

Expanding the Clinical Indications for α_1 -Antitrypsin Therapy

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α_1 -Antitrypsin (AAT) is a 52-kDa circulating serine protease inhibitor. Production of AAT by the liver maintains 0.9–1.75 mg/mL circulating levels. During acute-phase responses, circulating AAT levels increase more than fourfold. In individuals with one of several inherited mutations in AAT, low circulating levels increase the risk for lung, liver and pancreatic destructive diseases, particularly emphysema. These individuals are treated with lifelong weekly infusions of human plasma-derived AAT. An increasing amount of evidence appears to suggest that AAT possesses not only the ability to inhibit serine proteases, such as elastase and proteinase-3 (PR-3), but also to exert antiinflammatory and tissue-protective effects independent of protease inhibition. AAT modifies dendritic cell maturation and promotes T regulatory cell differentiation, induces interleukin (IL)-1 receptor antagonist and IL-10 release, protects various cell types from cell death, inhibits caspases-1 and -3 activity and inhibits IL-1 production and activity. Importantly, unlike classic immunosuppressants, AAT allows undeterred isolated T-lymphocyte responses. On the basis of preclinical and clinical studies, AAT therapy for nondeficient individuals may interfere with disease progression in type 1 and type 2 diabetes, acute myocardial infarction, rheumatoid arthritis, inflammatory bowel disease, cystic fibrosis, transplant rejection, graft versus host disease and multiple sclerosis. AAT also appears to be antibacterial and an inhibitor of viral infections, such as influenza and human immunodeficiency virus (HIV), and is currently evaluated in clinical trials for type 1 diabetes, cystic fibrosis and graft versus host disease. Thus, AAT therapy appears to have advanced from replacement therapy, to a safe and potential treatment for a broad spectrum of inflammatory and immune-mediated diseases.

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INTRODUCTION

By use of transgenic sheep that carry the genetic sequence for human α_1 -antitrypsin (AAT) under the control of mammary gland promoter, biologically active human AAT has been generated in milk and purified and introduced to humans by intravenous infusion (1). By using this method, it is estimated that a population of 4,500 sheep would be able to provide in a single year 5,000 kg (~11,000 lb) of human AAT. Unfortunately, the reaction of individuals who participated in the infusion trial was one of rapid onset of fever due to the mounting of human anti-sheep antibodies against residual sheep α_1 -antichymotrypsin. The trial was discontinued. The original goal

of this endeavor was to provide sufficient AAT to treat an increasing number of patients who are diagnosed with low circulating levels of AAT. The current source of AAT for augmentation therapy is human plasma-derived affinity-purified AAT. Yet, whereas the purpose of augmentation therapy is to avoid the progression of lung emphysema, efficacy studies that assess this goal are incomplete (reviewed in [2]).

Parallel studies that examine various attributes of human AAT depict the molecule as more than just an antiprotease. AAT appears to effectively interfere with inflammatory responses and protect from cell death in an impressive variety of *in vivo* (Table 1) and *in vitro* (Table 2)

experimental models (3). A noteworthy example includes the blockade of inflammatory cytokine release from human peripheral blood mononuclear cells (PBMC) (4). Specifically, AAT decreases the production of important inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , two prototypical upstream mediators of inflammation. AAT also lowers the levels of the chemokines IL-8 and monocyte chemoattractant protein (MCP)-1, two major chemokines in the trafficking of inflammatory cells. Whereas the activity of proinflammatory cytokines appears to consistently diminish in the presence of elevated AAT, the release of antiinflammatory mediators increases. The endogenous inhibitor of IL-1 activity, IL-1 receptor antagonist, is upregulated by AAT in human blood cells (5). Similarly, IL-10 levels have been shown to increase by AAT in various experimental conditions (4,6–9). When examining the cellular targets of AAT, one finds that these primarily include members of the innate immune system, such as macrophages and neutrophils, as well as

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Table 1. Selected *in vivo* biological activities of AAT.

<i>In vivo</i> model	Source and dose of AAT	Outcomes	Reference
<i>Modulation of adaptive immunity</i>			
Islet allograft immune response	Aralast, 60 mg/kg; matrigel-embedded islets	Graft survival prolonged, immune cell infiltration reduced, intragraft insulin content increased, intragraft VEGF transcript levels elevated	10
	Aralast, 60 mg/kg Plasmid-derived hAAT, 450 μ g/mL plasma levels	Grafts accepted, immune tolerance achieved, Tregs localized at graft sites, systemic and local IL-1Ra elevated	8 148
Islet autoimmune response	Aralast, 60 mg/kg; adeno-associated delivery of recombinant AAT	Islet function preserved, immune tolerance achieved, auto- and alloreactive grafts accepted	7,84,159
Cell allograft immune response	Aralast, 60 mg/kg	Day 1–5 immune cell infiltration reduced, including macrophages, neutrophils, T cells and NK cells	10
CIA	Prolastin, 60 mg/kg	Delayed disease onset, lower disease score	114,115
EAE	Mice transgenic for hAAT, constitutive 0.2 μ g/mL plasma levels	Decreased disease incidence, lower disease score, increased Treg proportions in lymphoid compartments	6
GVHD (MHC disparate bone-marrow transplantation)	Aralast, 1–4 mg/dose	Attenuation (posttreatment) and prevention (pretreatment) of GVHD, reduced expansion of alloreactive T cells, enhanced recovery of Tregs, reduced serum levels of proinflammatory cytokines and superior survival	11,12
<i>In vivo</i> leukocyte infiltration			
ThG-elicited peritoneal infiltration	Aralast, 60 mg/kg	Infiltrating macrophages and neutrophils diminished	10
Acute myocardial infarction	Aralast, 60 mg/kg	Myocardial leukocyte infiltration diminished	125
EAE	Tissue-specific transgenic hAAT, 0.2 μ g/mL plasma levels	Decreased spinal leukocytic infiltration	6
<i>In vivo</i> innate responses			
Systemic LPS challenge (mice)	hAAT plasmid-derived, 450 μ g/mL plasma levels	Antiinflammatory serum cytokine profile, for example, elevated IL-1Ra and IL-10 and greater levels of foxp3 Tregs	8
Lung LPS challenge (rabbits)	hAAT Shanghai Biological Production Institute, 120 mg/kg	Lung function and arterial blood gases improved, bronchoalveolar neutrophil elastase, TNF- α and IL-8 reduced	3
<i>In vivo</i> cell injury			
Toxic β -cell injury	Aralast, 60 mg/kg	48-h cell death reduced, insulin release preserved	10,98
Acute myocardial injury after LAD occlusion and myocardial infarction	Aralast, 60 mg/kg	Reduced infarct size, decreased caspase-1 tissue levels, reduced post-infarct remodeling	125

CIA, Collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; LAD, left anterior descending; ThG, thioglycolate.

