Alpha-1-antitrypsin Therapy Ameliorates Acute Colitis and Chronic Murine Ileitis

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Background: Fecal alpha-1-antitrypsin (AAT) clearance has been a marker of clinical disease severity in inflammatory bowel diseases (IBDs) for many years. Although 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. Although recent studies have highlighted the pleiotropic anti-inflammatory functions of AAT, including reducing proinflammatory 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 and 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 through reduced secretion of proinflammatory cytokines, inflammatory cell infiltration, and reduced tissue injury. AAT also increased intestinal restitution after chemically induced colitis. AAT significantly 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 through 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.

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Key Words: mucosal, inflammation, inflammatory bowel disease

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 proinflammatory cytokines within intestinal tissues. Clinically, there is an unmet need for newer therapies as the efficacy of tumor necrosis factor (TNF)-α neutralization induces remission at best in 50% to 70% of patients and sustained remission drops significantly at 1 year.[2]

Although the direct correlation between fecal alpha-1-antitrypsin (AAT) and disease activity in CD has been well documented for approximately 30 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, enterocytes,[5] 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 from 2-fold to 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 extrapulmonary and extrahepatic sites. AAT protects pancreatic beta cells from apoptosis,[7] inhibits proinflammatory cytokine production[8] and metalloproteinases,[9] reverses diabetes in non-obese diabetic mice,[10] induces immune

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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 10 times less frequent than IBD in North America, a retrospective study demonstrated that 9 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 an acute chemically induced (i.e., dextran sulfate sodium [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 ileitis[17] 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 patients with CD consistent with a potential compensatory anti-inflammatory mechanism.

MATERIALS AND METHODS

Induction of Chemically induced Colitis

Mice were administered DSS (3% wt/vol; 36–50 kDa; MP Biomedicals, Solon, OH) in drinking water ad libitum for 7 days. Water alone was used for vehicle groups. DSS groups received intraperitoneal 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 to 4 was given for each parameter, with a maximum DAI score of 12. At the time of killing, 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 to 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 hours in complete RPMI media (supplemented with 5% fetal bovine serum, 100 IU penicillin, and 100 μg/mL streptomycin; Invitrogen, Carlsbad, CA).

Recovery from DSS Colitis

Mice were treated with DSS for 9 days, replacing with fresh DSS every 3 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 intraperitoneal injections of either 2 mg hAAT (Aralast NP; Baxter or Prolastin C; Talecris) or human serum albumin on 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 intraperitoneally for 9 days with 2 mg/day hAAT (Prolastin C; Talecris) or human serum albumin. Terminal ileal tissues were harvested for histological evaluation of inflammation and leukocyte isolation before flow cytometric assessment.

Lymphocyte and Epithelial Cell Isolation

Single-cell suspensions were obtained by passage of the MLN or spleen through a 100-μm cell strainer. Splenic red blood cells were lysed by incubation in ammonium chloride lysing reagent (ACK Lysis Buffer; Invitrogen). 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-μm 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 (C9722; Sigma–Aldrich, St. Louis, MO) for 20 minutes 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 before 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 FACSCantoII system (Beckton-Dickinson Immunocytometry Systems, San José, CA). Results were expressed as percentage 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 cm^2) were cultured for 24 hours in Dulbecco's modified Eagles medium (without sodium pyruvate; Cellgro, Manassas, VA, supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 IU penicillin, and 100 μg/mL streptomycin; Invitrogen).

Culture supernatants were then analyzed for the presence of cytokines with reduced background protein levels compared with 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-μm pore; Costar, Cambridge, MA) for 7 to 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 hours posttreatment using an Evom (World Precision Instruments, Sarasota, FL).

Endothelial Cell Permeability Assay

Human microvascular endothelial cells (Biological Products Branch, Centers for Disease Control, Atlanta, GA) were incubated at 37°C and 5% CO_2 and cultured as recommended.[19] Macromolecule permeability was measured as previously described[20] with slight modifications. Briefly, human microvascular endothelial cells were seeded on 0.4-μm 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 lipopolysaccharide (10 μg/mL; List Laboratories, Campbell, CA), hAAT (0.5 mg/mL), or both. Following either 6 or 24 hours LPS treatment, fluorescein isothiocyanate-labeled dextran 70 kDa (FD70, 7 μM; Sigma–Aldrich) was added to the upper chamber of each insert. At time points of 20, 40, and 60 minutes, 50 μl of sample was 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 fluorescein isothiocyanate-dextran sugar was used as a surrogate marker of intestinal permeability. Mice received 200 μl of fluorescein isothiocyanate-labeled dextran 4 kDa (FD4; 20 mg/kg; Sigma–Aldrich) by gavage, and serum samples taken 4 hours later were assayed by a fluorimeter.

