Neutrophils have been implicated in the pathogenesis of alpha-1 antitrypsin deficiency (AATD) since the first descriptions of the disease. Neutrophil proteinases can cause all lung manifestations of AATD, from small airways destruction, to emphysema, to chronic bronchitis and airflow obstruction. Initially, it was proposed that neutrophil functions were normal in AATD, responding in an initially physiological manner to a high burden of pulmonary inflammation. More recent studies have shed new light on this, describing changes in neutrophil responses (a modulation of usual cellular functions) in the presence of inflammation or infection which might enhance tissue damage while impeding bacterial clearance, providing some evidence to support there being an AATD neutrophil phenotype.

Many facets of neutrophil function in AATD can be explained by the loss of alpha-1 antitrypsin (AAT) in diverse biological processes. If this were the only reason for altered neutrophil functions, one would predict similar disease presentation across affected people. However, this is not the case. Despite similar (low) levels of AAT, lung disease is extremely variable in AATD, with some patients suffering a significant burden of lung disease and some much less, irrespective of smoking habits and, in some cases, despite augmentation therapy. This review will explore how complex neutrophil responses are and how they are altered with age, inflammation and AATD. Further, it will discuss the need to understand more completely which aspects of AATD-associated disease are driven by neutrophils and how patients more susceptible to neutrophil dysfunction could be identified to potentially stratify treatment approaches.

Introduction

Alpha-1 antitrypsin (AAT) is the classical circulating anti proteinase in humans and its central function is to inhibit neutrophil proteinases (such as neutrophil elastase [NE] and proteinase 3 [PR3]). Such protection is warranted; neutrophil proteinases have substantial capacity to damage tissues, are able to degrade all components of the extracellular matrix and have significant effects on cell function and signaling. The actions of NE are outlined in Table 1. Proteinase
activity is associated with the development and progression of the many facets of chronic obstructive pulmonary disease (COPD), including emphysema, small airways damage and destruction, chronic bronchitis, acute bacterial infections and bacterial colonization. The damaging effects of neutrophil proteinases are not only felt in the lungs. These proteinases are also implicated in smoking-associated skin damage as well as arterial hypertension (including pulmonary arterial hypertension).

AAT limits rather than prevents neutrophil proteinase-related damage, as it binds proteinases on a one-to-one molar basis and concentrations of NE and PR3 far exceed that of AAT at the site of a degranulating neutrophil. This leads to an area of obligate tissue damage around each degranulating cell until concentrations of the proteinases are reduced by diffusion into the local tissue environment. AAT preferentially binds NE rather than PR3 suggesting the obligate tissue damage caused by PR3 could exceed that of NE, although the disease associations of PR3 are less well studied.

In addition to the anti-proteinase function of AAT, it is increasingly recognized that AAT has multiple non-proteinase-mediated effects, reviewed elsewhere in this series of articles. In brief, these are anti-inflammatory and immunomodulatory in nature and include reducing free radical production and associated damage, binding sterically to interleukin 8 (CXCL8) and leukotriene B4 (LTB4), reducing the signals which can initiate neutrophil migration towards inflammation and reducing cytokine release by macrophages, monocytes and neutrophils. AAT also has anti-microbial/viral properties, impacts on T and B cell functions and has been implicated in reducing inflammation-mediated apoptosis, including in pancreatic B cells suggesting effects of deficiency may be far more wide-ranging than on neutrophils alone.

