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Alpha1-antitrypsin therapy ameliorates acute colitis and chronic murine ileitis

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Abstract

Background—Fecal alpha-1-antitrypsin (AAT) clearance has been a marker of clinical disease severity in inflammatory bowel diseases (IBD) for many years. While AAT deficiency is more often associated with lung and liver pathologies, AAT-deficient patients with concomitant IBD have been shown to develop more aggressive disease and rapid progression to surgery. While recent studies have highlighted the pleiotropic anti-inflammatory functions of AAT, including reducing pro-inflammatory cytokine production and suppressing immune cell activation, its potential therapeutic role in IBD has not been described.

Methods—The therapeutic potential of human AAT administration was assessed in murine models of IBD including new-onset and established chemically-induced colitis as well as spontaneous chronic murine ileitis. Histological assessment of inflammation, cytokine secretion profiling and flow cytometric evaluation of inflammatory infiltrate were performed in each model. The effect of AAT on intestinal barrier function was also examined both *in vitro* and *in vivo*.

Results—AAT attenuated inflammation in small and large intestinal IBD models via reduced secretion of pro-inflammatory cytokines, inflammatory cell infiltration and reduced tissue injury. AAT also increased intestinal restitution following chemically-induced colitis. AAT significantly

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decreased intestinal permeability *in vitro* and *in vivo* as part of a protective mechanism for both acute and chronic models of IBD.

Conclusions—Our findings describe a beneficial role for AAT in IBD models via suppression of cytokine production and enhanced intestinal barrier function. This raises the possibility that AAT supplementation, which has a long history of proven safety, may have a therapeutic effect in human IBD.

Keywords

mucosal; inflammation; inflammatory bowel disease

Introduction

The inflammatory bowel diseases (IBD), i.e. ulcerative colitis (UC) and Crohn's disease (CD), are likely initiated by environmental factors (e.g. bacterial antigens), which trigger a dysregulated immune response in genetically predisposed hosts.¹ This response is characterized by imbalanced effector and regulatory mechanisms reflected by a preponderance of pro-inflammatory cytokines within intestinal tissues. Clinically, there is an unmet need for newer therapies as the efficacy of TNF α neutralization induces remission at best in 50-70% of patients and sustained remission drops significantly at 1 year.²

While the direct correlation between fecal alpha-1 antitrypsin (AAT) and disease activity in Crohn's disease has been well documented for approximately thirty years,³ the possibility that this increased loss of AAT might be detrimental in IBD has not been fully explored. AAT is a 418 amino acid serine protease inhibitor, primarily synthesized and secreted by hepatocytes.⁴ It is also transcribed and secreted by neutrophils, macrophages, monocytes, enterocytes⁵ and Paneth cells.⁶ Its best-described function is to protect tissues from the proteolytic activity of neutrophil elastase. This is particularly evident in the lung, where AAT deficiency results in panacinar emphysema.⁴ Despite being the most abundant protease inhibitor in the circulation, it increases 2-3-fold in response to lung inflammation, suggesting that rather than being constitutive; AAT expression can be upregulated in response to inflammatory cues. A growing body of evidence demonstrates that AAT plays additional roles at extra-pulmonary and extra-hepatic sites. AAT protects pancreatic beta cells from apoptosis⁷, inhibits pro-inflammatory cytokine production⁸ and metalloproteinases,⁹ reverses diabetes in NOD diabetic mice¹⁰, induces immune tolerance during islet allograft transplantation^{11, 12} and prolongs islet allograft survival in mice and transplanted organs from graft-versus-host disease.¹³

Although AAT deficiency is approximately ten times less frequent than IBD in North America, a retrospective study demonstrated that nine out of 10 patients with concomitant UC and AAT deficiency were refractory to medical therapy and required colectomy, suggesting that endogenous AAT may have a protective role in the intestine.¹⁴ Yet, to our knowledge this concept has not been explored in preclinical models of IBD. Given the pleiotropic anti-inflammatory effects of AAT, we hypothesized that administration of human plasma-derived AAT (hAAT) might attenuate experimental IBD.