B lymphocytes and dendritic cells. In contrast, responses of purified T lymphocytes are consistently unaffected by AAT (7,8,10–13), allowing for a variety of responses to IL-2, as well as to concanavalin A and anti-CD3/CD28 stimulation, to persist. This cell-specific discretion, together with the ability to

protect tissues from injury, sets AAT in a unique niche among modulators of the immune system, a separate entity to other antiinflammatory agents and classic immunosuppressants.

As an acute-phase protein, AAT rises in the circulation approximately fourfold and remains elevated for a week to 10 d.

These levels are reproduced clinically by so-called replacement or augmentation therapy, using affinity-purified human AAT (14). The standard protocol involves lifelong weekly infusions that result in a typical profile of systemic AAT: a spike that reaches fourfold the normal values in the first half of the week and a decline

Table 2. Selected *in vitro* biological activities of AAT.

<i>In vitro</i> assay	Cellular targets	Source and concentration	Outcomes	Reference
Cell function				
Glucose-stimulated insulin secretion	Mouse islets	Aralast, 0.25–0.5 mg/mL	Cytokine-dampened insulin release restored	10
	Human islets	Aralast, 0.5 mg/mL	Impure islet culture insulin release improved	160
	β -cell lines	Prolastin, 0.125–1 mg/mL	Insulin release improved	82
Collagen I production during wound healing	Skin fibroblasts	Human AAT (Calbiochem), 0.1 μ g/mL	Production increased	161
Cell survival				
Proinflammatory cytokine toxicity	Primary mouse islets	Aralast, 0.25–0.5 mg/mL	LDH release diminished	10
	Rat INS-1 cell line	Prolastin, 0.5 mg/mL	Cell death reduced	82
β -cell-specific toxin (streptozotocin)	Murine MIN-6 cell line	Prolastin, 0.5 mg/mL	Apoptosis reduced	98
Caspase-3-induced apoptosis	Primary lung alveolar endothelial cells	hAAT (Calbiochem), 0.05 mg/mL	Apoptosis reduced	60
LPS and ischemia-induced injury	Adult cardiac myocyte cell line HL-1	Aralast, 4.0 mg/mL	Cell death reduced	125
Immune cell cytokine production				
LPS stimulation	Human PBMC	Prolastin, 0.5 mg/mL	Reduced proinflammatory cytokine release	162
Heat-inactivated <i>S. epi.</i> stimulation	Human PBMC	Aralast, 8.0 mg/mL	Reduced proinflammatory cytokine release	4
Steady-state	Nonstimulated murine B cells	Prolastin, 0.5 mg/mL	Steady-state BAFF production decreased	114
LPS stimulation	Human neutrophils	Human neuronal cell line-derived recombinant hAAT, 0.5 mg/mL; prolastin, 0.25 mg/mL	Reduced TNF- α release	163
Mixed lymphocyte reaction	Human PBMC	Aralast, 0.1–0.5 mg/mL	Reduced IL-32	11
Mixed lymphocyte-DC reaction	Murine OT-II T cells and OVA-loaded DC	Aralast, 0.5 mg/mL	Reduced IL-6, elevated IL-2 and elevated IL-10	9
Immune cells not directly targeted by AAT				
<i>In vitro</i> immunization	Mouse splenocytes	Prolastin, 0.5 mg/mL	Intact T-cell clumping, proliferation, response to IL-2 and IFN γ release	114
Concanavalin A stimulation	Mouse splenocytes	Aralast, 0.5 mg/mL		10
CD3/CD28 stimulation	Purified mouse T cells	Aralast, 0.5 mg/mL		7,12

BAFF, B-cell activating factor; DC, dendritic cell; *S. epi.*; *Staphylococcus epidermidis*.

toward background levels before the next weekly infusion is afforded. Administration of AAT under these parameters reveals both excellent patient safety and patient compliance (15).

The attempts to generate transgenic sheep for producing human AAT and

other methods for AAT mass production represent a longstanding effort to generate much AAT for individuals who suffer from genetic AAT deficiency. However, the case for human AAT therapy outside this particular indication appears to be stronger than ever. In this review, an up-

date is provided on recent findings that relate to the ability of AAT to protect tissues as well as block unwanted inflammatory processes and modify the immune system in a beneficial and safe manner. According to the published studies presented above, one can readily appreciate

that AAT has a potential benefit for an impressive broad spectrum of human diseases, reigniting the requirement for yet greater supplies of human plasma-derived AAT, human AAT generated by recombinant techniques or, at a minimum, biologically active AAT fragments.

AN OVERVIEW OF α_1 -ANTITRYPSIN

AAT is a 52-kDa glycoprotein that earned its name by virtue of being the major serum trypsin inhibitor, occupying the α -1 globulin fraction of electrical current-separated serum proteins (16). AAT belongs to the 1,500-member family of serine protease inhibitors (SERPINs), thus also termed SERPINA1. Other acronyms may include A1AT and A1PI. Nevertheless, as discussed below, some nonserrine proteases are also inhibited by AAT, and, in addition, some activities of AAT may be unrelated to protease inhibition altogether, an important aspect to consider as far as its widely accepted mechanism of action and release criteria upon purification for clinical purposes.