AAT Detection

Human AAT messenger RNA transcript was measured in complementary DNA from control and patients with CD on the TissueScan complementary DNA 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 pathological information provided by Origene Technologies for each sample. Paraffin-embedded sections from archived human tissue of patients definitively diagnosed with(out) CD 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 the manufacturer's instructions.

Statistics

Statistical analyses were performed using Student's *t* test or 2-way analysis of variance with Bonferroni posttests with GraphPad Prism Data Analysis software (GraphPad Software, La Jolla, CA). Data were expressed as mean ± standard error of the mean. 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 preclinical 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 killing (day 7) decreased to 84.4 ± 1.0% of initial body weight in vehicle-treated mice compared with 93.7 ± 0.9% in hAAT-treated

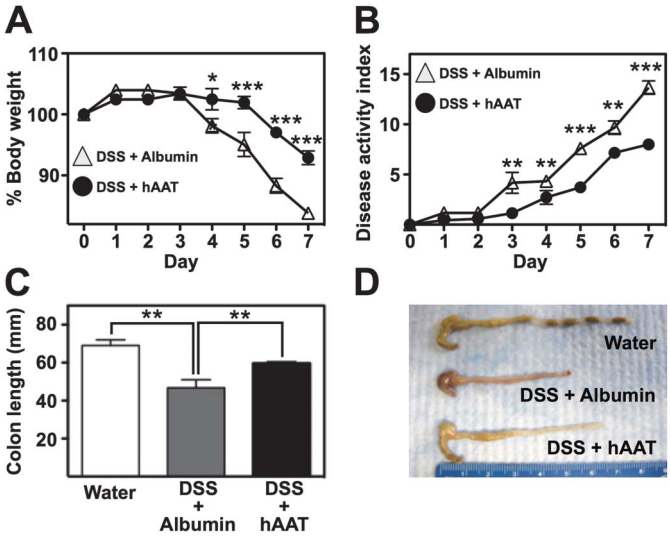

FIGURE 1. Alpha-1-antitrypsin protected mice from acute colitis. A, Assessment of DSS-induced weight loss in mice treated with albumin or hAAT. B, Measurement of clinical disease activity including weight loss, fecal consistency, and rectal bleeding of albumin and hAAT-treated DSS-challenged mice. C, 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-treated and hAAT-treated mice. Data expressed as mean ± standard error of the mean, n = 6 mice per group; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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 the performance of a dose response to hAAT in DSS colitis (data not shown). Based on its optimal performance, the dosage of 2 mg/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 ± 0.03%) following DSS compared with albumin controls (0.7 ± 0.05% in DSS; $P < 0.01$), and similarly, $CD19^+$ B cells decreased in hAAT-treated (3.3 ± 0.5%) mice relative to DSS controls (4.4 ± 0.2%; $P < 0.05$) (Fig. 2D), although 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 Proinflammatory 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