In the current studies we first assessed the therapeutic potential of hAAT in an acute chemically-induced (i.e. DSS) model of colitis, which is predominantly mediated by innate immune mechanisms and is characterized by superficial ulcerations limited to the colonic mucosa: 2 features that are shared with human UC.^{15, 16} We next assessed the benefit of hAAT administration in a more clinically relevant model of recovery from established chemically-induced colitis. Thereafter we demonstrated a protective effect in the SAMP1 chronic model in which mice spontaneously develop idiopathic segmental, transmural ileitis¹⁷ that recapitulate many features of CD. We characterized the impact of AAT administration on intestinal barrier function a feature that reflects both cause and consequence of intestinal inflammation in these models. Finally we demonstrated an upregulation of AAT in Crohn's patients consistent with a potential compensatory anti-inflammatory mechanism.

Materials and Methods

Induction of chemically-induced colitis

Mice were administered DSS (3% w/v; MP biomedical, USA; 36-50 kDa) in drinking water ad libitum for 7 d. Water alone was used for vehicle groups. DSS groups received i.p. injections of either 2 mg/day hAAT (Aralast NP, Baxter, Thousand Oaks, CA or Prolastin C, Talecris, Durham, NC) or human serum albumin. Mice were weighed daily and a disease activity index (DAI) was calculated for each mouse, based on weight loss, occult blood, and stool consistency. A score of 1–4 was given for each parameter, with a maximum DAI score of 12. At the time of sacrifice, spleen, mesenteric lymph nodes (MLN) and segments of colonic tissue were excised for flow cytometric analysis of leukocyte subsets. Colon sections (distal to cecum) were also excised, opened longitudinally and fixed in 10% buffered formalin; paraffin embedded, cut into 3-5 µm sections and stained with hematoxylin/eosin. The severity of colitis was assessed by a pathologist (P.J.) blinded to the treatment groups, as per published methods [18, 19]. Additionally colon lengths were recorded and colon explants cultured for 24 h in complete RPMI media (supplemented with 5 % FBS, 100 IU penicillin and 100 µg/ml streptomycin; Invitrogen).

Recovery from DSS colitis

Mice were treated with DSS for 9 d, replacing with fresh DSS every three days. Once mice had reached the desired target weight loss (15%), DSS was withdrawn. Subsequent recovery of weight was recorded daily and used as a surrogate indicator of mucosal healing. Treatment groups received daily i.p. injections of either 2 mg hAAT (Aralast NP, Baxter, Thousand Oaks, CA or Prolastin C, Talecris, Durham, NC) or human serum albumin upon establishment of disease. Due to the lack of frequent bowel movements during the initial phase of recovery from DSS colitis, DAI were not calculated for those studies.

Treatment of spontaneous chronic ileitis

Twenty-week-old SAMP1 mice from a colony bred by Dr Rivera-Nieves were treated i.p. for 9 days with 2 mg/day hAAT (Prolastin C, Talecris, Durham, NC) or human serum albumin. Terminal ileal tissues were harvested for histological evaluation of inflammation and leukocyte isolation prior to flow cytometric assessment.

Lymphocyte and epithelial cell isolation

Single-cell suspensions were obtained by passage of the MLN or spleen through a 100- μ m cell-strainer. Splenic red blood cells were lysed by incubation in ammonium chloride lysing reagent (ACK Lysis Buffer, Invitrogen, Carlsbad, CA). Intestinal segments were opened along the mesentery and rinsed of luminal contents with PBS before cutting into 1 cm sections in PBS containing 15 mM HEPES and 1 mM EDTA and processed with vigorous agitation on a vortex mixer. The tissue was then passed through a 70 μ m tissue strainer and the process repeated until the wash remained clear. The remaining lamina propria (LP) was digested in 1 mg/ml Collagenase Type VIII (Sigma Aldrich, C9722) for 20 min in an orbital shaker at 270 rpm and 37°C. Tissues were vortexed briefly and filtered to remove any remaining undigested material and cells counted prior to flow cytometric evaluation.

Flow cytometry

Cells from indicated compartments were incubated with fluorescent rat anti-mouse antibodies including against: mouse CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), Nk1.1 (PK136), CD11c (N418), MHCII (M5/114.15.2), F4/80 (BM8), Siglec F (E50-2440) and GR-1 (RB6-8C5). Cells were washed and fixed with 2% paraformaldehyde and analyzed using the BD FACSCanto™II system (Beckton-Dickinson Immunocytometry Systems, San José, CA). Results were expressed as % of total live cells recovered from indicated organs. Absolute numbers of cells were described separately as cellularity of the intestinal tissue. Post-analyses were performed using FLOWJo software (Tree Star Inc., Ashland, OR).