Table 1. The Actions of Neutrophil Elastase on Host Tissues

<table>
<thead>
<tr>
<th>Degradation/ Cleavage</th>
<th>Elastin</th>
<th>Collagen types I, II, III, IV, VI, VIII, IX, X, and XI Fibronectin</th>
<th>Laminin</th>
<th>Aggrecan</th>
<th>Cleaves epithelial-cadherin (E-cad), an important intercellular junction protein.</th>
<th>Inactivates endogenous proteinase inhibitors such as α2 antiplasmin, α1 antichymotrypsin, and tissue inhibitors of metalloproteinases</th>
<th>Cleaves the hinge region of IgA</th>
<th>Cleaves complement C3bi, forming a functional opsonin mismatch</th>
<th>T lymphocyte surface antigens</th>
<th>Cleaves anti-inflammatory molecules such as sirtuin 1 which are implicated in aging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>Matrix metalloproteinase 2, 3, 9,</td>
<td>Cathespin B</td>
<td>Enhances epithelial and endothelial secretion of inflammatory mediators including interleukin 8</td>
<td>Enhances macrophage secretion of pro-inflammatory cytokines including leukotriene B4</td>
<td>Decreases secretory leukoproteinase inhibitor (SLPI) secretion</td>
<td>Increases elastin secretion</td>
<td>Neutrophil/α-1 antitrypsin complexes are chemotactic for neutrophils</td>
<td>Cleaves intercellular adhesion molecule 1 (ICAM-1)</td>
<td>Disruption and detachment of epithelial cells</td>
<td>Reduces ciliary beating of columnar epithelium</td>
</tr>
</tbody>
</table>
| Modification of Inflammatory Mediators | Neutrophil/α-1 antitrypsin complexes are chemotactic for neutrophils | Cleaves intercellular adhesion molecule 1 (ICAM-1) | Disruption and detachment of epithelial cells | Reduces ciliary beating of columnar epithelium | Increases MUC5 AC protein content (a gel-forming mucin that contributes to the viscoelastic properties of mucus) | Increases cellular oxidative stress | This table is not exhaustive, but provides an overview of the pro-inflammatory and tissue damaging effects of neutrophil elastase.  

Alpha-1 antitrypsin deficiency (AATD) is a condition of low circulating levels of AAT, caused by mutations in SERPINA1 and inherited in an autosomal and...
Neutrophils in Health and Inflammation

Neutrophils are among the first line of effector cells to be recruited in inflammation, irrespective of cause and are also the most abundant leukocyte, accounting for 70% of all circulating white blood cells. They are short-lived, with a high basal production of $1-2 \times 10^{10}$ neutrophils/day in health; increasing to approximately $10^{12}$ during inflammatory challenges.\(^{34}\) Their mobilization from the bone marrow is tightly controlled with neutrophil C-X-C chemokine receptor type 4 (CXCR4) and bone marrow stromal cell C-X-C motif chemokine 12 (CXCL12) interactions causing cell retention or cell return to the bone marrow and increasing surface expression of neutrophil CXCR2 causing neutrophil release into the circulation.\(^{35,36}\) Once in the circulation, neutrophils are able to navigate towards the source of inflammation using only small gradients in inflammatory cytokines. They do this through internal amplification of signals via positive feedback loops, with cell polarization (a clear “front” and “back” morphology, facing the inflammatory source) central to this process.\(^{37}\) To enable migration through dense extracellular matrices, neutrophils utilize granules containing high concentrations of proteinases such as NE and PR3. NE and PR3 have significant proteolytic activity and the radius of tissue damage is up to 8 times greater than that of the granule itself for NE\(^{38}\) and even more for PR3\(^{18}\) due to quantum proteolysis and AAT enzyme kinetics, leaving an obligate trail of damaged tissue. Once at the site of inflammation, neutrophils are avid and unrestricted phagocytes, engulfing tissue debris, pathogens and damaged cells, but also recruit other neutrophils to the source of inflammation while phagocytosis is still required. They form dense cellular clusters or “swarms” of cells in the presence of LTB4, with the swarms maintained through auto and paracrine amplification of LTB4 secretion. Swarm size (and thus the potential for bystander tissue damage) is contained/ limited by repeated neutrophil to neutrophil contact through 2 main integrins; lymphocyte function-associated antigen 1 (LFA-1) and macrophage 1 antigen (MAC-1) interactions. The combination of localized LTB4 and close cell contact through integrins appears to both drive swarm formation but also limit the size of the swarm, to prevent local tissue damage beyond the inflamed tissue.\(^{39}\)