Circulating AAT is controlled mainly by the liver. Hepatocytes are responsible for the steady-state constitutive circulating levels of 0.9–1.75 mg/mL AAT, although these ranges might slightly vary in the literature. Hepatocytes are also responsible for IL-1/IL-6-inducible AAT production during inflammation. Levels of AAT also increase in the circulation during normal pregnancy (17) and in the process of aging (18). The half-life of AAT in the circulation is 3–5 d (16). In contrast, lung type II alveolar epithelial cells are primarily responsible for interstitial AAT in the lung (19). In addition to hepatocytes and alveolar epithelial cells, AAT is expressed by monocytes and macrophages, neutrophils, endothelial cells, human intestinal paneth cells, endometrial cells and other types of epithelial cells, as well as by human pancreatic islet α and δ cells. This list of cell sources likely does not contribute to systemic AAT levels but rather to local, inflammation-driven and hypoxia-driven inducible AAT levels.

Disorders in the levels of circulating AAT allow for the degradation of lung tissue that leads to the characteristic manifestation of pulmonary emphysema (20). The decline in circulating AAT levels below 0.5 mg/mL represents a severe form of deficiency, affecting 1 in 1,600–5,000 Caucasian individuals of western European origin (21). A commonly encountered mistaken concept is that individuals with genetic AAT deficiency do not synthesize AAT and that administration of AAT to individuals with normal AAT levels might be problematic. Yet the molecule is readily produced by hepatocytes and some amounts do appear in the circulation in patients with AAT deficiency. However, most of the protein forms aggregates inside the producing hepatocytes, resulting in endoplasmic reticulum stress and cellular damage that can progress into hepatocyte autophagy and liver organ injury (22). Indeed, AAT deficiency represents the most common inherited condition that leads to liver transplantation in infants, children and adults. The presence of a null variation in humans has been reported in two families, yet, intriguingly, its recently developed animal counterpart in the form of a genetically engineered knockout mouse for AAT proves nonviable (23). Unlike the AAT knockout mouse, the phenotype of the elastase knockout mouse is near normal (24). The reason for nonsustainable life in the AAT knockout mouse remains unknown.

Molecular Profile of AAT

AAT is comprised of 394 amino acids. The prototypical antiproteolytic function of AAT is contained within a nine-amino acid reactive center loop (RCL) that stems out of the globular structure and is comprised of a primary sequence that forms “the perfect bait” for a highly specific sequence-directed set of proteases. Elastase, for example, will bind to the RCL in an attempt to cleave a targeted peptidic bond between amino acids 358 and 359, only to remain irreversibly bound to its cleaved product. As a consequence, cleaved AAT undergoes refolding and a binding site

becomes exposed that exhibits high affinity to a receptor for the newly formed AAT:elastase complex, termed SERPIN:enzyme complex (SEC) receptor (25). Cells that express the SEC receptor will internalize the inactive complex, as readily occurs in hepatocytes. Interestingly, the domain that is required for AAT to bind to the SEC receptor after an encounter with a serine protease is a pentapeptide with the sequence FVFLM, located at positions 370–374 (26). These five amino acids are located within a 36-amino acid C-terminal stretch, spanning amino acids 359–394. The so-called C-36 peptide was shown to attract neutrophils (27), as well as activate monocytes (28,29) and display atherogenic properties (30,31). The sequence is highly conserved among SERPINs and, intriguingly, is also mostly hydrophobic. This latter trait allows the C-36 peptide to engage with hydrophobic lipid molecules, such as cholesterol. The remainder portion of AAT outside the RCL and the C-36 peptide appears to have no reported binding partners, yet is strikingly conserved within members of the SERPIN family.

Molecular Targets of AAT Closely Relate to Inflammation

Protease inhibition by AAT reduces inflammation. For example, AAT binds to and inactivates elastase, trypsin and proteinase-3 (PR-3), the activity of which includes proteolytic cleavage of a specific cassette of membrane protein receptors called protease-activated receptors (PARs). In the presence of *inactivated* proteases, such as in the presence of excess AAT, PARs lack their primary trigger for activation. Once cleaved, PARs undergo a conformational change and initiate an intracellular signaling cascade. The role of PAR-1 through PAR-4 during inflammatory responses, as well as during innate and adaptive immunity, appears essential (32). For example, PAR-1 awaits an N-terminal cleavage event to expose a self-binding site within one of its loops, which will cause rapid increase in intracellular calcium levels and subsequent MCP-1 release. Similarly, PAR-2 has its

N-terminal self-binding site unavailable until cleaved by serine proteases; the receptor then facilitates release of IL-1 β , IL-6, IL-8 and TNF- α and increases neutrophil motility. Dendritic cells mature on activation of PAR-2; accordingly, bone marrow cells from mice that lack PAR-2 fail to mature under *in vitro* protocols (33). Protease-activated receptors are also implicated in cardiovascular diseases (34), implying that intervening with their activation by AAT may interfere with relevant pathological pathways. PARs are also implicated in inflammatory gastrointestinal and mucosal disorders (32), since they are expressed in gut epithelial cells, mast cells, nerve cells and smooth muscle cells. In patients with inflammatory bowel disease, PAR-1 is expressed at high levels and PAR-1 antagonism ameliorates inflammation in the respective animal model (35). Each of the four PARs is expressed by cells of the central nervous system, and their activating proteases can be produced locally or enter through a breached blood-brain barrier, such that might be encountered in central nervous system-related pathologies (35). In an animal model for multiple sclerosis, a role for PAR-2 was established; accordingly, mice that lack PAR-2 displayed diminished disease progression (36). That said, a role for AAT in controlling protease-related events outside the cells does not preclude the presence of other aspects of AAT activity—some intracellular and some altogether unrelated to the activity of proteases.

Some targets for AAT inhibition are, surprisingly enough, not serine proteases. Aggrecanase-1 (ADAMTS-4) is involved in the pathogenesis of rheumatoid arthritis and was recently shown to be inhibited by AAT (37). AAT also targets the metalloproteinase MMP-9 (gelatinase B) (38), an important IL-1-inducible protease that is suspected of contributing to the progression of cardiovascular disease, as well as to rheumatoid arthritis, chronic obstructive pulmonary disease (COPD) and multiple sclerosis (39). Inhibition of calpain-1 by AAT was recently reported (40). Calpain-

1 processes the precursor of IL-1 α , a proinflammatory intracellular IL-1 family member that is constitutively expressed in nearly all epithelial cells. It was recently established that blockade of calpain-1 by AAT results in neutrophil polarization and random migration (40).