Cytokine production assays

To assess cytokine secretion from the inflamed intestines, tissue explants (0.5 cm²) were cultured for 24 h in DMEM (without sodium pyruvate, Cellgro Manassas, VA, supplemented with 5 % FBS, 2 mM glutamine, 100 IU penicillin and 100 μ g/ml streptomycin; Invitrogen). Culture supernatants were then analyzed for the presence of cytokines with reduced background protein levels compared to tissue homogenates using the highly sensitive Quansys Cytokine Assay system (Logan, UT). Results were then expressed relative to the initial wet weight of the intestinal tissue explants.

Transepithelial electrical resistance assays

Human colonic epithelial cells (T84; a gift from Dr. Stephen Keely, UCD, Dublin, Ireland) were cultured as previously described.¹⁸ Briefly T84 cells (0.5×10^6) were cultured on 6.5 mm polyester permeable Transwell® membranes (0.4 μ m pore; Costar, Cambridge, MA) for 7-10 days to allow for formation of a monolayer with appropriate barrier function, as measured by transepithelial electrical resistance (TEER). Quadruplicate wells were incubated on both sides with control (media alone), hAAT (0.5 mg/ml), DSS (3%) or combined treatment. TEER measurements were taken 6 h post treatment using an Evom (World Precision Instruments, Sarasota, FL).

Endothelial cell permeability assay

Human microvascular endothelial cells (HMEC; Biological Products Branch, Centers for Disease Control, Atlanta, GA) were incubated at 37°C and 5% CO₂ and cultured as

recommended.¹⁹ Macromolecule permeability was measured as previously described²⁰ with slight modifications. Briefly, HMEC were seeded on 0.4µm Transwells as above at 1×10^6 cells per insert and allowed to reach confluency. Inserts were placed in Ca^{2+} -containing Hanks buffered saline solution with or without LPS (10µg/ml, List Laboratories; Campbell CA), hAAT (0.5mg/ml) or both. Following either 6 or 24 h LPS treatment, FITC-labeled dextran 70kD (FD70; 7µM; Sigma Aldrich) were added to the upper chamber of each insert. At time points of 20, 40, and 60 minutes, 50µl of sample were collected and fluorescence measured using a Cytofluor 2300 fluorimeter (Millipore Corp, Bedford, MA). FD70 concentrations were determined from serial dilution and flux rates calculated by linear regression. Data were then normalized and plotted as fold increase over the observed flux rate in untreated endothelial monolayers.

In vivo intestinal permeability assays

Flux of FITC-dextran sugar was used as a surrogate marker of intestinal permeability. Mice received 200µl of FITC-labeled dextran 4kD (FD4; 20mg/kg; Sigma-Aldrich) by gavage and serum samples taken 4 h later were assayed by a fluorimeter.

AAT detection

Human AAT mRNA transcript was measured in cDNA from control and Crohn's disease patients on the TissueScan™ cDNA array (Origene Technologies, Rockville, MD) using Taqman probes (Life Technologies, Grand Island, NY) for both AAT and β -actin as an endogenous control. Disease distribution was determined using clinical and pathologic information provided by Origene for each sample. Paraffin-embedded sections from archived human tissue of patients definitively diagnosed with(out) Crohn's disease were obtained under research protocols approved by the Colorado Multi-Institutional Review Board. These were immunostained using the monoclonal anti-human AAT antibody (1102), Thermo Fisher Scientific (Rockford, IL), according to manufacturers instructions.

Statistics

Statistical analyses were performed using Student t test or two-way ANOVA with Bonferroni posttests with Graphpad Prism Data Analysis software (GraphPad Software, La Jolla, CA). Data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was set at a p value <0.05 .

Results

hAAT protects mice from clinical signs of colitis

Based on the known anti-inflammatory role of hAAT and the association between loss of AAT and disease severity, we chose to assess the therapeutic benefit of AAT supplementation in murine pre-clinical models. We began by assessing the impact of hAAT administration on acute murine colitis. Mice treated with hAAT lost less weight in response to DSS administration compared with albumin-treated mice. Weights at time of sacrifice (day 7) decreased to $84.4 \pm 1.0\%$ of initial body weight in vehicle-treated mice compared with $93.7 \pm 0.9\%$ in hAAT-treated mice ($p < 0.001$) (Fig. 1A). Additionally, at day 7 we observed a reduction of the DAI from 13.7 ± 0.6 in albumin-treated mice to 8.0 ± 0.6 in

hAAT-treated mice ($p<0.001$) (Fig. 1B) and a 20 percent decrease in colonic shortening in hAAT-treated mice compared with albumin-treated mice ($p<0.01$) (Fig. 1C). These differences were evident macroscopically (Fig. 1D). The concentration of hAAT used for these and subsequent studies was chosen based on performance of a dose-response to hAAT in DSS colitis (not shown). Based on its optimal performance, the dosage of 2mg/ml/day was therefore administered in subsequent therapeutic studies.

