

The Pathology of Asthma: What Is Obstructing Our View?

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Keywords

asthma, mucus, airway obstruction, galectin-10, neutrophils

Abstract

Despite the advent of sophisticated and efficient new biologics to treat inflammation in asthma, the disease persists. Even following treatment, many patients still experience the well-known symptoms of wheezing, shortness of breath, and coughing. What are we missing? Here we examine the evidence that mucus plugs contribute to a substantial portion of disease, not only by physically obstructing the airways but also by perpetuating inflammation. In this way, mucus plugs may act as an immunogenic stimulus even in the absence of allergen or with the use of current therapeutics. The alterations of several parameters of mucus biology, driven by type 2 inflammation, result in sticky and tenacious sputum, which represents a potent threat, first due to the difficulties in expectoration and second by acting as a platform for viral, bacterial, or fungal colonization that allows exacerbations. Therefore, in this way, mucus plugs are an overlooked but critical feature of asthmatic airway disease.

INTRODUCTION

Asthma is a chronic inflammatory airway disease that universally penetrates the globe, across all societies, ages, genders, health statuses, and social statuses. More than 360 million people on the planet have been diagnosed with asthma, a syndrome that encompasses a huge spectrum of disease, with symptoms of shortness of breath, chest pain, wheezing, and coughing that vary in intensity over time (1). Despite the susceptibility of most individuals to asthma, the prevalence differs vastly across the world, with high-income countries such as Sweden, Australia, and the United Kingdom reporting up to 20% of the population clinically diagnosed with asthma and experiencing symptoms of wheezing (2). A defining, and debilitating, feature of the disease is its chronic duration, most often associated with reversible airway obstruction that is responsive to inhaled bronchodilators and treatment with inhaled steroids. Some 10–15% of patients, however, progressively develop permanent loss of lung function over the course of life. While treatments are available that help many asthma patients bring their symptoms under manageable control, there is a subset of asthmatics who suffer severe and often progressive disease, sometimes with multiple yearly exacerbations. Severe asthmatics experience frequent symptoms despite high-intensity treatment (3) and can account for up to 60% of the direct health-care costs of asthma despite representing only 4–10% of the asthmatic population (4). Indeed, even exacerbations in mild to moderate asthmatics can lead to hospitalizations. While the social burden of asthma is more difficult to measure, this is quantified by disability-adjusted life years lost (23.7 million in 2016), costing more than \$82 billion in the United States alone (5) and projected to rise in future years (6).

Asthma was once considered one disease with one universal treatment, a view that now has been largely abandoned (1). To understand molecular drivers of underlying inflammation and symptoms, and with the ultimate aim of administering the right drug to the right patient, huge clinical patient data sets from multiple research centers were clustered. This resulted in the new classification of up to five asthma endotypes, which describe a subtype of a condition defined by a distinct pathophysiological mechanism, often leading to the identification of treatable traits, such as bronchial hyperreactivity (BHR) (1, 7, 8). The best-known endotype is type 2 high asthma, which is marked by the presence of circulating and sputum eosinophils and a prominence of adaptive immune system Th2 T lymphocytes or type 2 innate lymphocytes (ILC2s) that produce the interleukins (ILs) IL-4, IL-5, and IL-13. However, for the most part, the other endotypes are difficult to detect in point-of-care testing, and by necessity asthma is therefore still divided largely by the presence or absence of type 2 inflammation. The type 2 asthma endotype accounts for roughly half of all asthmatic patients, but within the severe asthma patient category, up to 70% of patients demonstrate features of it (9). Type 2 inflammation is also prevalent in some comorbidities of asthma, such as chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) and allergic bronchopulmonary aspergillosis (ABPA). CRS is associated with similar chronic inflammation of the upper airways and is estimated to affect up to 50% of severe asthmatics, with 65% of CRSwNP patients having comorbid asthma (10). There is also a strong link between patients with severe asthma and skin test and immunoglobulin E reactivity to fungal allergens (such as *Aspergillus fumigatus*) and bacteria such as *Staphylococcus aureus* (11, 12), and, particularly in warm and humid climates, fungi can be cultured from the airways of asthma, ABPA, and CRSwNP patients (13).

Within the heterogeneity and diversity of asthma etiology and pathophysiology, patients will frequently be diagnosed based on clinical evidence of recurrent respiratory symptoms, including coughing, wheezing, shortness of breath, and chest tightness. This is substantiated by demonstrating airflow limitation and reversible airway obstruction with assessment of lung function, almost always accompanied by bronchial hyperresponsiveness to nonspecific triggers. Therefore,

it can be postulated that airway obstruction is at the heart of this lung disease. Despite this unifying feature of asthma and other type 2 diseases, the mechanisms of airway obstruction in asthma are still incompletely understood. Up to now, asthma research and the development of powerful treatments has been centered around inflammation, smooth muscle contraction, and bronchoconstriction as the main culprits for airflow limitation. While undoubtedly inflammation triggered by inhalation of an antigen, air pollution, or viral infection are primary initiators of and contributors to many features of asthma, emerging data are demonstrating that even with the most sophisticated inflammation prevention strategies, the chronic remodeling and airway obstruction is not always alleviated and continues to impact the quality of life of the patient, sometimes leading to irreversible airway obstruction (14, 15). A consistent, but poorly studied, observation in severe asthmatics, patients with CRSwNP, and certainly those with complicating ABPA is obstruction of airways with mucus plugs (16). Many of these patients eventually also develop bronchiectasis and bronchiolectasis, characterized by irreversible damage and dilatation of the conducting airways (17).