In overwhelming infection or inflammation, neutrophils can release their granule contents extracellularly, and primary and secondary granules contain a wealth of bacteriocidal proteins including myeloperoxidase, proteinases, defensins and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which form reactive oxygen species (ROSs).\(^{40}\) ROSs have antimicrobial activity but also...
are utilized in cellular signaling and in the formation of neutrophil extracellular traps (NETs), which are able to entrap bacteria in a net which is significantly larger than the cell, exposing bacteria to high concentrations of antimicrobial peptides. Degranulation, NETosis and ROS release have all been associated with tissue damage, and so to limit the potential for unwarranted neutrophil activity, neutrophils exist in 3 states, quiescent, primed and activated, with the ability to move between states, depending on the environment. Of note, recent elegant studies suggest that the narrow and tortuous pulmonary capillary bed might act as a venue for neutrophil depriming. Here, the mechanical manipulation of neutrophils through narrow vessels appears to assist with the cell entering a more quiescent state. This suggests a healthy lung environment is needed for adequate cell de-priming to occur.

**Neutrophil Maturity and Phenotype**

Circulating neutrophil numbers are not constant throughout the day. Neutrophils are released from the bone marrow during the resting phase of an animal’s life (night, for most humans), with absolute numbers peaking at this time. Aged neutrophils are then cleared before the waking phase of the day, expressing more CXCR4 and migrating to areas of higher concentrations of CXCL12 (including the bone marrow) which are cleared following apoptosis. There is some evidence that neutrophil function changes as the cells age with more aged cells having an increased capacity to phagocytose bacteria and to produce more NETs and ROSs but this requires further study.

To add to this complex picture, it is increasingly recognized that neutrophils may be more heterogenous in functional responses than previously appreciated. Neutrophils are transcriptionally active and experimental models have described an increasing number of neutrophil phenotypes which have different functional characteristics. Studies have described anti-inflammatory neutrophils, involved in tumor clearance and dampening of T cell responses via MAC1 signaling. A subset have been shown to reverse transmigrate back into the systemic circulation and there also appears to be a pro-angiogenic neutrophil, characterized by increased MMP-9 release. This has been reviewed recently.

**Host Aging and Neutrophil Responses**

As well as potential changes in function as the neutrophil ages, there are also changes in neutrophil function as the host ages, and as AATD is a genetic condition, this may have relevance in disease pathogenesis across the life course. In healthy infants, isolated neutrophils have been shown to display reduced migratory accuracy, reduced degranulation in the presence of a stimulus, preserved phagocytosis and preserved ROS generation but reduced NET generation compared to young adults. There are also changes in neutrophil function at the other extreme of age, with old age associated with impaired migration due to dysregulated and excessive PI3K activity, reduced NET generation, reduced phagocytosis and increased spontaneous ROS production but decreased ROS production following stimulation. These changes in function (especially those associated with host aging) could predispose towards less effective pathogen clearance but increased bystander tissue damage, and these deficits may be compounded even more during severe infections. Usually, the combination of inflammation and hypoxia prolongs the lifespan of neutrophils, priming and activating them to initiate pathogen clearance. In sepsis, especially in the very young and very old, neutrophil function is globally reduced, a state which appears to persist long term. Furthermore, there is an increased incidence of neutropenia during infection due to blunted responses to granulocyte colony stimulating factor (G-CSF) resulting in both a reduction in cell mobilization from the bone marrow without an increase in the survival of activated and mature cells. However, in some instances these functions appear resuable, offering the potential for therapeutic manipulation.

Hence, a number of factors might impact neutrophil function; the age of the neutrophil, the age of the host, the state of the neutrophil (activated, primed or quiescent) and potentially the phenotype of the neutrophil (classical, pro-angiogenic, anti-inflammatory) before one considers the impact of disease.
Neutrophilic Inflammation and Neutrophil Functions in Alpha-1 Antitrypsin Deficiency

