרת את הפגיעה בכבד, לבלב, ריאות וכן את רמת הלויקופניה בדם בעקבות זיהום בטני. שרידות העכברים שנחשפו ל hAAT הייתה גבוהה יותר לאחר 24 שעות והעומס החיידקי הופחת. מספר שעות לאחר הזיהום hAAT הגביר באופן בלתי צפוי את רמות מתווכי הדלקת וכן את

כמות הניוטרופילים באזור הזיהום. אולם, 72 שעות לאחר הזיהוםhAAT הפחית את כמות מתווכי הדלקת.S-NO-hAAT נמצא בריאות הדלקתיות של העכברים הטרנסגנים. בתרבית, ללא ליפופוליסכריד, hAAT הפחית את רמות ה- TNFα פי 1.9, בעוד ש- S-NO-hAAT העלה אותם פי 4.3, בהשוואה לתאים לא מטופלים. בנוכחות ליפופוליסכריד hAAT וכן GSNO הפחיתו משמעותית את רמות ה- TNFα, בעוד ש- S-NO-hAAT לא השפיע עליהן. אינקובציה של התאים עם S-NO-hAAT למשך שש שעות גרמה לעלייה בביטוי שעתוק הגנים IL-1β, TNFα, KC, IL-6 וגם רמות iNOS ו- TLR2 (פי 1.3-3.6 באופן יחסי לתאים לא מטופלים). S-NO-hAAT הפחית במידה מסוימת את כמות החיידקים בצורה ישירה, אולם הפחתת כמות החיידקים בצורה העקיפה תוך כדי שפעול תאים, הושג בכמות S-NO-hAAT נמוכה הרבה יותר, על ידי אינקובציה לפני או אחרי ההדבקה בחיידקים. מבנית, S-NO-hAAT נמצא כרגיש יותר לטמפרטורה גבוהה.

מסקנות. hAAT מפחית את העומס החיידקי לאחר זיהום. hAAT מראה השפעה דואלית תלוית זמן אשר מאופיינת בסילוק החיידקים וכן בהגנה מפני נזק מערכתי מפני דלקת לא מבוקרת. עם זאת, נראה כי הדואליות של hAAT הינה תלוית קונטקסט המערבת ניטרוזילצייה בסביבה עשירה בחנקן חד חמצני תוך כדי שיפעול מקרופגים להשמדת החיידקים. לעומת זאת, הפעילות נוגדת הדלקת מתרחשת רחוק מאזור הזיהום. המנגנון מערב שפעול P38 באופן לא ידוע; יתכן כי הניטרוזילצייה גורמת לשינוי מבני אשר גורם להעברת הסיגנל לפעילות הגנתית אפקטיבית יותר. תוצאות אלו מאששות כי טיפול מתמשך בתוספת hAAT הינו בטוח ויכול להיכלל כטיפול מונע לבעלי סיכוי לזיהומים חיידקיים.

הצהרת תלמיד המחקר עם הגשת עבודת הדוקטור לשיפוט

אני החתום מטה מצהיר/ה בזאת: (אנא סמן):

__✓__ חיברתי את חיבורי בעצמי, להוציא עזרת ההדרכה שקיבלתי מאת מנחה/ים.

__✓__ החומר המדעי הנכלל בעבודה זו הינו פרי מחקרי מתקופת היותי תלמיד/ת מחקר.

___ בעבודה נכלל חומר מחקרי שהוא פרי שיתוף עם אחרים, למעט עזרה טכנית
הנהוגה בעבודה ניסיונית. לפי כך מצורפת בזאת הצהרה על תרומתי ותרומת שותפי למחקר, שאושרה על ידם ומוגשת בהסכמתם.

תאריך _____ שם התלמיד זיו קנר חתימה

_____

העבודה נעשתה בהדרכת

פרופ׳ אלי לואיס

מחלקה לביוכימיה קלינית ופרמקולוגיה

פקולטה למדעי הבריאות

חקירת מנגנון הפעילות האנטי-חיידקית של אלפא-1-אנטיטריפסין

מחקר לשם מילוי חלקי של הדרישות לקבלת תואר "דוקטור לפילוסופיה"

מאת

זיו קנר

הוגש לסינאט אוניברסיטת בן גוריון בנגב

אישור המנחה _____

אישור דיקן בית הספר ללימודי מחקר מתקדמים ע"ש קרייטמן

_____

י"א באדר ב', תשע"ו 21.3.16

באר שבע