Since many recent reviews have already summarized the importance of the immune system and inflammation in driving asthma pathology (18, 19), we focus this review on the importance of mucus as a cause of persistent airway obstruction and a trigger for disease perpetuation. None of these components of airway disease exist in isolation, however, and understanding the complex interplay of the immunological, physiological, and epithelial processes should be the ultimate goal in understanding the pathophysiology of asthma. Such knowledge will undoubtedly help us understand how we can integrate clinical measurements of disease with therapeutics to provide the best possible care.

THE OBSERVATION OF MUCUS PLUGS IN FATAL ASTHMA

Wheezing or the presence of a whistling breath sound during expiration is a frequent self-reported clinical sign of asthma that points toward airway narrowing. In a survey of 181,000 people worldwide, including those with doctor-diagnosed asthma (2), more than half of participants responded positively to this question, indicating the prevalence of asthma symptoms caused by airway obstruction. In its most innocuous form, airway obstruction is reversible and causes a feeling of breathlessness and wheezing only in certain situations, such as during exercise and cold air exposure. In more severe forms of the disease, airway obstruction becomes irreversible, even with maximal treatment, and can completely occlude multiple airways and result in death. Mucus plugs were identified as a cause of death in asthmatics by clinicians in the 1880s and have been repeatedly observed since (20–23) (**Figure 1**). In a study of 93 fatal asthma cases, 95% of patients had abnormal luminal obstruction on autopsy, with half of patients having >80% of their airway blocked by mucus (24). The outstanding pathological vignette of lungs from these patients was “the failure of clearance of bronchial secretions” (25, p. 32). Further descriptions of the lungs detailed “numerous gray, glistening mucus plugs scattered throughout the airway passages” (25, p. 28). A literature review of 160 fatal asthma cases in 1957 reported that the “pathological findings in most cases were the bronchial tree plugged with sticky mucus” and concluded that “most writers are agreed that the chief cause of death is asphyxia due to bronchial obstruction. . . most authors attach much more importance to bronchial secretion than to bronchospasm” (26, p. 198). The term endobronchial mucus suffocation was given by Lynne Reid to describe “the typical findings in the patient who dies in status asthmaticus. The large airways, and even the small are often stuffed with secretions so viscid that the mucus can be removed only by cutting the plugs” (27, p. 415).

Further postmortem studies have used silicone casts to analyze the location of airway obstruction and the degree of airway truncation in the airways of patients who died from asthma (fatal