Airway neutrophilia is a feature of AATD but this could reflect the inflammatory environment of the lung, an intrinsic difference in how the cell functions (a modulation of function), or a combination of both. Studies which have sampled the lungs of patients with AATD describe significant inflammation, and sputum from patients with AATD is highly chemotactic, with increased levels of the neutrophil chemoattractants CXCL8 and LTB4 and high neutrophil counts suggesting these cells are responding to the these chemoattractants by passing from blood into the airways, although (as in usual COPD levels vary between even quite closely matched PiZZ patients. However, studies have focused on individuals with impaired lung function, and there is little in the literature to describe the lung environment of those without significant lung disease or younger patients. This significantly lessens our understanding of what might be the initiating damaging signals in AATD lung disease. It is known that the presence of inflammation affects cell functions and chronic inflammation is likely to have long term impact on the local environment, amplifying the complex inflammatory milieu through a number of mechanisms including epigenetic change. In established disease it is difficult to identify AATD-specific changes versus those present due to chronic inflammation. A study of end-stage AATD patients compared to end stage non-AATD COPD highlights this, assessing host lung samples from 2 groups of patients at transplant. There were no differences in cellular studies between the upper or lower zones of the lung, despite differing locations of emphysema, potentially questioning the contribution of chronic inflammation versus disease specific pathology. A birth cohort of screened AATD patients and control participants suggested no abnormalities in lung function were present in any of the 16 year olds screened, but by the time the same cohort had reached 22 years of age, approximately 14% of the AATD patients had chronic bronchitis and exertional dyspnea and most had a decline in lung function that was above the expected for health. Furthermore, most individuals had evidence of small airways dysfunction and apical emphysema (evidenced by reduced lung density) in their late thirties all of which can be consequences of increased neutrophil activity although inflammation was not studied in these studies.

The increased ability of AATD neutrophils to migrate towards AATD sputum does not just reflect the increased chemoattractant burden but is amplified by the lack of functional AAT. AAT can deter excessive neutrophil recruitment both by binding directly to CXCL8, preventing CXCL8 binding to CXCR1 on neutrophils and by preventing ADAM17: Fc receptor FcyRIIib interactions on the surface of neutrophils, which would normally initiate downstream chemotaxis signaling events. The increased levels of LTB4 would lead to an increased tendency of AATD neutrophils to “swarm” within inflamed tissues. However, neutrophil proteinases can cleave LFA-1 and MAC1, required to limit swarm size and in the presence of AATD, the combination of both enhanced LTB4 and reduced integrin interactions could lead to uncontained neutrophil swarming, and significant tissue damage. Since migration is associated with proteinase and ROS release, the swarming cells would cause more obligate tissue damage and more LTB4 release, in a vicious cycle of injury, irrespective of the initiating cause. In keeping with this, there is clear evidence of increased neutrophil proteinase activity in AATD, with footprints of both NE and PR3 present systemically, with relationships with proteinase burden and disease severity, even in never smoking individuals. There is also an increased burden of ROSs in AATD, and AAT modulates neutrophil ROS production elicited by N-formylmethionine-leucyl-phenylalanine and CXCL8 in a dose dependant manner.

Phagocytosis appears to be reduced in AATD, with a number of studies showing a reduced ability of AATD neutrophils to engulf different pathogens including non-typeable *Haemophilus influenzae*, a bacteria of particular significance in lung disease. There are few studies of NET formation in AATD. One study using the non-physiological stimulant phorbol myristate acetate described that NET formation was not altered in AATD, however, this study is yet to be replicated using disease relevant stimuli. Finally, studies have described accelerated neutrophil apoptosis in AATD.

In total, this would support an aggressive neutrophil phenotype in AATD but one with limited capacity to clear bacteria, and this would be in keeping with the neutrophil-associated lung damage present in AATD.
and the high numbers of exacerbations experienced by some within this patient group. The damaged lung tissue present in so many patients with AATD may make any functional activation of neutrophils worse, as the damage to the pulmonary capillary network may impede de-priming, creating a vicious cycle of disease. Figure 1 provides a visual overview of these processes.