hAAT ameliorates histological evidence of tissue injury

Treatment with hAAT generated a significant decrease in tissue damage indices, from 2.9 ± 0.1 in vehicle-treated mice to 1.8 ± 0.4 in hAAT-treated animals ($p<0.05$) (Fig. 2A). The decreased tissue damage was reflected by improved villus architecture and reduced goblet cell hyperplasia, as seen in representative micrographs (Fig. 2C). Although adaptive immunity plays a less important role than innate immune cells in this acute model, we observed a significant decrease in the percentage of CD4⁺ T cell in the colonic LP of hAAT-treated mice ($0.4 \pm 0.03\%$) following DSS compared to albumin controls ($0.7 \pm 0.05\%$ in DSS; $p<0.01$) and similarly CD19⁺ B cells decreased in hAAT-treated ($3.3 \pm 0.5\%$) mice relative to DSS controls ($4.4 \pm 0.2\%$; $P<0.05$) (Fig. 2D), though these differences were insufficient to affect overall cell numbers significantly. Thus hAAT administration decreased histological evidence of tissue damage and decreased lymphocytic infiltration during acute colitis.

hAAT suppresses colonic pro-inflammatory cytokine production in DSS-treated mice

Having demonstrated attenuation of colitis with hAAT, we then cultured colonic explants of mice treated with DSS and albumin or hAAT and along with tissues from untreated controls (water) and measured the cytokines released into the supernatants. IL-1 β secretion from colonic explants of DSS colitis mice was significantly higher than from water controls. This increase was significantly attenuated in hAAT-treated mice (3.5 ± 1.3 pg/ml/mg) exposed to DSS relative to vehicle-treated DSS mice (9.1 ± 2.7 pg/ml/mg; $p<0.05$) (Fig. 3). Similarly, IL-6, which was induced in DSS, was significantly reduced in hAAT-treated tissues (6.1 ± 1.1 ng/ml/mg) compared with vehicle-treated controls (10.0 ± 1.0 ng/ml/mg; $P<0.05$). MCP-1 was significantly higher from colitis explants and once again this was significantly decreased in hAAT-treated colitic mice (175.5 ± 57 pg/ml/mg) relative to vehicle controls (420.8 ± 43.6 pg/ml/mg; $p<0.01$). Finally, CXCL1 was increased in response to DSS but was lower in hAAT-treated tissues (4.2 ± 1.2 ng/ml/mg) compared with vehicle-treated DSS colitis (6.6 ± 0.6 ng/ml/mg; $p<0.05$). Therefore, the inflammatory cytokine response induced by DSS colitis was significantly blunted by hAAT administration.

hAAT accelerates mucosal healing after DSS-induced injury

We then studied whether hAAT might have an effect, not only on the prevention but also in the recovery from chemically-induced colonic injury. Upon establishment of inflammation of the colonic mucosa, DSS was discontinued and body weight was monitored (Fig. 4A). Treatment with hAAT significantly increased weight recovery of mice through day 13 when the difference in mean weight loss between treatment groups differed from 10 ± 0.9 in AAT-treated mice vs. 15.6 ± 0.6 in vehicle-treated mice ($n=5$ /group; $p<0.01$) (Fig. 4A). The increased weight gain by hAAT-treated mice coincided with a significant reduction in colon

shortening from 53 ± 2 mm in vehicle-treated mice vs. 69 ± 3 mm in hAAT-treated mice ($n=5$; $p<0.05$) (Fig. 4B). Quantification of cells from DSS-treated mice demonstrated that mice treated with hAAT had a significant increase in the total numbers of cells in the spleen compared with from $50 \times 10^6 \pm 8.5 \times 10^6$ vs. $70 \times 10^6 \pm 8 \times 10^6$ ($P<0.05$; $n=5$) (Fig. 4C) and MLN from $14 \times 10^6 \pm 3 \times 10^6$ in vehicle-treated mice to $27 \times 10^6 \pm 5 \times 10^6$ in hAAT-treated mice ($p<0.05$) (Fig. 4C). In contrast, there was a significant decrease in inflammatory infiltrate in the colonic lamina propria of DSS-treated mice from $4 \times 10^6 \pm 5 \times 10^5$ in vehicle-treated mice vs. $2 \times 10^6 \pm 5.6 \times 10^5$ in hAAT-treated mice ($p<0.05$) (Fig. 4C).