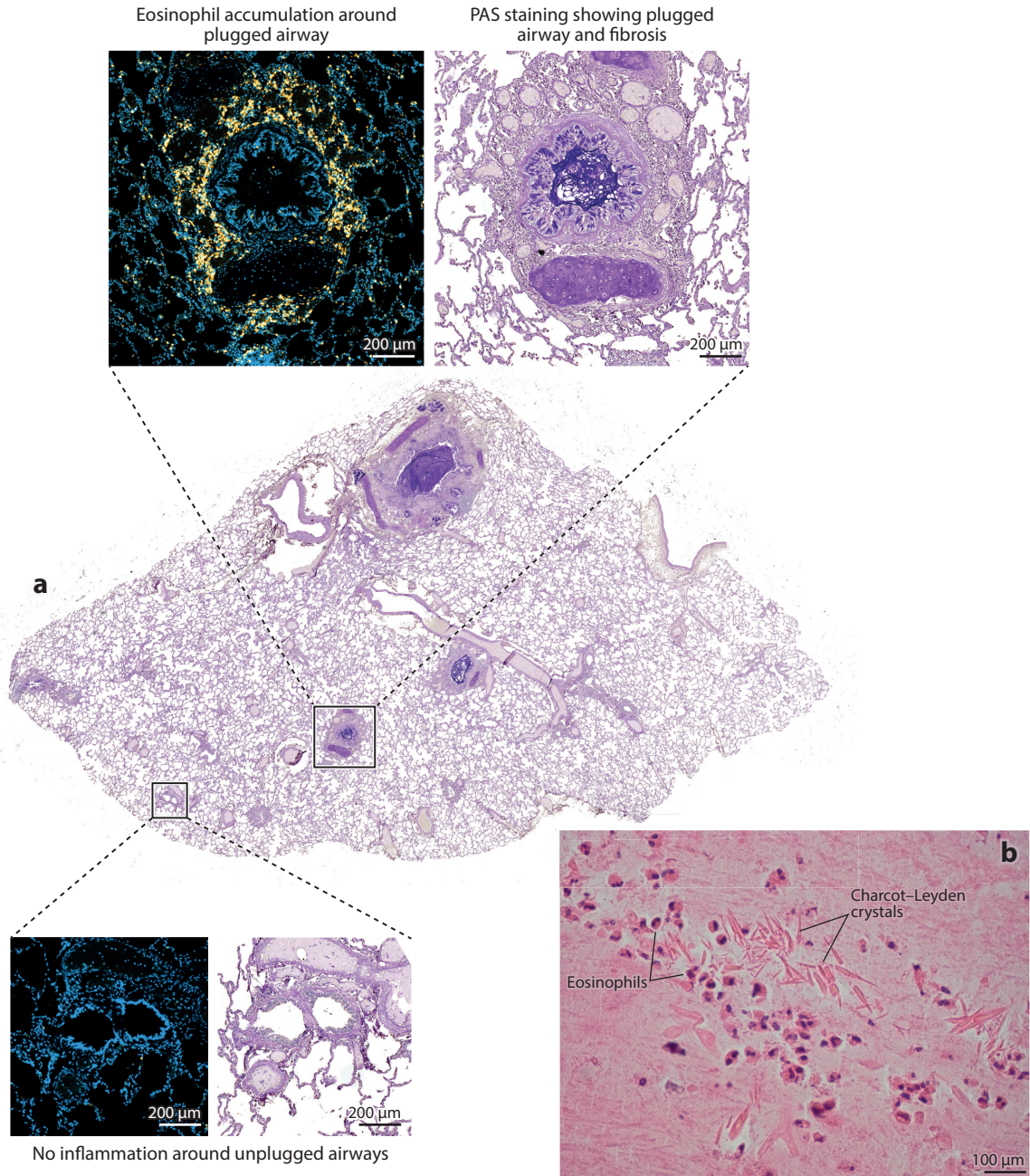


Figure 1

Mucus plugs are a characteristic of severe and fatal asthma and contain eosinophils and Charcot–Leyden crystals. (a) Lung sections taken from a fatal asthmatic (provided by Walter E. Finkbeiner, MD, PhD, Univ. of Calif. San Francisco, Pathology Dept.) and stained with PAS to indicate mucus plugs are shown. Alternate sections were stained with galectin-10 (orange) and DAPI (blue) to indicate eosinophils and nuclei, respectively. (b) H&E staining of a sputum smear obtained from a patient with allergic bronchopulmonary aspergillosis, demonstrating the presence of abundant eosinophils and Charcot–Leyden crystals (provided by Prof. Dr. Jo Van Dorpe, UZ Gent). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; PAS, periodic acid–Schiff.

asthma) versus asthmatic patients who died from nonpulmonary causes (nonfatal asthma). This study demonstrated that the larger, proximal airways were lost from the lung casts of fatal asthmatics, whereas the casts from nonfatal asthmatics showed blockage of the smaller distal airways. All asthmatic patients in this study suffered from a loss in fractal dimension, which can be attributed to ventilation heterogeneity or a loss of space-filling ability in the lung. Interestingly, this loss of fractal dimension was significantly correlated with the area of mucous glands and smooth muscle hyperplasia in these patients but not with the local eosinophilic infiltration or duration of asthma (28).

MUCUS PLUGS IN SEVERE ASTHMA

Autopsy studies demonstrate not only the overwhelming contribution of mucus plugs to fatal asthma but also indicate the persistent and progressive nature of airway obstruction, from mild to moderate disease. A recent study using computed tomography (CT) scans to identify mucus plugs in the lungs of severe asthmatics showed not only that the same mucus plugs could be identified in the lungs of patients over the course of 3 years but also that many of these patients progressed to a more severe score of mucus plugging that was unresponsive to bronchodilators and steroids (16). In this study, and others (14), the severity of mucus plugging was well correlated with low lung function and irreversible airway obstruction that was no longer responsive to inhaled bronchodilators or to inhaled or systemic steroids. The progressive loss of lung function and the development of irreversible airway obstruction is heavily accelerated in asthmatics compared with healthy controls and can be further enhanced by several parameters associated with severe asthma, including duration of disease, a low baseline forced expiratory volume in the first second (FEV1), high propensity to exacerbations, male sex, smoking history, dose and insensitivity to systemic steroids, and presence of eosinophils in the sputum (**Figure 1**) (29). Not only did these studies identify mucus plugs, but they also identified immune factors that correlated with patients with severe plugging, including features of type 2 immunity such as sputum eosinophilia, high IL-5 and IL-13, and, as a derived biomarker, a high concentration of nitric oxide in exhaled air, a well-known sign of IL-13 bioactivity on lung epithelial cells (14, 16). Studies such as these, using innovative imaging techniques combined with inflammatory profiling to identify airway obstruction in asthmatics, are much needed, as they offer the possibility of rapidly improving our understanding of the distribution and frequency of airway obstructions inside the lung.