Binding Targets That Are Unrelated to Protease Inhibition

Binding of IL-8. AAT directly binds to the major neutrophil chemoattractant IL-8 (41). Blockade of IL-8 may provide benefit during diabetic retinopathy, sickle cell disease, transfusion-related acute lung injury, acute respiratory distress syndrome, renal microvasculopathy, acute coronary artery syndrome and stroke. In parallel, neutrophils are targeted by AAT, primarily by the blockade of granule- and membrane-contained serine proteases (42). Added to the finding of reduced IL-8 release in the presence of AAT (40), it is not unexpected that lack of AAT, as occurs in lungs of individuals with genetic AAT deficiency, results in excessive mobilization of neutrophils into the parenchyma.

Binding of heat shock proteins. In general, heat shock proteins (HSPs) fit the definition of dual-function molecules: inside the cells, they chaperone proteins for proper folding, yet when they leak out from a failing cell membrane, for example, during necrosis, they function as immune adjuvants and participate in inflammatory responses, whether alone (43) or in complex with other inflammatory mediators (44). Elevated levels of HSP70, for example, were found to be present in the plasma of type 1 diabetic individuals but not in plasma from healthy individuals (45). Affinity chromatography followed by immunoprecipitation and immunoblot analyses of HSP-enriched, plasma-purified fractions revealed that HSP70 is closely linked to AAT in these patients. Whether it is its functional role as a chaperone of aberrant proteins that has HSP70 clinging to plasma AAT, or perhaps an inherent ability of HSP70 to directly engage with AAT, remains to be confirmed. Nevertheless, the potential in-

jurious properties of extracellular HSP70, particularly in the context of islet β -cell biology, may favor its blockade.

Direct association of AAT with lipid rafts and low-density lipoproteins. AAT was shown to localize within lipid rafts in human monocytes (46). In an elegant study in which cells from AAT-deficient individuals were investigated, the lipid raft-bound form of AAT was shown to originate from the circulation, and not the cell cytoplasm, an important aspect when considering a clinically relevant administration route. Interestingly, a direct association was demonstrated between AAT and cholesterol; the association of AAT with monocytic membranes is enhanced by free cholesterol, and AAT appears to deplete lipid raft cholesterol as well as inhibit oxidized low-density lipoprotein (LDL) uptake. In addition, studies point at a direct relationship between AAT and apolipoprotein B on LDL particles (47,48), and AAT-LDL complexes have been detected in human atherosclerotic lesions (48). In support of a therapeutic implication for such an association, a protective role for AAT in atherosclerosis was demonstrated in the Lipid Coronary Angiography Trial that evaluated male participants after coronary bypass surgery (49); the authors of the trial concluded that low AAT levels are associated with increased occurrence of atherogenesis.

Binding to human immunodeficiency virus entry proteins. Initially described by Shapiro *et al.* (50), infection of whole human blood *in vitro* with human immunodeficiency virus (HIV)-1 does not occur, whereas whole blood from AAT-deficient patients is readily infected (50). This observation suggests that endogenous levels of AAT prevent infection of HIV-1. In fact, adding exogenous human AAT to latently-infected cell lines reduced production of HIV-1 *in vitro* (49). Thus, steady-state levels of AAT appear to provide a certain degree of HIV-1 infection inhibition. Subsequently, a screening study of ultra-filtered human plasma and protein sequencing revealed that, indeed, the major plasma-derived inhibitor

of HIV-1 is circulating human AAT (51). Accordingly, it was later demonstrated that HIV-1 infection is associated with reduced serum AAT concentrations (52). A C-terminal 26-residue peptide fragment of AAT, the result of internalized and cleaved AAT, was found to be a direct inhibitor of viral infection (53). Recently, a 10-d intravenous monotherapy course using a peptidic derivative of the C-terminal of AAT reduced viral RNA levels in treatment-naive, HIV-1-infected individuals without causing adverse effects (54). Such activity of AAT is essentially devoid of serine protease inhibition.

Induction of vascular endothelial growth factor. In various studies, AAT has been shown to induce vascular endothelial growth factor (VEGF) production (55–57) and to prevent proteolytic degradation of VEGF (58). In addition, AAT was shown to facilitate smooth muscle myocyte migration and proliferation (59) and reduce endothelial cell and smooth muscle cell apoptosis (55,56,60,61). In fact, the pathological hallmark of AAT deficiency, pulmonary emphysema, can be observed in animal models in which the VEGF signaling pathway is blocked in the presence of normal local levels of AAT (62–64), suggesting that AAT deficiency-related emphysema is the result of insufficient AAT-induced VEGF, and a subsequent collapse of the capillary bed. Indeed, the promoter for AAT contains the hypoxic response element for HIF-1 α , and AAT is upregulated during hypoxia (65). Interestingly, such association cannot be reproduced by mimics of serine protease inhibitors.

Thus, whereas AAT is a textbook example of actions afforded by its antiprotease properties, increasing amounts of data are emerging that support the concept by which domains within AAT that are independent of protease binding can provide clinically beneficial functions (66).

Cellular Targets of AAT

As early as 1978, it was reported that AAT does not engage with T lymphocytes, but rather brings along diminished

T-cell responsiveness in the context of particular experimental setups in an indirect manner (13). As confirmed in several recent reports, AAT indeed does not interfere with the biological activity of IL-2 (7,8,10–12), allowing T cells to release interferon (IFN)- γ and proliferate in an undeterred manner in the presence of IL-2. With the benefit of having intact IL-2 signaling, T regulatory cells (Tregs) can readily differentiate, particularly when considering that levels of IL-6 are reduced by AAT (4,9,10), a prerequisite for directing of naive T cells away from Th17 (67). Additionally, Th17 cells rely on an intact IL-1 pathway (68), a willing cytokine-related target of AAT. Thus, the expansion of Tregs in animal models that incorporate AAT distinguishes the therapeutic efficacy of AAT from IL-2-directed immunosuppression.