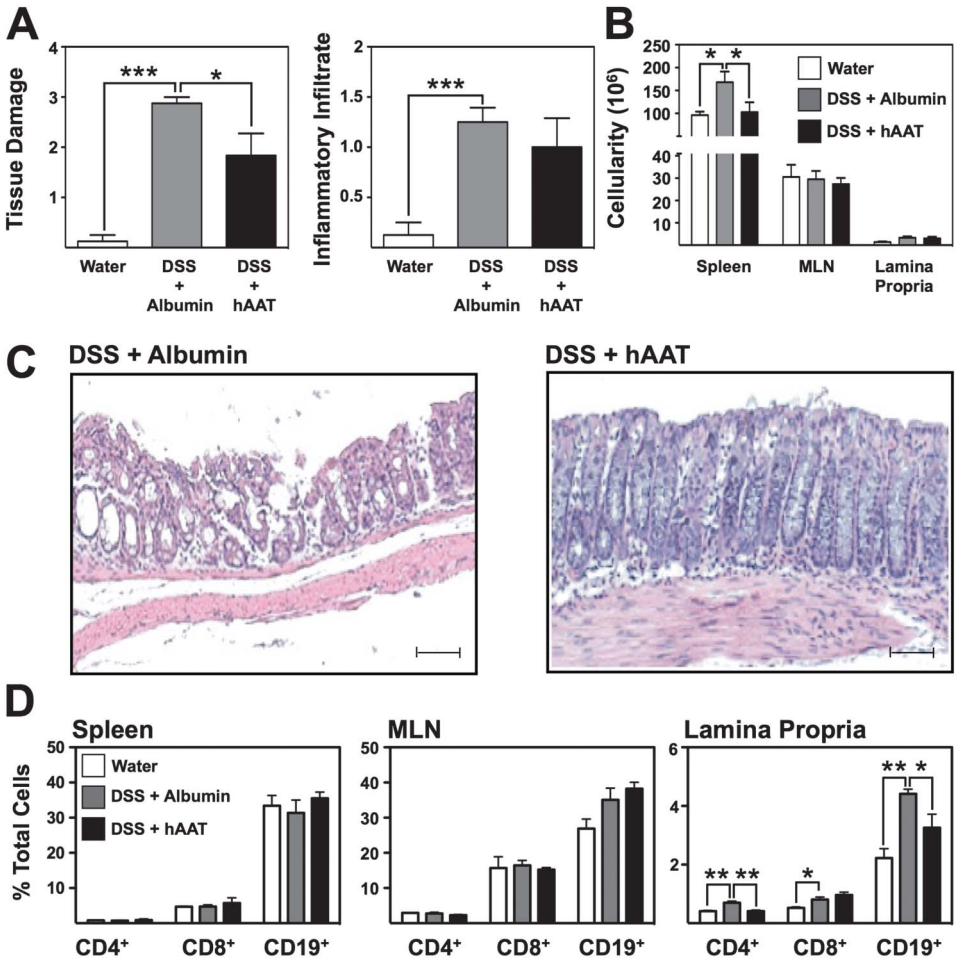

FIGURE 2. hAAT attenuated histological indices of colitis. A, Histological scores of tissue damage and inflammatory cell infiltrate assessed by a trained pathologist in a blinded fashion. B, Quantification of cellularity from indicated organs of water, albumin-treated DSS mice, or mice receiving both 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 LP assessed by multicolor flow cytometry. Data are expressed as mean ± standard error of the mean; *$P < 0.05$. Results are representative of 3 independent experiments with at least 6 mice per group in each experiment.

or hAAT, along with tissues from untreated controls (water) and measured the cytokines released into the supernatants. Interleukin (IL)-1β 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; $P < 0.05$). Monocyte chemotactic protein-1 (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. On 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 versus 15.6 ± 0.6 in vehicle-treated mice (n = 5 per 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 versus 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 $50 \times 10^6 \pm 8.5 \times 10^6$ versus $70 \times 10^6 \pm 8 \times 10^6$ ($P < 0.05$; 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 LP of DSS-treated mice from $4 \times 10^6 \pm 5 \times 10^5$ in vehicle-treated mice versus $2 \times 10^6 \pm 5.6 \times 10^5$ 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 versus 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 the release of IL-1β (6.2 ± 1.5 pg/mL/mg versus 0.8 ± 0.4 pg/mL/mg; $P < 0.01$) (Fig. 4E) and interferon γ (3.5 ± 1.5 pg/mL/mg versus 0.4 ± 0.1 pg/mL/mg; $P < 0.05$). Similarly, treatment with hAAT significantly decreased the 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 versus 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 versus 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.

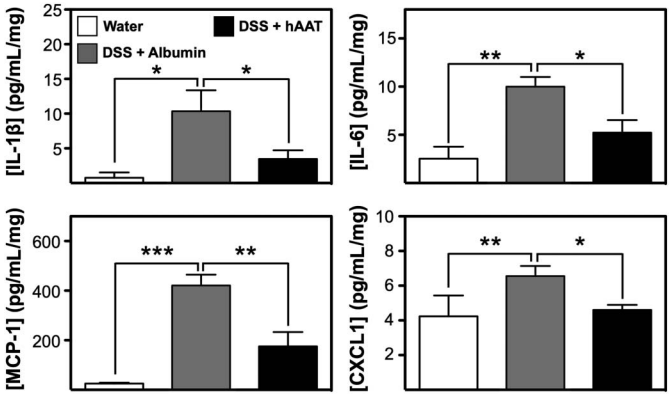

FIGURE 3. hAAT reduced cytokine secretion by colonic explants. Assessment of cytokines released by colon tissue explants cultured for 24 hours in complete media from DSS-treated albumin or hAAT-treated mice compared with control (water) mice. Data expressed as mean ± standard error of the mean, n = 6 per group, *$P < 0.05$.