Previously, the decline in (postbronchodilator) FEV1 has been used as a measurement of loss of lung function. While this, as a general parameter, can be informative in understanding severity of disease and extent of airway obstruction, more precise measurements are needed to understand how this disease manifests within the airways. The use of hyperpolarized ³He magnetic resonance imaging (MRI) has permitted a high-resolution investigation of airway obstruction, revealing specific focal defects in the lungs of asthmatics, referred to as ventilation heterogeneity (VH) (30, 31). Ventilation heterogeneity has been shown to be an independent determinant of airway hyperresponsiveness and a good predictor of exacerbation frequency (32, 33). Importantly, while treatments such as bronchodilation have been shown to improve VH in asthmatic patients, significant ventilation defects persist in patients with uncontrolled eosinophilia, even following bronchodilation (14). Teague et al. (34) elegantly examined the immune cell profile of specific regions of airway obstruction in children by using bronchoalveolar lavage of areas of VH as identified by ³He MRI. This investigation revealed that higher numbers of eosinophils, but not neutrophils, were found in poorly ventilated regions compared with well-ventilated regions. Understanding the inflammation in regions of poor ventilation will inform treatment options, but these types of studies should also be performed during times of exacerbation, as other granulocytes may contribute to excessive inflammation and bronchoconstriction during this period.

Going forward, it will be important to understand the effect of type 2 treatments on this aspect of disease. Generally speaking, whereas biologicals targeting type 2 immunity mainly affect the annual rate of exacerbations and reduce the need for steroid treatment in patients with severe asthma, their effects on improving irreversible airway obstruction is less clear. At least 24 weeks of treatment with the IL-4R α antagonist dupilumab or the depleting IL-5R α antibody benralizumab can improve FEV1, but it is unclear whether this is the result of resolution of mucus plugs (35, 36). How biologicals affect VH has been addressed by one study by Svenningsen et al. (37), albeit in a small number of patients. While type 2 biologicals were able to largely improve disease in terms of VH, there were multiple caveats to their efficacy, including their effects on eosinophilic inflammation, and residual ventilation defects remained in 7 out of 10 patients. This outcome was also seen in another study using benralizumab, in which the mucus plugging of two-thirds of severe asthmatics did not improve after a single administration (15). A wider use of these measurements will contribute to a general understanding of airway obstruction and to clinical decisions regarding combinatorial treatment to alleviate the chronic and persistent features of airway obstruction, which are classic for patients with type 2 disease.

While imaging studies are undoubtedly providing an invaluable insight into focal ventilation defects in asthma, allowing for a much more precise understanding compared with that of FEV1, they cannot definitively assess the form of airway obstruction. Airway obstruction can result from occlusion of the airways by mucus but also because of airway wall thickening, inflammation, or edema (18). Endobronchial optical coherence tomography studies have also demonstrated a strong phenotype of mucosal buckling in asthmatics but not in healthy controls. This result is speculated to be a consequence of bronchoconstriction and is strongly correlated with loss of lung function (38). In severe asthmatics, airway walls become thicker as a percentage of the total airway, coinciding with a smaller lumen (38) and correlating with a lower lung volume (39). A significant narrowing of airways is seen in asthmatics, particularly in airways <2 mm in diameter, with a third of asthmatics showing ~30% of their airways completely closed at baseline (40). The stiffening that occurs around a remodeled airway, due to the deposition of extracellular matrix proteins under the basement membrane and smooth airway muscle hyperplasia (41, 42), can also have a drastic impact on airflow limitation by narrowing airway caliber. According to Poiseuille's law, an increase of airway resistance of 16-fold can result from a reduction of the radius of the airway lumen by only 50%. While this reduction already presents a problem at baseline, the importance of this phenomenon is exaggerated during exacerbations, as less airway narrowing is required in asthmatics to result in either a substantial drop in lung function or airway closure (40). This can create a high propensity for exacerbations and even lead to death.

THE BIOLOGY OF MUCUS PLUGS IN ASTHMA

In the healthy lung, mucus plays a cardinal role in maintaining healthy barrier function and gas exchange. Mucus and ciliated cells have been found in the oldest living ancestors of vertebrates, and even corals dedicate a huge portion of their net carbon fixation to mucus production (43). To prevent the constant irritation of the epithelial barrier and activation of homeostatic and/or resident immune cells by pollutants or allergens, mucus acts to trap irritants that are conveyed in the 7 L of air that humans intake every minute. Mucus lines the conducting airways down to the terminal bronchioles and forms a crucial component of the mucociliary blanket, which propels mucus upward to the top of the trachea against gravity by the ciliated epithelium. Mucus in the healthy lung is mostly composed of water (97%) and a complex mixture of mucins, other proteins, salts, lipids, and cellular debris. The composition of mucus is extremely important and must be well regulated to ensure a liquidity that allows proper functioning of this system.

A major barrier to the research of mucosal obstructions in type 2 diseases is the lack of suitable models outside of the human airway and the difficulty of retrieving patient samples. Human cultures of epithelial cells are certainly relevant and useful for dissecting the molecular processes that occur in the airways but do not recreate the 3D organization of airways, in which these cells respond and communicate to a plethora of immune, neural, and endocrine signals over an extended period. While mouse models using allergen challenge can replicate some features of asthma, they are usually relatively acute and, furthermore, miss vital features of airway remodeling. Even in chronic and sustained models of allergic airway challenge, important features of human disease such as mucus production and airway reactivity return to normal after cessation of stimulus, although increased collagen deposition and airway smooth muscle are maintained (44). This outcome likely reflects major differences in the anatomy of the lung (45) between mice and humans as well as important differences in the immune compartments of these species (46).