However, it would be too simple to assume that the burden of disease in AATD merely reflected increased neutrophil activity. The 18-fluorodeoxyglucose (18FDG) positron emission tomography-computed tomography (PET-CT) studies provide data of pulmonary glucose uptake, which has been shown to relate to neutrophil activity in animal models.94,95 PET-CT scans of patients with COPD have shown 18FDG uptake which was greater in emphysematous regions of the lung, and correlated with physiological measures of disease severity.96 However, in AATD, pulmonary 18FDG uptake was similar to healthy controls, which did not support an active neutrophil signal in AATD emphysematous regions of the lung. The reasons for this are unclear, but may reflect the emphysema being now “burned out,” with less cell activity than would have been seen in an early disease, differences in neutrophil activity in AATD and non-AATD related emphysema, or that neutrophilic inflammation was not the driver of disease in this subset of patients. This has yet to be confirmed, but initial studies suggest there are functional differences in non-AATD COPD and AATD neutrophils, including less accurate migration in non-AATD COPD.8 What is clear is that neutrophils in AATD are not merely “activated” (or their survival would be enhanced) or “older cells” (or their phagocytosis would be preserved), or cells from an older host (or their ROS production during a challenge would be blunted).

Figure 1. Interactions Between Alpha-1 Antitrypsin Deficiency, Lung Disease and Neutrophil Functions

AAT has multiple effects on neutrophils and the inflammatory lung environment which, when deficient, could enhance lung disease.
1. The AATD lung environment is highly chemotactic to neutrophils, with raised concentrations of chemokines including CXCL8 and LTB4 causing cell priming and activation and increasing their recruitment.
2. Migration is associated with a release of proteinases and ROS, to enable movement through complex tissues, leading to obligate tissue damage which cannot be easily contained, due to low AAT levels.
3. High levels of LTB4 could induce neutrophil swarming while proteinase-associated cleavage of integrin receptors could prevent containment of that swarm, again increasing the area of tissue damage.
4. Proteinase cleavage of complement and immunoglobulins lead to failure of opsonophagocytosis, impeding bacterial clearance.
5. Increased ROS release supplement the inflammatory local.
6. Local pulmonary capillary damage prevents the depriming of neutrophils, so activated cells are present systemically.

AAT=alpha-1 antitrypsin; AATD=alpha-1 antitrypsin deficiency; LTB4=leukotriene B4; ROS=reactive oxygen species

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Impacts of Treatment

There is evidence that augmentation therapy can restore or improve some aspects of neutrophil function, supporting the suggestion that at least some aspects of neutrophil dysfunction are directly related to low functional levels of AAT. These include augmentation causing increased AAT binding to CXCL-8 and the neutrophil membrane, decreased FcyRIIIB release from the neutrophil membrane and reducing CXCL-8: CXCR1 interactions, with normalization of chemotaxis. Augmentation therapy has also been associated with a reduction in systemic and pulmonary cytokines, including tumor necrosis factor alpha (TNFα) and LTB4 (which might theoretically limit swelling), an improvement in bacterial killing and a reduction in cellular apoptosis. In keeping with this, augmentation therapy has been associated with a reduction in systemic PR3 activity, with this falling from 287nM to 48.6nM in a recent study. Not all neutrophil functions have been tested to date, and it is unclear whether treatment with augmentation merely corrects the proteinase/antiproteinase balance in the immediate environment or has a more profound effect on neutrophil function or phenotype.

Potential Drivers of Functional Change

What none of the available studies have been able to address so far is why there is such variability in disease burden in AATD, even within never smoking PiZZ patients. There is evidence that neutrophil functions associated with disease vary significantly between AATD patients, and this might in part explain some differences in disease presentation, with some patients having highly injurious neutrophil activity and some less so. For example, in studies of a neutrophil elastase activity footprint, there was a 3.75 fold difference between the highest and lowest NE activity levels between patients with severe AATD deficiency. Similarly, in a recent study, the PR3 activity footprint showed an 18-fold difference between the highest and lowest values in patients with PiZZ AATD. The reasons for this are unclear, as little is known about the variability of neutrophil proteinase release and activity in health, or with age. However, in the prementioned studies, the differences in proteinase activity could not fully explain lung disease severity, rates of decline or be attributed to smoking status. It might be that those with the greatest level of proteinase activity also suffer the most inflammatory burden but understanding which came first (the inflammation or the change in neutrophil function) will be challenging. Furthermore, most studies of AATD have focused on systemic cells and neutrophils from the lung compartment may differ in response and require study.