Decreased colonic shortening and colonic cell infiltrate was accompanied by decreased inflammatory indices from 2.4 ± 0.2 to 1.7 ± 0.1 ($p<0.05$) and tissue damage index of 2 ± 0.3 vs. 0.9 ± 0.2 ($p<0.01$; Fig. 4D). Multiplex analysis of cytokine release from colonic explant cultures identified a significant hAAT-mediated decrease in release of IL-1 β (6.2 ± 1.5 pg/ml/mg vs. 0.8 ± 0.4 pg/ml/mg; $p<0.01$) (Fig. 4E), IFN- γ (3.5 ± 1.5 pg/ml/mg vs. 0.4 ± 0.1 pg/ml/mg; $p<0.05$). Similarly, treatment with hAAT significantly decreased release of TNF α (0.8 ± 0.2 pg/ml/mg to 0.1 ± 0.1 pg/ml/mg; $p<0.01$) and IL-17 (3.3 ± 1.6 pg/ml/mg vs. 0.3 ± 0.1 pg/ml/mg; $p<0.05$). Finally release of MCP-1 from colonic explants was significantly reduced by hAAT administration (131.3 ± 38.0 pg/ml/mg vs. 64.3 ± 15.2 pg/ml/mg; $p<0.05$). Thus, 2 weeks after injury we additionally observe changes in lymphocyte-derived cytokines, suggesting that AAT may not only attenuate acute inflammation but also chronic inflammatory processes.

hAAT administration ameliorates chronic ileitis in SAMP1 mice

To further test the hypothesis that hAAT attenuates chronic intestinal inflammation, we treated SAMP1 mice, a chronic ileitis model that is predominantly mediated by adaptive immune responses. hAAT attenuated chronic inflammation (4 ± 0.8 vs. 2.4 ± 0.5 ; $p<0.05$), villus distortion (4.6 ± 0.7 vs. 2.3 ± 0.5 ; $p<0.05$) and total inflammatory indices (14 ± 2 vs. 9 ± 0.5 ; $p<0.05$) (Fig. 5A) relative to albumin-treated controls. In addition, histological hallmarks of ileitis, such as leukocyte infiltration, goblet cell hyperplasia and muscularis hypertrophy, were noticeably decreased (Fig. 5B).

hAAT decreased CD4⁺ T cells in MLN and lamina propria of SAMP1 mice

We then examined whether hAAT might also decrease lymphocyte recruitment to the ileal lamina propria. Subset analysis of leukocytes from the spleen, MLN and ileal lamina propria of SAMP1 mice treated with hAAT or albumin demonstrated a significant decrease in the proportion of CD4⁺ T lymphocytes in both the MLN ($p<0.01$) (Fig. 6A, B) and lamina propria ($p<0.05$). In contrast, there were no significant changes in the proportions of CD8⁺ T lymphocytes, B cells, dendritic cells, macrophages, Nk cells or NkT cells.

hAAT decreased pro-inflammatory cytokines in SAMP1 ileum

Analysis of cytokine release from cultured ileal explants of SAMP1 mice treated with vehicle or hAAT demonstrated a significant decrease in TNF α (0.08 ± 0.03 pg/ml/mg vs. 0.02 ± 0.01 pg/ml/mg; $P<0.05$), IL-17 (0.04 ± 0.01 pg/ml/mg vs. 0 pg/ml/mg; $p<0.05$) and CXCL1 (0.4 ± 0.05 pg/ml/mg vs. 0.1 ± 0.06 pg/ml/mg; $p<0.01$) (Fig. 6C). Therefore treatment of chronic murine ileitis with hAAT attenuated inflammation, decreased lymphocyte

infiltration and suppressed pro-inflammatory cytokine secretion in a manner similar to that seen in the murine colitis models.