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 versus 2.4 ± 0.5; $P < 0.05$), villus distortion (4.6 ± 0.7 versus 2.3 ± 0.5; $P < 0.05$), and total inflammatory indices (14 ± 2 versus 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 LP. Subset analysis of leukocytes from the spleen, MLN, and ileal LP 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 LP ($P < 0.05$). In contrast, there were no significant changes in the proportions of CD8$^+$ T lymphocytes, B cells, dendritic cells, macrophages, natural killer cells or natural killer T cells.

hAAT Decreased Proinflammatory Cytokines in SAMP1 Ileum

Analysis of cytokine release from cultured ileal explants of SAMP1 mice treated with vehicle or hAAT demonstrated a significant

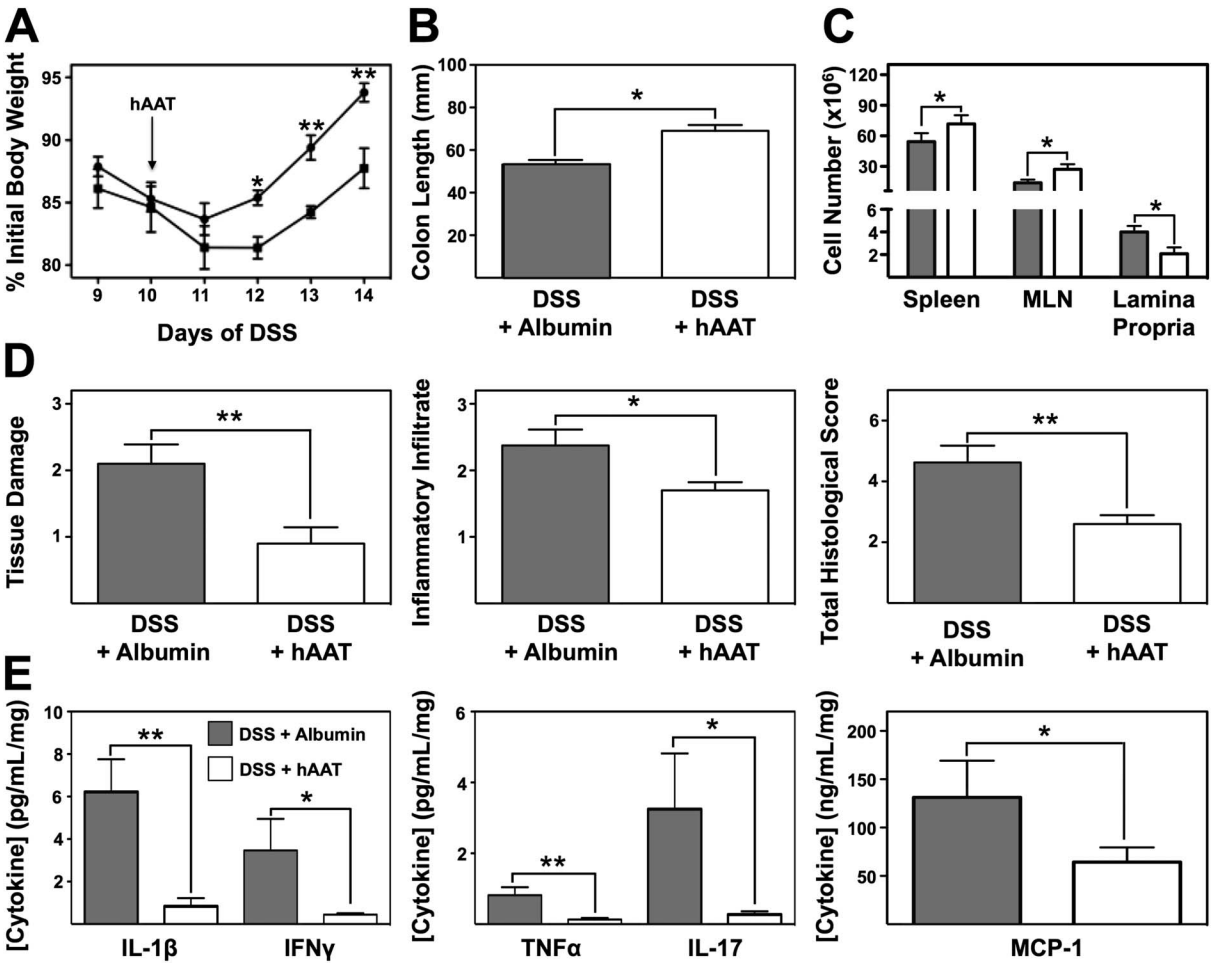

FIGURE 4. hAAT expedited recovery from established colitis. A, Weight change during recovery from DSS injury expressed as percentage change from day 0 of DSS administration. B, Postmortem assessment of colon length from colitic mice treated with (DSS + hAAT) or without hAAT (DSS + albumin). C, Quantification 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 ± standard error of the mean, n = 6 mice per group; *$P < 0.05$, **$P < 0.01$.