While patients with hypersecreting mucus diseases, such as cystic fibrosis, produce grams of sputum spontaneously, this is not necessarily the case in diseases such as asthma or ABPA. The use of hypertonic saline to aid the induction of sputum production also confounds the biophysical properties of sputum (47), which is then often contaminated with saliva. For example, while many studies of the local inflammatory environment during asthma use induced or spontaneous sputum to quantify airway eosinophilia, this induced form of mucus is by definition not representative of a plug that occludes the airways and cannot be expectorated. To properly study occlusions, tissue needs to be retrieved from the site of obstruction, a requirement that limits collection methods to invasive procedures, such as bronchoscopy, or retrieval from endotracheal tubes (48), as well as limiting the amount of sample that can be collected.

Pathogenic mucus from type 2 diseases, such as CRS or asthma, demonstrates a significantly higher elastic modulus (49–51), making it much stickier and harder to expectorate by coughing (50). A high elastic modulus indicates extensive cross-linking of the mucus, which results in an incredibly tenacious substance that cannot be moved by the mucociliary escalator, is hard to expectorate, and remains lodged in the airways.

Therefore, alterations in the quality, rather than merely the quantity, of mucus in type 2 diseases is a more relevant parameter of disease. While increased mucus production is certainly necessary and is associated with plugging, mucus hypersecretion and expectoration of sputum by itself should not be viewed as being synonymous with mucus plugs or considered a good clinical surrogate for them (23). When asthmatics do cough up sputum, they often recount that the mucus is highly elastic and tenacious, often comparing its aspect with dried glue. Mucus hypersecretion is a feature of bronchitis seen in many other airway diseases, such as chronic obstructive pulmonary disorder (COPD) and cystic fibrosis (CF), and even in acute settings of viral and bacterial infection. Notably, the extensive study of mucus plugs in asthmatics enrolled in the Severe Asthma Research Program (SARP) by CT scans found that symptoms failed to correlate with mucus hypersecretion and, vice versa, that patients with mucus hypersecretion were not prone to plugs (16).

Healthy mucus resembles a gel, in that it displays both liquid- and solid-like properties referred to as viscosity and elasticity, respectively. Viscosity is a liquid property and can describe the resistance to flow, which is often conferred by the number of molecules in a substance. Elasticity is description of the resistance to shear force and results from the cross-linking of molecules within a substance. The mucins present in mucus play key roles in determining both parameters. The two primary mucins found in the human and mouse lung are *MUC5B/Muc5b* and *MUC5AC/Muc5ac*. These have distinct expression patterns, with *MUC5B* being secreted at high levels in submucosal glands and secretory cells in the distal airways, whereas *MUC5AC* is secreted by goblet cells (52). Healthy lungs contain mostly *MUC5B*, but a common feature of asthmatic mucus is dysregulation of this ratio, with *MUC5AC* being dramatically upregulated (53), particularly in patients with an

eosinophilic asthmatic phenotype (53). High levels of *MUC5AC* staining are seen in the mucus plugs of fatal cases of asthma (22). The importance of *Muc5ac* in contributing to mucus plugging in mouse models was shown when mice deficient for this gene (*Muc5ac*^{-/-}) were challenged with *Aspergillus oryzae* extract to induce allergic inflammation and airway hyperreactivity (AHR). These mice had a 74% reduction in airway occlusions, seen to be mucus plugs, but no significant decrease in airway eosinophilia (54).