hAAT enhanced intestinal barrier function both *in vitro* and *in vivo*

Having demonstrated a significant anti-inflammatory effect of hAAT, we next sought to assess the impact of hAAT administration on intestinal barrier function, based on the shared role for barrier disruption in the mouse models studied. Polarized intestinal epithelial cells (T84) were exposed to DSS in the presence or absence of hAAT. Within 6 h of treatment DSS-induced a 60% reduction in barrier function as measured by TEER ($903 \pm 64 \Omega\text{cm}^2$) relative to vehicle. This decrease in TEER was attenuated by coadministration of DSS and hAAT ($1326 \pm 105 \Omega\text{cm}^2$), which was partially protective compared to DSS alone ($p < 0.05$) (Fig. 7A). Similarly, endothelial cells (HMEC) displayed decreased FD70 flux, an alternative surrogate marker of barrier function, in response to treatment with lipopolysaccharide (LPS; 10 $\mu\text{g/ml}$) following hAAT pre-treatment (1.4 ± 0.1 vs. 0.8 ± 0.1 ; $p < 0.01$) (Fig. 7B).

We then assessed whether hAAT may also reduce barrier permeability in diseased animals, as measured by detection of FD4 in serum 4 h post-FD4 gavage. In SAMP1 mice with established ileitis (20-weeks-of-age) detection of serum FD4 was lower in mice treated for 9 days with hAAT (0.8 ± 0.1 ng/ml) compared with vehicle controls (1.2 ± 0.1 ng/ml; $p < 0.05$) (Fig. 7C) consistent with decreased intestinal permeability in these mice. Thus, hAAT has a protective effect on barrier function both *in vitro* and *in vivo*.

Upregulation of AAT in intestinal tissue of IBD patients

While AAT has long been used as a surrogate marker of intestinal inflammation in IBD, the expression of AAT in the intestine of IBD patients has not been demonstrated to-date. Here we describe the increased expression of AAT in the intestinal crypts of CD patient tissue by immunohistochemistry (Fig. 8A). This increased protein expression coincided with a significant up-regulation of AAT mRNA from CD patients (2.4 fold; $n=18$; $p < 0.05$) relative to healthy controls. This difference was even more striking in inflamed ileal tissue from CD patients (3.3 fold; $n=7$; $p < 0.001$) (Fig. 8B). Finally to account for the cross-species anti-inflammatory effect of hAAT we compared protein sequences and demonstrated that AAT is highly conserved between mouse and human. Therefore, AAT is upregulated in intestinal tissue of Crohn's patients consistent with attempting to offer protection against overactive inflammation.

Discussion and Conclusions

Inflammatory bowel disease affects several million people and the most effective therapies (anti-TNF α antibodies) work at best in 70% of patients²¹. Thus, novel therapies are needed. Here, we demonstrate that human alpha-1-antitrypsin administration protects mice from acute colitis, enhances recovery from established colitis and attenuates spontaneous chronic ileitis. hAAT-mediated attenuation of inflammation coincided with decreased cytokine production and reduced inflammatory infiltrate within the affected intestine.

Intestinal inflammation has been associated with a significant loss of AAT In the feces²² and fecal AAT levels positively correlate with disease severity in Crohn's disease.^{22, 23} Although stool AAT has been seen merely as a surrogate marker of decreased intestinal barrier integrity, its loss may also result in considerably lower local AAT levels. Given the pleiotropic anti-inflammatory effects of the molecule, this deficit may have negative consequences in IBD. For example, patients with AAT deficiency and concomitant IBD develop significantly worse inflammation and are considerably more likely to require colectomy.¹⁴ Although administration of AAT attenuates inflammation in a number of autoimmune murine models^{10, 13}, whether this molecule may attenuate intestinal inflammation had not been explored previously.

We began by examining this question in an acute injury model of colitis, which results from the administration of DSS. DSS-induced epithelial damage results in predictable impaired barrier function^{24, 25}, increased bacterial translocation and prominent macrophage and neutrophil infiltrates. A key role for endogenous AAT is to neutralize neutrophil elastase. Previous studies had demonstrated increased intestinal elastase activity in both murine models and patients with IBD.^{26, 27} This is consistent with the neutrophilic infiltrates, which contribute to the pathogenesis of IBD. Moreover, specific chemical inhibition of neutrophil elastase attenuates DSS-induced colitis^{26, 27}, thus attenuation by hAAT in this model was predictable, based solely on its effect on neutrophil elastase. We attempted to address the relative contribution of neutrophil elastase to the anti-inflammatory effect of hAAT in DSS colitis, through antibody depletion of neutrophils. Yet, neutrophils are such important mediators of inflammation in DSS colitis that it was not possible to differentiate between the beneficial effect of hAAT alone, compared with the effect of neutrophil depletion (data not shown).