How are T cells affected by AAT without being direct cellular targets? Innate cells appear to undergo marked changes in the presence of added AAT. Neutrophils, in particular, are disarmed (40,42,69). In fact, the local shortage of AAT in lungs of patients with genetic AAT deficiency are readily associated with an influx of neutrophils with injurious consequences (20). Dendritic cells and macrophages are modified; dendritic cells become semi-mature (9), a state associated with reduced co-stimulatory abilities, excess IL-10 production and facilitation of antigen-specific Treg expansion (8). Interestingly, Ozeri *et al.* (9) demonstrated that AAT promotes semi-mature IL-10-producing and readily migrating dendritic cells, allowing the cells to reach the draining lymph nodes and exert their tolerogenic functions. In this study, an intriguing uncoupling of inflammation-mediated elevation in the dendritic cell chemoattractant receptor (CCR7) was observed, whereby AAT appears to have allowed for persistent CCR7 surface expression but had downregulated other dendritic cell inflammatory markers (9). Indeed, in the whole animal and, specifically, in lymph nodes during an antigenic event, IL-10 production rises as a consequence of AAT ther-

apy (6–9). Moreover, *ex vivo* AAT-treated graft-derived dendritic cells were shown to communicate a tolerogenic profile across to the host dendritic cells, causing the host cells to increase IL-10 production (9). B cells represent a lymphocyte with a biological response profile that is closer to the innate system than to the adaptive immune system; indeed, B-cell activation is, in part, inhibited by AAT (70).

These cell types are the topic of ongoing research with particular attention toward a tissue-protective antiinflammatory tolerogenic function, such that would drive Tregs to predominate without the T cells ever engaging directly with AAT.

AAT DEFICIENCY POINTS TO POTENTIAL INDICATIONS FOR AAT THERAPY

AAT deficiency is largely underdiagnosed. An asymptomatic drop in levels of AAT to as low as 85% of normal circulating levels can readily be detected in healthy individuals by random evaluation (71). Moreover, serum AAT concentrations may not be representative of the functional capacity of the antiprotease aspect of AAT, since inactivated forms might falsely ascribe to normal “immunogenic” levels of the molecule, as detected by Western blot or enzyme-linked immunosorbent assay (72). It is also suggested that failure to elevate AAT under physiological conditions might present as a novel relative functional deficiency. Systemic conditions that have been associated with AAT deficiency include panniculitis, vasculitis, pancreatitis, glomerulonephritis, bronchiectasis and asthma; all are characterized by excessive inflammation (73). In more recent literature, polymorphism studies revealed several unexpected diseases that might be associated with moderate AAT deficiency (non-M polymorphisms), such as fibromyalgia, mood disorders and intense creative energy (74,75). Although frank deficiency in AAT renders the patient eligible for AAT augmentation therapy, none of the conditions above are included as labeled indications for AAT.

ANTIINFLAMMATORY THERAPIES POINT TO NOVEL INDICATIONS FOR AAT THERAPY

The role of inflammation in the pathogenesis of several diseases cannot be overstated. Yet, some illnesses have not been associated with inflammation and, subsequently, appear to have attracted therapeutic approaches using antiinflammatory agents. Blockade of inflammatory pathways by these agents in clinical trials has provided proof of concept for the potential benefit that would be gained by incorporation of AAT into treatment protocols.

Type 2 Diabetes

Type 2 diabetes is associated with the failure of insulin to communicate intracellular signals upon engagement with its receptor. The current therapeutic approach primarily centers around diet and exercise, plus manipulation of liver cells to halt endogenous glucose production and attempts to enhance insulin sensitivity and insulin release; these approaches are joined by few and recent agents that also target inflammation (76). The rationale for the latter approach is that insulin signaling is negatively affected by active inflammatory signals. Under physiological conditions, inflammation evolves into local and systemic insulin resistance, creating a temporary and desired rise in circulating glucose. Blockade of inflammatory pathways might thus salvage insulin responsiveness. Indeed, a reduction in the levels of glycated hemoglobin (HbA_{1c}) has been reported in a clinical trial that examined the effect of a derivative of aspirin, salsalate (77). In a similar manner, blockade of IL-1 by using the IL-1 receptor antagonist anakinra (78), and also by using an antibody to IL-1 β (79), provides consistent outcomes. In addition, it has become widely accepted that islet β -cell injury is inherent to disease pathogenesis. Chronic high glucose and fatty acid levels exert direct β -cell toxic effects (80), and IL-1 β has been shown to be highly toxic for β cells (81). Protection of islets from inflammatory cytokine-mediated injury has been

widely reported in both mouse and human islets (7,8,10,82–86). Support for an apparent association between the lack of protection by AAT and type 2 diabetes may be found in a recent report that describes low circulating AAT levels in 50% of type 2 diabetic patients (87). It remains to be determined whether insulin signaling and systemic glucose control can improve in the presence of added AAT, and whether AAT can ameliorate β -cell injury in the case of chronic elevated glucose or fatty acid levels and can thus benefit type 2 diabetic patients.

Type 1 Diabetes

Formerly termed “juvenile diabetes,” type 1A autoimmune diabetes harbors an elaborate antigen-specific attack on β cells. Thus, protection of islets from injury would appear to be the obvious target in type 1 diabetes, yet many trials have been primarily directed at the T-cell-mediated autoimmune arm of disease. In the advent of an apparent failure of recent clinical trials that incorporate removal of T cells using anti-CD3 antibodies (88), the fact that blockade of inflammation appears as effective, if not more effective, in the ideal context than immunosuppression is of great importance. A factor absent in classic immunosuppression that predominates in antiinflammatory approaches may be a metabolic one, such that facilitates insulin receptor responsiveness. IL-1 is a target in this context, both as a relevant provocateur of inflammation and also as a direct and potent β -cell toxic agent (89). In a recent clinical trial, IL-1 blockade was evaluated in 15 children within 1 wk of type 1 diabetes diagnosis using daily anakinra for 28 d. As a consequence, compared with controls, insulin requirement was reduced for the duration of 4 months (90).

Nonfunctional circulating AAT has been shown to exist in most individuals with type 1 diabetes (72,91–95). The loss-of-function relates, most probably, to excessive nonenzymatic glycation. Moreover, in the autoimmune animal model for type 1 diabetes, the nonobese diabetic (NOD) mouse, AAT levels were half the

levels found in the majority of other wild-type mouse strains (96). Indeed, preclinical data show a consistent benefit with AAT in the protection of islets and modification of immune systems across multiple models of diabetes (7,8,10,84,96–98). Furthermore, after NOD mice revert to normoglycemia by a 14-d course of AAT, grafted autoreactive β cells are accepted in the animals in the absence of subsequent requirement for exogenous AAT (7).