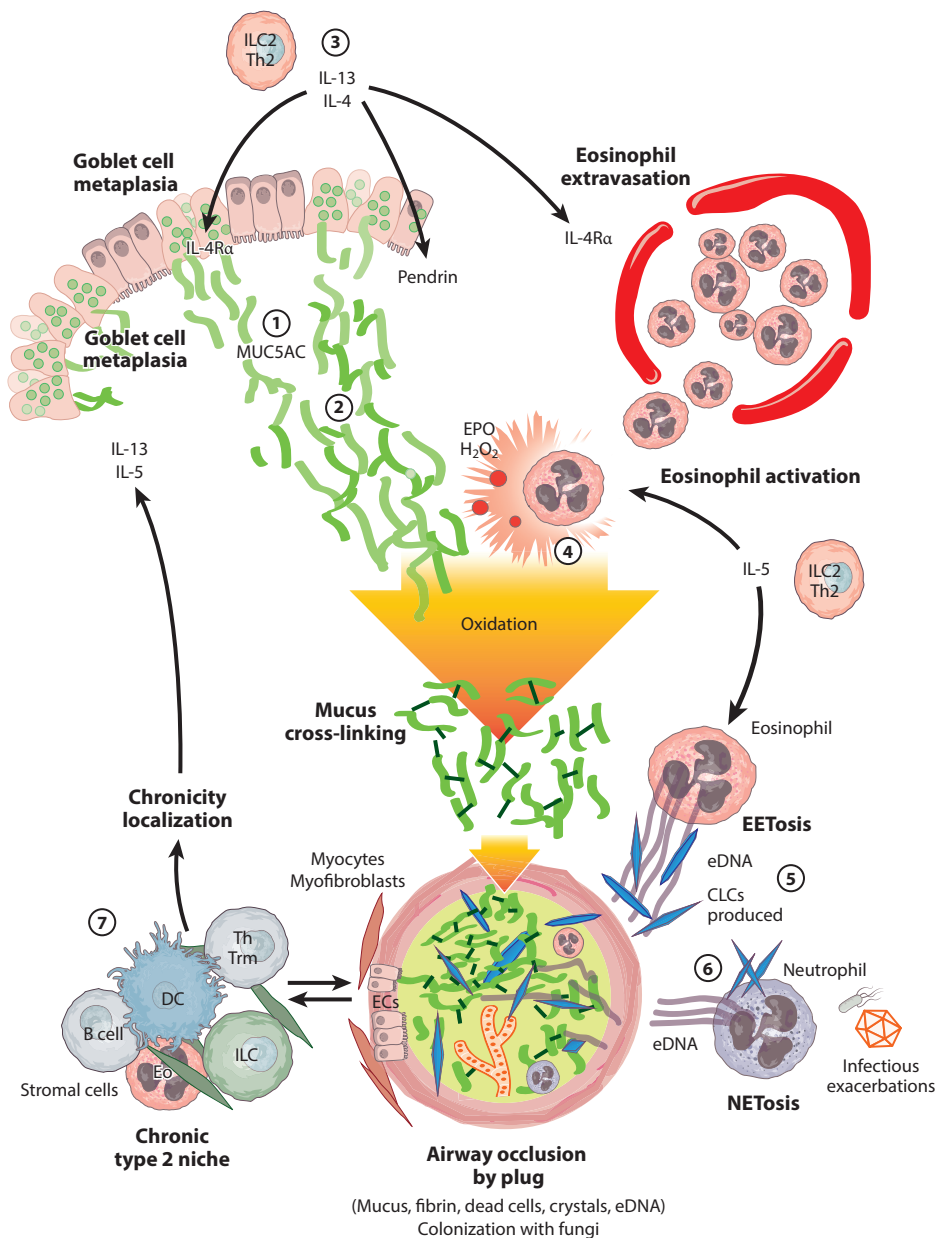
The well-demonstrated alteration in *MUC5AC:MUC5B* ratio is not merely a signature of type 2 inflammation but has profound significance on the biophysical and inflammatory properties of the mucus. These two mucins have distinct structural and functional attributes, and *MUC5AC* is capable of actively contributing to the pathogenic tenacity of mucus. Scanning electron microscopy demonstrates clear ultrastructural differences of these mucins. *MUC5B* exists as long linear polymers that do not extensively interact with each other. A distinguishing feature of *MUC5AC* is how highly branched it is, which translated into a stickier, and stiffer, layer, as measured by dissipation monitoring on a quartz crystal microbalance (Figure 2) (55). These distinct morphological networks can also be observed by lectin staining in porcine airways (56). The specific tethering of *MUC5AC* to the epithelium was demonstrated in an ex vivo human airway epithelial cell system, in which extensive surface washing of the cells following IL-13 induction removed only *MUC5B* (22).

CAUSE OF BIOPHYSICAL ALTERATIONS IN MUCUS PLUGS IN ASTHMA

Mucins are subject to extensive cross-linking due to cysteine-rich domains at both their amino (N) and carboxyl (C) termini. The branching of *MUC5AC* can be reduced by dithiothreitol, indicating disulfide bonds contribute to multimer assembly of these mucins (55). The production of oxidative species, such as thiocyanate, by peroxidases in granulocytes, such as eosinophil peroxidase in eosinophils, has been shown to cross-link thiol groups to stiffen the elastic modulus of gels similar to mucus plugs (Figure 2) (16). The reversal of these disulfide cross-links can reduce the elastic modulus of mucus and make it easier to expectorate (20). Furthermore, it has been demonstrated that mucus from acute asthmatics contains high levels of albumin, which inhibits proteases involved in the degradation of mucins, resulting in a high elastic modulus (50). Mucins can be further modified at their glycan residues by processes such as fucosylation. The fucosyltransferase *Fut2* is upregulated in the mouse epithelium during house dust mite (HDM) challenge and has been shown to contribute to acute allergic inflammation (57). In humans, *FUT2* is required to form H antigens on mucin glycans in O-secretor individuals. The O-secretor phenotype is enriched in asthma patients relative to controls, and these individuals demonstrate a significantly higher propensity to exacerbations and a lower FEV1 (58), suggesting a potential role for fucosylation in human asthma.