decrease in TNF-α (0.08 ± 0.03 pg/mL/mg versus 0.02 ± 0.01 pg/mL/mg; $P < 0.05$), IL-17 (0.04 ± 0.01 pg/mL/mg versus 0 pg/mL/mg; $P < 0.05$), and CXCL1 (0.4 ± 0.05 pg/mL/mg versus 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 proinflammatory 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 hours of treatment, DSS induced a 60% reduction in barrier function as measured by TEER (903 ± 64 Ωcm^2) relative to vehicle. This decrease in TEER was attenuated by coadministration of DSS and hAAT (1326 ± 105 Ωcm^2), which was partially protective compared with DSS alone ($P < 0.05$) (Fig. 7A). Similarly, endothelial cells (human microvascular endothelial cells) displayed decreased FD70 flux, an alternative surrogate marker of barrier function, in response to treatment with lipopolysaccharide (10 μg/mL) after hAAT pretreatment (1.4 ± 0.1 versus 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 hours 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.

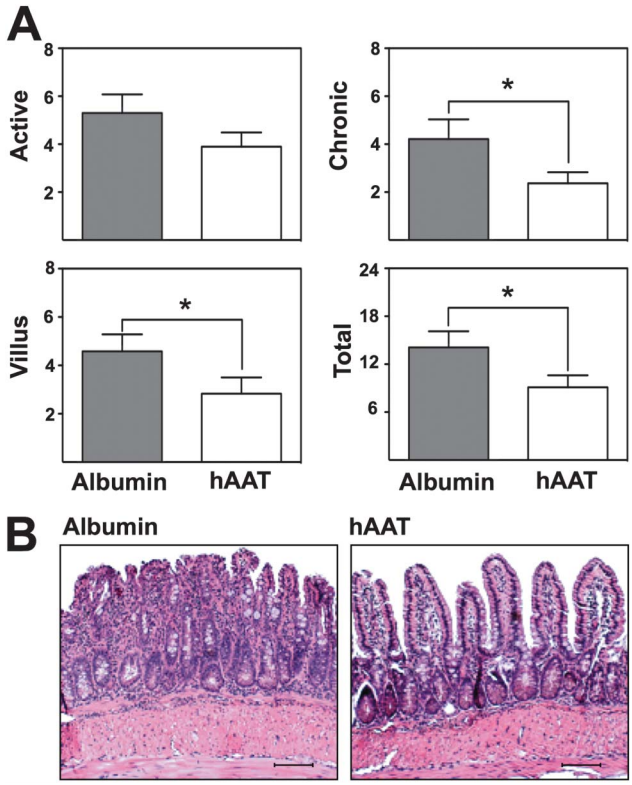

FIGURE 5. hAAT attenuated ileitis in SAMP1 mice. A, Histological assessment of 20-week-old SAMP1 mice treated with albumin or hAAT. Data expressed as mean ± standard error of the mean, n ≥ 6 mice per group from a single study reflective of multiple independent studies; *$P < 0.05$. B, Representative micrographs showing ileal tissue morphology in SAMP1 mice from albumin and hAAT-treated animals. Black scale bars indicate 100 μm.