The fact that AAT is endogenously produced under inflammatory stimuli by both mouse and human islet cells (99,100) strengthens its relevance for type 1 diabetes. Protection of grafted human islets for individuals with type 1 diabetes may thus incorporate AAT as both an islet protector and a tolerogenic agent, addressing several of the current multiple goals for successful long-term human islet transplantation outcomes. As for recently diagnosed type 1 diabetic patients, three clinical trials are currently running that study AAT in youngsters with type 1 diabetes (National Institutes of Health clinical trial registry NCT01304537, NCT01319331 and NCT01183468), all incorporating as part of their inclusion criteria that residual islet function be measurable before initiation of treatment with weekly intravenous infusions of AAT.

COPD

Inflammation in COPD, both acute and chronic, is key in disease progression. Whereas environmental assaults, such as cigarette smoke, dust or pollution directly contribute to an inflammatory flare, the process of airway inflammation ensues long after the trigger is gone. An association between COPD and AAT may not require that a genetic deficiency in AAT be present in patient background, since evidence for protease/antiprotease imbalance can be found also in COPD patients with normal AAT levels (101). Pharmacologically speaking, lungs present with a practical benefit: inhaled approaches have gained advances in the introduction of antiinflammatory agents. Inhaled nonsteroidal antiinflammatory drugs, for example, create signifi-

cant improvement in disease parameters (102). The effects of inhaled AAT in mice are consistent with protection from inflammation and tissue damage caused by cigarette smoke (69). With the recent development and approval of an inhaled preparation of AAT (103), one can expect local lung concentrations of AAT to be achieved at low doses, sparing both systemic delivery of AAT and cost.

Cystic Fibrosis

Even in the absence of demonstrable infection, inflammation is evident in lungs of cystic fibrosis patients (104). Antiinflammatory agents have proven effective, at most, yet at times result in adverse effects such as in the case of chronic oral corticosteroids. Given by inhalation, some agents display a wider therapeutic index (102). As such, inhaled AAT has proven effective in reducing inflammation in cystic fibrosis patients in multiple reports (studies compiled in [105]). The effect of AAT on neutrophils, both as an inhibitor of IL-8 function (42) and also as an avid inhibitor of injurious neutrophil serine proteases, supports its use in this particular clinical indication.

Graft versus Host Disease

A limiting factor in the success of hematopoietic stem cell transplantation, graft versus host disease (GVHD) remains difficult to control. Immunosuppression, intended to blunt the aggressive immune response against the host tissues, exposes the individual to opportunistic infections and impairs graft-versus-leukemia responses. There is a need to develop immune modulating agents that can allow T-cell-mediated graft-versus-leukemia responses while sparing the recipient from an injurious inflammatory and immunological assault. Given the ability of AAT to modify dendritic cells toward a tolerogenic phenotype and to facilitate the expansion of Tregs (6,8,9), together with its potent antiinflammatory profile and outstanding safety record, AAT is an attractive candidate to address acute GVHD. Several

lines of evidence support this approach. Of particular interest, a recent report describes an elevated loss of AAT during intestinal GVHD as a measure of GVHD severity in children (106). In addition, evidence points to a significant involvement of IL-1 during the progression of GVHD, albeit mostly in advanced stages of the pathology (107); in 16 of 17 patients with steroid-resistant GVHD, a 7-d continuous intravenous infusion of recombinant IL-1 receptor antagonist reduced the severity of the disease (108). However, the sole blockade of IL-1 during the development of GVHD was less effective (109). Because cell injury appears to fuel GVHD (110), the ability of AAT to provide tissue protection may result in a synergistic advantage to cytokine modification. Indeed, in several animal models for GVHD, AAT provided clear benefit in immune profile, animal weight and cohort survival (11,12). Its role in blocking serine protease PR-3-related IL-32 activation further denotes a possible mechanism for protection during GVHD, particularly in light of the recent report of elevated blood IL-32 mRNA transcripts in 10 acute GVHD patients compared with 5 GVHD-free allogeneic hematopoietic cell transplant recipients (11).

Rheumatoid Arthritis

The progression of rheumatoid arthritis (RA) involves the maintenance of an inappropriate inflammatory process by immune cells (111). Biologics include systemic or local blockers of TNF- α and IL-1 β , and an anti-IL-6 receptor antibody that neutralizes the effector function of IL-6 (111). These particular cytokines are inhibited by AAT at several levels, including both their release and function, suggesting that AAT may serve to prevent the positive inflammatory feedback loop that appears to perpetuate the disease. In fact, a relationship between AAT inactivation and TNF- α concentrations in the synovial fluid of patients with rheumatoid arthritis was described (112). In addition, inhibition of neutrophil elastase has been shown to interfere with

disease progression in respective animal models (113). Thus, it is not unexpected that AAT was recently shown to delay arthritis development in a mouse model, both in the form of injected human material and in the form of adenoviral plasmid-derived circulating human AAT (114,115). In the particular case of IL-1 processing, the role of non-caspase-1 extracellular processing of the IL-1 β precursor has been shown to incorporate enzymatic targets of AAT, such as elastase, cathepsin G and PR-3 (107). In this particular context, gout is another excellent candidate that would benefit from blockade of IL-1 activities by AAT in joints.

Multiple Sclerosis

Inflammation is a therapeutic target in neurological disorders (116). In addition, the metalloproteinase MMP-9 appears to take part in the pathogenesis of multiple sclerosis (MS) and has been shown to be a target for AAT inhibition (38). Whether insufficient AAT plays a role during the disease is not yet established, although mutation analysis has detected the presence of inactive alleles of AAT in individuals with MS (117,118). Additionally, mice that express human AAT in their circulation are protected from disease course in the respective mouse model of multiple sclerosis (6). In that study, the small proportion of AAT-treated animals that did display initial signs of pathology after disease induction overlapped the timing of the control sick group as far as initial signs of neuronal damage, but then exhibited a rapid regression in symptoms. Another support for the relevance of AAT therapy for MS patients is the detection of biologically active elevated levels of AAT in the cerebrospinal fluid of patients with MS (119).