In addition to cross-linking, many factors such as cytokines and epithelial growth factors can induce the upregulation of mucins in asthma. Mucins are normally stored in secretory granules, which can be released within seconds in response to insults as part of an ancient defense mechanism (43). Mucus production has been seen to be upregulated in humans following segmented allergen challenge of asthmatics but not healthy controls (38, 59). However, persistent alteration of mucin gene expression occurs during chronic asthma, with IL-13 being a major inducer of the *MUC5AC* mucin. IL-13, produced locally by immune cells such as innate ILC2 cells and Th2 T cells, signals through the IL-4R α on epithelial cells. This evokes a signaling cascade via signal transducer and activator of transcription 6 (STAT6) that results in increased expression of the transcription factors SAM-pointed domain-containing Ets-like factor (SPDEF) and the Forkhead

box (FOX) transcription factor. At the same time, IL-13-induced STAT6 also controls the epithelial expression of an anion solute carrier pendrin (encoded by *SLC26A4*) that transports anions into the airway secretions and contributes to mucus abnormalities by altering the airway surface liquid hydration (60). The importance of these factors has been very clear from mouse modeling. Transgenic overexpression of *Spdef* or *Foxa3* in the airway secretory lineage was sufficient to induce mucous cell metaplasia (61), and *Spdef* and *Slc26a4* are required for allergen-induced mucous cell metaplasia. Administration of IL-13 to the airway or transgenic overexpression of IL-13 in airway



(Caption appears on following page)

Figure 2 (Figure appears on preceding page)

A hyperactivated type 2 niche surrounds mucus plugs. (①) MUC5AC is substantially upregulated in goblet cells and becomes the predominant mucin in type 2 inflammation. (②) MUC5AC is an incredibly branched mucin, which creates a highly cross-linked mucus gel, resulting in a stickier and stiffer mucus layer. (③) The type 2 cytokines IL-4 and IL-13, initially supplied by ILC2s and subsequently by Th2 cells, signal through IL-4R α to broadly activate type 2 inflammation, including the upregulation of MUC5AC, as well as promote eosinophil extravasation. (④) EPO can catalyze the reaction between H₂O₂ and halides, such as hypothiocyanous acid, to generate oxidants capable of cross-linking mucus, which makes it more sticky. (⑤) Eosinophils recruited to the airways become activated to produce extracellular traps (a form of programmed cell death termed EETosis), resulting in the formation of CLCs. (⑥) CLCs recruit and activate neutrophils to produce NETs (a process known as NETosis), which can cause increased stickiness of airway mucus. (⑦) The presence of CLCs in an airway can drive persistent activation of immune cells and ECs to create a feed-forward loop that results in airway occlusion and chronic type 2 inflammation. Abbreviations: CLC, Charcot-Leyden crystal; DC, dendritic cell; EC, epithelial cell; eDNA, extracellular DNA; EET, eosinophil extracellular trap; Eo, eosinophil; EPO, eosinophil peroxidase; H₂O₂, hydrogen peroxide; IL, interleukin; ILC, innate lymphoid cell; NET, neutrophil extracellular trap; Th, T helper; Trm, T resident memory. Figure adapted with permission from Reference 138.

epithelial cells is sufficient to induce similar changes in mucin expression (62, 63). Airway cultures treated with IL-13 transport microvesicles less efficiently, without affecting ciliary beating (22).

Recent single-cell analysis of the human airways has demonstrated that IL-13 is also capable of inducing mucus production in other lineages of airway epithelial cells. An ex vivo culture of human airway epithelial cells demonstrated that even acute (2-day) IL-13 stimulation induced a switch of secretory cells into mucus-producing cells, with upregulation of MUC5AC and MUC2 (64). Interestingly, this was accompanied by alterations in pathways involved in mucin processing, such as glycosylation, solute carriers, and mucin-interacting proteins, suggesting an overall alteration in the mucus that is produced by these cells following IL-13 stimulation, potentially making mucus more tenacious. Furthermore, this switch to mucin production was at the expense of other functions, such as defense and production of cilia (64). Besides a shift in the composition of cells exposed to IL-13, novel populations in the asthmatic lung have been identified by single-cell sequencing. A population of mucous-ciliated cells, which express genes of both lineages (*MUC5AC*, *CEACAM5*, *FOXJ1*), was found, and these cells also upregulated genes downstream of NOTCH and IL-13 (65). Claudins are a family of tight-junction proteins that have an important role in preserving barrier function. Human asthmatic epithelial brushings show low levels of claudin-18, which is actively downregulated following IL-13 stimulation, possibly increasing the permeability of the epithelial barrier. Mice lacking claudin-18 show a significantly higher AHR in response to *Aspergillus* (66). Furthermore, the depletion of claudin-1 in epithelial cell cultures and mice results in an increased expression of *Muc5ac* and increased production of type 2 cytokines and mucus in an allergic mouse model (67).

Genome wide association studies of asthmatics have so far not been able to account for the relatively high heritability of asthma (estimates range from 40% to 90% heritability). However, notable genes with roles in mucus production, airway remodeling, and obstruction have been identified that associate with decreased lung function during asthma, including *MUC5AC*, *IL13*, *CHI3L1*, *TSLP*, and *TGFB1* (68). Two distinct groups of *MUC5AC* polymorphisms were recently identified with the potential to alter EGR1 binding (69). Additionally, during the functional validation of a risk variant of *MUC5AC*, it was seen that other genes involved in mucus production and secretion were also affected, indicating a *trans* action for this risk expression quantitative trait loci (70). In addition, the chitinase *CHI3L1*, also known as YKL-40 in humans and AMCase in mice, has single-nucleotide polymorphisms linked to asthma, specifically AHR and decreased lung function in studies of Hutterites and Japanese asthmatics, as well as in the SARP cohort (71).