Upregulation of AAT in Intestinal Tissue of Patients with IBD

Although AAT has long been used as a surrogate marker of intestinal inflammation in IBD, the expression of AAT in the intestine of patients with IBD has not been demonstrated to date. Here, we describe the increased expression of AAT in the intestinal crypts of tissue of patient with CD by immunohistochemistry (Fig. 8A). This increased protein expression coincided with a significant upregulation of AAT messenger RNA from patients with CD (2.4-fold; n = 18; $P < 0.05$) relative to healthy controls. This difference was even more striking in inflamed ileal tissue from patients with CD (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 patients with CD consistent with attempting to offer protection against over-active inflammation.

DISCUSSION AND CONCLUSIONS

IBD affects several million people and the most effective therapies (anti-TNF-α antibodies) work at best in 70% of patients.[21] Thus, novel therapies are needed. Here, we demonstrate that human AAT 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. Fecal AAT levels positively correlate with disease severity in CD.[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.

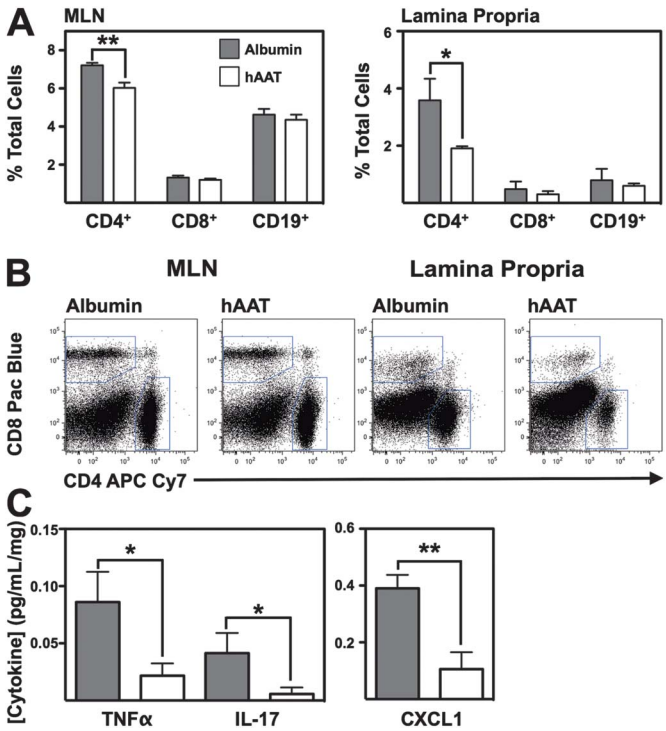

FIGURE 6. hAAT decreased CD4+ T cells and inflammatory cytokines in SAMP1 mice. A, The percentages of CD4+, CD8+, and CD19+ lymphocytes from indicated organs analyzed by flow cytometry. B, Representative dot plots showing percentages of CD4 and CD8 lymphocytes from the MLN and LP of SAMP1 mice treated 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 ± standard error of the mean, n = 6 per group, *$P < 0.05$, **$P < 0.01$.