Inflammatory Bowel Disease

Although several studies suggest an imbalance of protease activities during inflammatory bowel disease (IBD), correlation appears to exist between AAT deficiency and IBD only in populations with a severe type of genetic AAT deficiency

(PI*ZZ) (120,121). Nevertheless, considering that intestinal paneth cells are potent producers of inducible AAT (122), its involvement in IBD as being produced extra-hepatically may be relevant.

Ischemic Heart Disease

Approaches to diminish inflammatory pathways during myocardial injury show benefit in various parameters of cardiomyocyte viability and cardiac function (123). Whereas inflammation might display a positive role in cardiac repair and scar formation (124), the particular ability of AAT to block arms of inflammation while preserving tissue integrity may place AAT as an attractive antiinflammatory agent in the context of cardiac damage and repair. In addition, cells of the innate immune system take part in the injury that follows myocardial reperfusion, serving as another target for AAT inhibition. In a study reported by Toldo *et al.* (125), AAT-treated mice had significantly smaller infarct sizes (−30% on d 1 and −55% on d 7) compared with mice treated with albumin. Also, AAT treatment resulted in >90% reduction in caspase-1 activity in homogenates of hearts 24 h after ischemia reperfusion. Seven days after acute myocardial infarction, AAT-treated mice exhibited superior cardiac function. The increase in caspase-1 activity in cardiomyocytic HL-1 cells induced by lipopolysaccharide (LPS) and nigericin or after ischemia was reduced by >80% and cell death by >50% in the presence of AAT. As suggested by Daemen *et al.* (126), there is demonstrable protection from ischemia reperfusion injury in renal mouse models by AAT, including renal function. The authors of that study conclude that exogenous administration of AAT may provide new therapeutic means of treatment for ischemia reperfusion injury.

PREGNANCY COMPLICATIONS

AAT rises during normal pregnancy (17). An intriguing association appears to exist between AAT inactivity and preterm premature rupture of membranes. The relationship between trypsin activ-

ity in the amniotic fluid and premature rupture of membranes has been described in early reports (127), and AAT is found at subnormal levels in amniotic fluid obtained from patients with preterm premature rupture of membranes (128). The source of AAT was identified as human amniotic epithelial cells. Screening for levels of AAT is not a standard blood test in pregnant women, especially not at late stages of pregnancy. However, should an association between AAT and normal pregnancy be established, it might be strongly proposed to incorporate plasma AAT testing in the third trimester of pregnancy, at which point most normal pregnancies would exhibit circulating AAT levels greater than nonpregnant normal values (128).

SAFETY OF PROLONGED AAT THERAPY

Safety Demonstrated in Prolonged Clinical Trials with AAT

Although naturally existing, AAT might be considered a biological once introduced to patients over prolonged periods of time and in excess (similar to other naturally occurring molecules that are represented by drugs, such as CTLA-4 and IL-1 receptor antagonist). Yet experience with humans that receive AAT under protocols that are comprised of prolonged periods of time and excess material has proven safe. With a focus on the United States, extrapolation of data from population-based screening studies, evaluations of patients with COPD and genetic epidemiologic surveys lead to an estimated 60,000–90,000 Americans with the severe type of genetic AAT deficiency (PI*ZZ). However, AAT deficiency is highly underdiagnosed, and of the estimated numbers above, only about 6,000 will have been diagnosed as having AAT deficiency, as of the year 2009 (129). Administered to these patients once weekly for the past 3 decades, serum AAT levels increase to approximate the concentrations of an acute-phase response for the first part of the week and then decline to normal values in the second half of the week (14). Since these treated individuals exhibit no ad-

verse effects, the molecule is considered safe for chronic therapy. In addition, as suggested by Churg *et al.* (130), added benefit is demonstrated when administering AAT, even in the absence of AAT deficiency (130). Studies demonstrate high patient compliance, demonstrate no evidence of compromised host defense, such as opportunistic infections or reactivation of *Mycobacterium tuberculosis*, and actually report reduced frequency of pneumonia incidents (131). In addition, in the presence of elevated systemic levels of AAT in mouse models, tumor angiogenesis appears to be compromised (132) and metastasis appear to lose protease-dependent migratory capabilities (133).

AAT Is an Antibacterial

SERPINS possess antibacterial functions (134). Findings that relate to the ability of AAT to inhibit bacterial expansion include binding to the bacterial-essential furin (135), as well as undergoing S-nitrosylation in the presence of nitric oxide (136), both actions that are independent of protease inhibition. The observation that the virulence of *E. coli* is increased by IL-1 (137) suggests that blockade of IL-1 by AAT may interfere with bacterial growth, a finding corroborated by reduced numbers of live *S. pneumoniae* in infected mouse lungs (EC Lewis, unpublished observations). In this context, notably, the role of AAT in reducing bacterial load involves tissue modulation, that is, reducing the levels of tissue-derived bioactive IL-1. Indeed, the protection from LPS mortality in a model of 24-h pretreatment with low-dose systemic IL-1 is attributed to induction of acute-phase proteins (138). Clinical studies indicate that augmentation therapy with AAT for genetically deficient patients effectively attenuates microbial colonization, as well as the frequency and severity of acute COPD exacerbations (131,139–143). Moreover, the fact that cystic fibrosis patients benefit from AAT inhalation, even when introduced during active lung infections (*Staphylococcus* and *Pseudomonas*) (144), further supports the claim that host de-

fenses are intact, if not enforced, in the presence of elevated AAT levels.

ADDRESSING AAT FOR EXPANDED CLINICAL INDICATIONS

The 8th World Congress on Trauma, Shock, Inflammation and Sepsis - TSIS 2010, held in Munich, Germany, hosted a symposium titled "Alpha-1-Antitrypsin (AAT) as a Novel Therapeutic in Inflammatory Diseases." The symposium reviewed several indications for AAT augmentation therapy outside pulmonary emphysema and provided preclinical evidence for the effects of AAT in type 1 diabetes, GVHD, IBD and acute myocardial infarction. The Alpha-1 Foundation's 12th Gordon L. Snider Critical Issues workshop, entitled "New Formulations and Applications of α -1-Antitrypsin," provided preliminary findings for AAT therapy in transplant rejection, type 1 diabetes, IBD, rheumatoid arthritis, viral infection and cystic fibrosis. These scientific programs form a bridge between the vast body of recently published preclinical studies and the potential clinical use of AAT outside the context of genetic AAT deficiency.