Consistent with a principal role for innate immune mechanisms in this model, all of the cytokines that were decreased after hAAT treatment (IL-6, CXCL1, MCP-1) are produced by neutrophils and/or macrophages. In contrast, in human IBD the chronic nature of disease strongly implicates the adaptive immune response in the perpetuation of disease. Thus, we additionally investigated the potential therapeutic efficacy of AAT in SAMP1 mice, a spontaneous chronic murine model of ileitis in which effector CD4⁺ T cells play a central role.¹⁷ Drugs with potent anti-inflammatory effects in this model, as in human IBD, have pleiotropic anti-inflammatory activities acting through multiple mechanisms in concert. In that context we would have expected that the benefit of a molecule that strictly neutralizes neutrophil elastase should be limited. Our experiments demonstrate that this was not the case. Treatment with hAAT reversed chronic intestinal inflammation in these mice, independent of changes in neutrophil numbers but associated with decreased intestinal CD4⁺ T cells and consistent with previous anti-inflammatory studies in chronic murine ileitis²⁸. There was also a concomitant decrease in pro-inflammatory cytokines (i.e. TNF α and IL-17) secreted from the ilea of hAAT-treated mice.

Blockade of pro-inflammatory cytokines, such as TNF α , has proven highly effective in the treatment of IBD²⁹. This cytokine has multiple pro-inflammatory effects at sites of inflammation.^{21, 30} Pro-inflammatory cytokines increase intestinal permeability through targeting of intestinal epithelial³¹ and endothelial cells²⁰, which in turn increases

translocation of luminal antigens and exacerbates activation of resident leukocytes. Locally secreted cytokines also increase the endothelial expression of adhesion molecules³², perpetuating inflammation via increased leukocyte recruitment thereby further increasing pro-inflammatory cytokine secretion and worsening epithelial injury. This deficit in intestinal barrier integrity is closely associated with disease severity³³. The anti-inflammatory effect of AAT on the ileitis of SAMP1 mice, like that of TNF α inhibition, is most likely multifactorial, dependent in part on its inhibition of cytokine production and partly on the restoration of intestinal barrier function, both interconnected mechanisms which are critical in this model³⁴.

Given the clear therapeutic potential of AAT supplementation in murine models we next chose to assess levels of endogenous AAT in IBD patients. While we might have anticipated that IBD patients were deficient in this natural anti-inflammatory, our data demonstrates an increase in AAT at both mRNA and protein levels in IBD patient tissues. The localization of AAT appears consistent with previous description of paneth cell⁶ expression of AAT and also appears to occur to a greater extent in the subset of Crohn's patients with ileal involvement. It may be that the upregulation of AAT is an attempt to counteract the excessive loss of the anti-inflammatory AAT associated with IBD. Lessons learned from the IL-1 field have demonstrated that although IL-1 receptor antagonist, another endogenous anti-inflammatory molecule, is significantly upregulated in chronic diseases such as rheumatoid arthritis³⁵, nevertheless further supplementation has proven beneficial for the delay of joint erosion in these patients³⁶.

The potential anti-inflammatory role of AAT in preclinical models and human IBD had not been explored previously. Yet based on its known effect on neutrophil elastase a therapeutic effect on DSS-induced injury was anticipated. However, its anti-inflammatory role in a chronic model of ileitis is an entirely novel finding. The therapeutic benefit seen in these studies using an endogenous anti-inflammatory molecule, which lacks the immunogenicity of other biologicals and has a long-standing safety record, makes this a potential novel approach for the treatment of IBD.