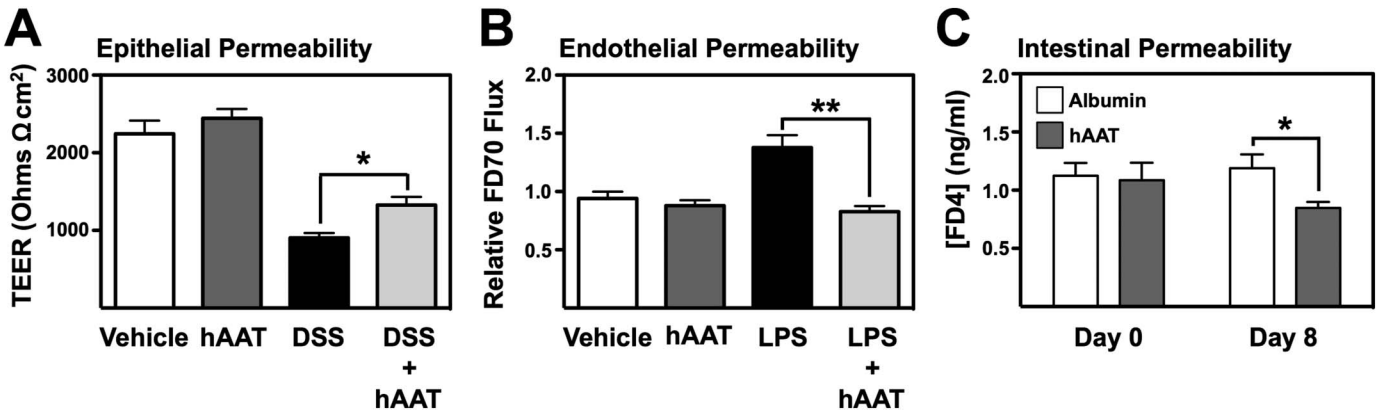

FIGURE 7. Intestinal barrier function is protected by hAAT administration. A, Measurement of TEER across confluent intestinal epithelial monolayers grown on permeable supports and treated with either vehicle or hAAT alone or with concomitant DSS injury as an indirect indicator of epithelial barrier function. B, Fluorometric assessment of fluorescein isothiocyanate-dextran flux across an endothelial cell monolayer treated with and without hAAT and concomitantly challenged with or without lipopolysaccharide (LPS). C, Fluorescent detection of fluorescein isothiocyanate-dextran, a surrogate marker for in vivo intestinal permeability, measured in serum of SAMP1 mice treated with albumin or hAAT. $*P < 0.05$, $**P < 0.01$.

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).

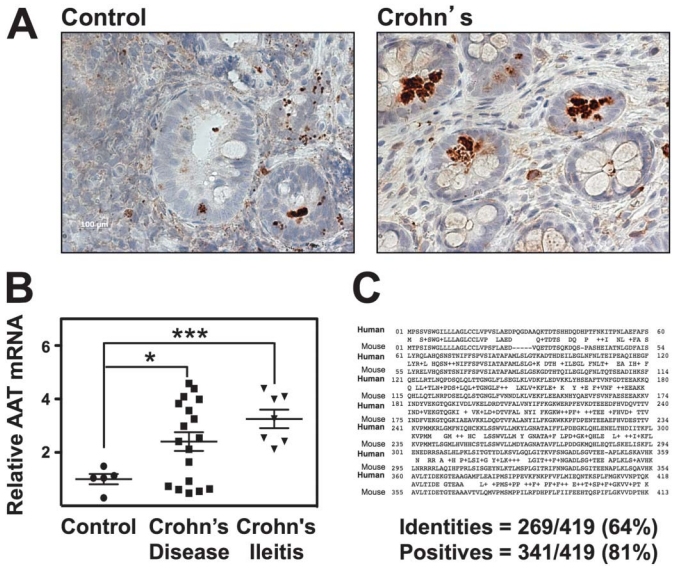

FIGURE 8. Increased expression of hAAT in the intestine of patients with IBD. A, Immunohistochemical detection of hAAT expression in control and patient with CD intestinal biopsies demonstrates an increased expression predominately localized to the intestinal crypts. B, Real-time PCR analysis of hAAT messenger RNA expression in human intestinal tissue demonstrates a significant upregulation 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. Data expressed as mean +/− standard error of the mean; $*P < 0.05$, $***P < 0.001$.

Consistent with a principal role for innate immune mechanisms in this model, all 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 though 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 ileitis.[28] There was also a concomitant decrease in proinflammatory cytokines (i.e., TNF-α and IL-17) secreted from the ilea of hAAT-treated mice.

Blockade of proinflammatory cytokines, such as TNF-α, has proven highly effective in the treatment of IBD.[29] This cytokine has multiple proinflammatory effects at sites of inflammation.[21,30] Proinflammatory cytokines increase intestinal permeability through

targeting of intestinal epithelial[31] and endothelial cells,[20] which in turn increases translocation of luminal antigens and exacerbates activation of resident leukocytes. Locally secreted cytokines also increase the endothelial expression of adhesion molecules,[32] perpetuating inflammation through increased leukocyte recruitment thereby further increasing proinflammatory cytokine secretion and worsening epithelial injury. This deficit in intestinal barrier integrity is closely associated with disease severity.[33] 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 model.[34]

Given the clear therapeutic potential of AAT supplementation in murine models, we next chose to assess levels of endogenous AAT in patients with IBD. Although we might have anticipated that patients with IBD were deficient in this natural anti-inflammatory, our data demonstrates an increase in AAT at both messenger RNA and protein levels in tissues of patient with IBD. The localization of AAT seems consistent with the previous description of paneth cell[6] expression of AAT and also seems to occur to a greater extent in the subset of patients with CD 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 arthritis,[35] nevertheless further supplementation has proven beneficial for the delay of joint erosion in these patients.[36]

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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