Importance of Activities Unrelated to Protease Inhibition

Evidence for functions of AAT that occur without the requirement of its RCL to bind to a serine protease is accumulating. Oxidized AAT (erroneously called "inactive") cannot inhibit elastase, yet exerts antiinflammatory activities (130). Modified forms of AAT cause a rise in cAMP and induce IL-10 release, even though they lack protease inhibitory activity (145). The concept of a protease-antiprotease imbalance was recently revisited, pointing at the possibility that in some disease models, AAT may function in an unrelated manner (66). Indirect evidence also exists. The C1 inhibitor is a SERPIN that displays antiinflammatory attributes, despite having targets of inhibition that are nonoverlapping with those of AAT (146). Similarly, the SERPIN antithrombin III exerts antiinflammatory activities, yet

shares little overlap with the serine proteases that are inhibited by AAT (147). These findings are important because release criteria for clinical-grade human AAT are uniformly based on the level of elastase inhibition per milligram of protein. Yet it is likely that during the purification steps from human plasma, the nonprotease-interacting domain(s) of AAT are compromised such that the antiinflammatory properties are reduced. In support of this concept, plasmid-derived human AAT (hAAT) (148) and transgenic hAAT (6) modulate the immune system at concentrations too low to afford elastase inhibition by the equivalent concentration of injected plasma-derived hAAT. In addition, knockout mice to metalloproteinase MMP-12 are protected from emphysema, despite the presence of elastase (62); in this study, TNF- α is reduced by the absence of MMP-12, further suggesting that the inhibitory effect of AAT on TNF- α is of benefit for tissue protection, regardless of the status of elastase. Moreover, in the same study, the outcome remained consistent when using oxidized AAT, which has a 2,000-fold lower association rate constant for neutrophil elastase. Interestingly, whereas elastase knockout mice do not develop emphysema, they do appear to have normal neutrophil development and recruitment. But perhaps even more importantly, they are resistant to lethal doses of LPS (24).

Sources of AAT

Pooled human plasma affinity purified AAT. Clinical-grade AAT is factory-lined for a rare genetic condition, found among human populations at a rate anywhere from 1:1,600 in Denmark to 1:5,000 in the U.S. Defined under "Medical Needs" in the review "Emerging drugs for alpha-1-antitrypsin deficiency" (149), COPD is the sole extension of clinical indications for AAT. Providing adequate supply of AAT for a common condition such as type 1 or type 2 diabetes will be challenging—even more so for multiple conditions

with varying requirements of duration of therapies.

Gene therapy. Being a single-gene disease, AAT deficiency is one of several well-studied human genes to be experimentally delivered by genetic manipulations (150). Moreover, evidence exists to show that native gene-derived circulating human AAT is superior to the plasma-purified material (148). Gene therapy may surpass the requirement of purification of AAT, as well as reduce the exposure of patients to human-derived material and supply the circulation with the native molecule. However, gene therapy still holds the downside of genetic manipulation in humans and the inability to control circulating AAT levels once introduced. Notably, a plasmid that contains a significant stretch of the human gene for AAT, including introns, exons and a generous expanse of the original promoter, has been generated (151) and introduced into animals. In these studies, the animals display enhanced protection of transplanted islets much like the findings obtained using the injected material (148).

Recombinant AAT. Recombinant AAT has been derived from plants, yeast, fungi, animals, insect cells, bacteria and mammalian cells and has been manipulated toward humanized systems, mutated at specific amino acids and conjugated with polyethylene glycol (PEG) (152). Bacterial nonglycosylated 44-kDa recombinant human AAT typically aggregates, is inactive and is also rapidly cleared from the circulation; the prolongation of its half-life by PEGylation may provide a solution to its inferiority (153). Inhaled recombinant AAT has been shown to ameliorate cigarette smoke-induced emphysema in mice (69), much like the injected material (130). Biologically active N-glycosylated human AAT was generated in mouse urine directed by the uromodulin promoter, raising yet another attractive option for the potential large-scale production of functional therapeutic human AAT in livestock (154). Nevertheless, none of the above approaches appears to appeal more to

patients in need than the traditional plasma-derived affinity-purified form of human AAT.

CONCLUSIONS

This review opened with the description of transgenic livestock that generate human AAT in milk (1). It is a dual challenge to introduce AAT into clinical use at the appropriate effective window of clinical opportunity, as well as to find a creative way by which to supply such a potentially broad demand. Any new AAT-based treatment agent will also be required to stand the challenge of elucidating the mechanism of AAT and to define a “grand unified theory,” should one mechanism account for the broad spectrum of its disease-modifying properties. The ~52-kDa glycoprotein that is decorated by a 9 amino acid-long protease-binding region most probably contains domains in the nonprotease-interacting conserved areas of the molecule that can account for the profoundly altered protease-independent *in vivo* activities. Since its first description in 1906 as an inhibitor of trypsin (155), a mechanism is yet to be identified for the protease-independent properties of AAT that modify gene expression profiles of pivotal beneficial molecules, such as VEGF and IL-1 receptor antagonist. That elastase activity is not the sole indicator of AAT activity is rapidly becoming an accepted concept.

Efficacy of AAT augmentation therapy in conditions other than pulmonary emphysema recently was addressed in an evidence-based analysis (156). Its use in conditions such as type 1 and type 2 diabetes; acute myocardial infarction and postinfarction remodeling; and GVHD, cystic fibrosis and IBD is currently being evaluated worldwide in controlled trials. Conditions such as stroke, Alzheimer’s disease, MS, vasculitis and organ and cell transplantations are also promising clinical indications. In light of the reduced production and activity of IL-1 β by AAT, diseases that are responsive to blockade of IL-1, IL-17 and TNF- α should also be considered as candidates for AAT therapy

(107). To date, unlike in the case of AAT, antiinflammatory approaches that aim at ameliorating acute and chronic conditions that depend on the combination of inflammation, hypoxia and tissue damage (157,158) often succeed in blocking the unwanted arms of the inflammatory response, yet are at risk of failing to sustain the imperative benefits of inflammation.

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DISCLOSURE

The author declares that he has no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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