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Abbreviations

AAT	alpha-1-antitrypsin
IBD	inflammatory bowel disease
CD	Crohn's disease
UC	ulcerative colitis
hAAT	human alpha-1-antitrypsin
LP	lamina propria
MLN	mesenteric lymph node
DSS	dextran sulfate sodium
DAI	disease activity index
HMEC	human microvascular endothelial cells
TEER	transepithelial electrical resistance
FD	fluorescein isothiocyanate (FITC) dextran

Figure 1. Alpha-1 antitrypsin protected mice from acute colitis

(A) Assessment of DSS-induced weight loss in mice treated with albumin or hAAT. (B) Measurement of clinical disease activity including weight loss, fecal consistency and rectal bleeding of albumin and hAAT-treated DSS-challenged mice. (C) Colon length of water control, albumin-injected DSS-treated mice and mice receiving DSS and hAAT. (D) Representative photograph of DSS-induced injury in albumin and hAAT-treated mice. Data expressed as mean±SEM, $n=6$ mice/group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 2. hAAT attenuated histological indices of colitis

(A) Histological scores of tissue damage and inflammatory cell infiltrate assessed by a trained pathologist in a blinded fashion. (B) Quantification of cellularity from indicated organs of water, albumin-treated DSS mice or mice receiving both DSS and hAAT. (C) Representative micrographs showing attenuated inflammation by hAAT treatment in DSS-colitic mice compared with albumin-treated controls. (D) Infiltration of T and B cells in the colonic lamina propria assessed by multicolor flow cytometry. Data are expressed as mean $\pm$ SEM; * P <0.05. Results are representative of 3 independent experiments with at least 6 mice/group in each experiment.

Figure 3. hAAT reduced cytokine secretion by colonic explants

Assessment of cytokines released by colon tissue explants cultured for 24 hr in complete media from DSS-treated albumin or hAAT-treated compared with control (water) mice. Data expressed as mean $\pm$ SEM, $n=6$ /group, * $P<0.05$.

Figure 4. hAAT expedited recovery from established colitis

(A) Weight change during recovery from DSS injury expressed as percentage change from day 0 of DSS administration. (B) Postmortem assessment of colon length from colitic mice treated with (DSS + hAAT) or without hAAT (DSS + albumin). (C) Quantification of cellular infiltrate in indicated organs from colitic mice treated with hAAT or albumin. (D) Histological assessment of colitis from albumin or hAAT-treated mice during recovery from colitis. (E) Measurement of secreted cytokines from colonic explants of colitic mice treated with albumin or hAAT. Data expressed as mean $\pm$ SEM, $n=6$ mice/group; * $P<0.05$, ** $P<0.01$.

Figure 5. hAAT attenuated ileitis in SAMP1 mice

(A) Histological assessment of 20-week-old SAMP1 mice treated with albumin or hAAT. Data expressed as mean $\pm$ SEM, $n = 6$ mice/group from a single study reflective of multiple independent studies; $P < 0.05$. (B) Representative micrographs showing ileal tissue morphology in SAMP1 mice from albumin and hAAT-treated animals. Black scale bars indicate 100 μ m.

Figure 6. hAAT decreased CD4⁺ T cells and inflammatory cytokines in SAMP1 mice
 (A) The percentages of CD4⁺, CD8⁺, CD19⁺ lymphocytes from indicated organs analyzed by flow cytometry. (B) Representative dot plots showing percentages of CD4 and CD8 lymphocytes from the MLN and lamina propria of SAMP1 mice treated with albumin or hAAT. (C) Expression of cytokine secretion from ileal tissue explants from SAMP1 mice treated with albumin or hAAT. Data expressed as mean±SEM, $n=6$ /group, * $P<0.05$, ** $P<0.01$.

Figure 7. Intestinal barrier function is protected by hAAT administration

(A) Measurement of transepithelial electrical resistance (TEER) across confluent intestinal epithelial monolayers grown on permeable supports and treated with either vehicle or hAAT alone or with concomitant DSS injury as an indirect indicator of epithelial barrier function. (B) Fluorometric assessment of FITC-dextran flux across an endothelial cell monolayer treated with and without hAAT and concomitantly challenged with or without lipopolysaccharide (LPS). (C) Fluorescent detection of FITC-dextran, a surrogate marker for *in vivo* intestinal permeability, measured in serum of SAMP1 mice treated with albumin or hAAT. * $P < 0.05$, ** $P < 0.01$.

Figure 8. Increased expression of hAAT in the intestine of IBD patients
 (A) Immunohistochemical detection of hAAT expression in control and Crohn's disease patient intestinal biopsies demonstrates an increased expression predominately localized to the intestinal crypts. (B) Real time PCR analysis of hAAT mRNA expression in human intestinal tissue demonstrates a significant up-regulation of hAAT in the intestine of IBD. (C) Sequence analysis of hAAT expression compared with that of mouse AAT demonstrates strong homology by basic local alignment.