There may be other reasons why lung disease penetration is so variable in AATD. Lifestyle is obviously important, and it has been known for many years that smoking cigarettes with AATD accelerates lung disease, just as a high burden of cigarette exposure is an important risk factor for non-AATD COPD.

Age is a risk factor for lung disease in AATD and many patients with AATD survive to an older age, making it important to understand the impact of AATD in old age. Most studies of older adults describe low-grade, systemic inflammation including in proinflammatory cytokines such as interleukin 6 and TNFα. TNFα is especially important in AATD, as AAT inhibits TNFα converting enzyme (TACE; ADAM-17) activity and TNF receptor 1 (TNF-R1) expression, reducing the conversion of pro-TNF to soluble TNFα, preventing the self-propagating autocrine signaling of the secreted TNF-a. How we age is extremely heterogeneous, with huge variations in the presence of the hallmarks of cellular and tissue aging (which are genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis) and it might be that the presentation of AATD is a reflection of our overall ability to age well. In keeping with this, it is increasingly recognized that patients with AATD frequently have other diseases (multimorbidity or 2 or more conditions in the same individual) and potentially these may influence general health and lung function, by increasing inflammatory burden or altering immune cell function. There is huge interest in trying to identify both factors of susceptibility and resilience to chronic disease to provide mechanistic insight which could be therapeutically targeted, but these are likely to be highly complex, reflecting both genetic and environmental interactions. Such genetic and environmental factors may also impact on neutrophil function in AATD. Emerging studies are already identifying the impact of epigenetic modifications in neutrophils, and these long-lived changes in cellular function have been shown to impact on inflammatory pathways and signals. These studies will need to be
expanded upon, to fully understand the place of this complex cell in a complex disease.

Finally, not all patients with AATD may have active neutrophilic inflammation as a predominant signal, and inflammatory studies using lung and systemic samples would help stratify patients so that treatments could be better targeted.

**Conclusions**

For years the central role of neutrophils and neutrophil proteinases have been described in AATD pathogenesis, and there is increasing evidence of neutrophil dysfunction in AATD, a modulation of the classically described neutrophil functions, which could contribute to the burden of disease for many patients. However, this has been a challenging area of study. Neutrophils are short-lived, difficult to genetically modify and quick to activate when manipulated or removed from their host environment. Furthermore, neutrophil function is likely to change within the compartment where the cell is sampled (bone marrow, blood, lung) and sample collection can be invasive. However, studies to date suggest that the combination of a lack of AAT, lung damage and inflammation is likely to be associated with a particularly aggressive (in terms of tissue damage) but ineffective (in terms of bacterial clearance) neutrophil phenotype. However, neutrophil responses are complex, reflecting the age of the cell, the age of the host, the potential phenotype of the cell and the chronic inflammatory environment the cell is in.

We have a sound understanding of the impact of neutrophil inflammation and proteinases in experimental models, which relate to the clinical manifestations of AATD. However, it is increasingly clear that AATD is a complex disease, with heterogeneity in disease onset, pathology and progression and a mechanistic understanding of why some patients develop severe (or no) lung disease is vital. Identifying which patients have disease driven by neutrophils will allow us to stratify our therapeutic approach, both for augmentation therapy with AAT and beyond. This is especially timely now, as proteinase inhibitors are under assessment in AATD. Being able to select the patients most likely to benefit from neutrophil-based therapeutics will be critical if these therapies are to progress into clinical practice. However, even in those patients, caution is needed, so as to normalize, and not inhibit a biological system so crucial to host defense. If we are able to achieve this, not only will AATD patients benefit, but so will many other people with chronic illnesses which are driven by excessive or ill-targeted neutrophil responses.

**Declaration of Interest**

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Neutrophils in Alpha-1 Antitrypsin Deficiency


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