There also appear to be profound epigenetic changes induced in the epithelium of asthmatics (72, 73). The significance of epigenetic changes is their persistence, particularly if these occur in the basal stem cells of the epithelium. A short exposure to IL-13 results in the methylation of thousands of sites, most heavily at genes associated with asthma (74). This may suggest that epithelial cells become easily imprinted, priming them for exaggerated disease.

EOSINOPHILS AND MUCUS PLUGS

A dominant cell type in type 2 inflammation that is strongly linked to poor lung function and exacerbation frequency in asthma is the eosinophil (1, 16, 18, 75). Eosinophilic inflammation associated with decreased FEV1 has remarkably even been observed in apparently healthy individuals (76, 77).

Eosinophils are a canonical feature of type 2 inflammation and have roles across the spectrum of disease, inducing inflammation, mucus production, and airway remodeling in asthma (78). Severe asthmatics can be stratified by high numbers of circulating eosinophils (often >300 cells per microliter of blood), but eosinophils exert their main effector functions once they are within tissues. These specialized granulocytes secrete several toxic proteins, such as major basic protein (MBP), eosinophil cationic protein, and eosinophil-derived neurotoxin, as well as reactive oxygen species. Oxidants can also be generated following the reaction of eosinophil peroxidase with hydrogen peroxide in the presence of halide substrates such as bromide or chloride, but particularly hypothiocyanous acid (16). These oxidants and basic proteins can directly damage the epithelium and also have a profound impact on the pathogenicity of mucus, making it extremely tenacious by oxidating cysteines and inducing cross-links between polymers, increasing the elasticity of mucus (**Figure 2**) (16). MBP has been demonstrated to decorate mucus plugs and damaged epithelium in studies of fatal asthma (79). In mouse models, the overexpression of IL-5 and eotaxin-2 resulted in a synergistic recruitment of lung eosinophils, which already demonstrated substantial mucin gene induction and mucus plugging, concurrent with AHR, even in naive mice (80).

An additional response of eosinophils during inflammation is a form of programmed cell death termed EETosis [eosinophil extracellular trap (EET) cell death]. In a comparable fashion with neutrophils producing neutrophil extracellular traps (NETs), a large quantity of nuclear DNA is extruded in the proximity of the dying cell. In contrast to NETs, the intracellular granules are released in intact form. EETs have been identified in the tenacious mucus of patients with CRS and ABPA (**Figure 2**) (81, 82). The DNA produced by neutrophils in NETs has a potent effect on increasing the elasticity of CF sputum (83, 84), and EETs may perform a similar function in type 2 diseases. Additionally, EETs have been shown to be 5 times thicker and more persistent than NETs, retaining their shape for more than a week (81). Eosinophils derived from the blood of severe eosinophilic asthmatics were more prone to ex vivo induced EETosis than eosinophils from nonsevere asthmatics, and patients with highly reactive eosinophils had a low FEV1 (85). EETs were also capable of activating eosinophils and mast cells, and inducing IL-6 and IL-8 production by human epithelial cells, demonstrating their ability to perpetuate inflammation (85). Similarly, EETs have been shown to induce the neuropeptide CGRP from pulmonary neuroendocrine cells, contributing to allergic inflammation (86). Mucins are also ligands for receptors on innate immune cells and macrophages, and, generally speaking, tonic signals through lectin receptors dampen the function of these cells to maintain homeostasis (87). However, purified mucins have also been shown to induce mouse eosinophil apoptosis through engagement of sialic acid-binding immunoglobulin-like lectin F (Siglec-F) (88).

The genetic or antibody-mediated depletion of eosinophils in mouse models has varying efficacies on improvement of AHR and mucus production during allergic inflammation, suggesting

a context-dependent role for eosinophils, often acting in concert with IL-13 or IL-4 (89–91). A similar picture is seen in humans treated with eosinophil depletion antibodies; while some patients demonstrate improvements in ventilation heterogeneity with mepolizumab or benralizumab, this is not universal (15, 37). Indeed, heterogeneity in tissue eosinophilia is being uncovered, with distinct populations of IL-3R^{hi}CD62^{lo}CD69^{hi} eosinophils found in patients with asthma and CRS (92, 93). Even following mepolizumab treatment—in which blood eosinophils are effectively depleted—~50% of airway eosinophils remain (94). The distinct phenotype of eosinophils in the nasal and lung tissues of patients with asthma and CRS may reflect a reliance on local factors, such as components of the extracellular matrix (95).

EOSINOPHIL-DERIVED PROTEIN CRYSTALS AND MUCUS PLUGGING

Another abundant eosinophil protein that contributes to the severity of asthma is galectin-10. Galectin-10 is the fifth most abundant eosinophil protein (96), representing 7–10% of the cytoplasmic content of these cells (97). While the intracellular role of this protein is elusive in eosinophils, it has been shown to intracellularly localize with eosinophil-derived neurotoxin and aid in granulogenesis (98). Importantly, following eosinophil activation and EETosis, galectin-10 can autocrystallize extracellularly to form Charcot–Leyden crystals (CLCs), which are often found embedded in upper or lower airway mucus collections (99). CLCs were initially identified by Jean-Martin Charcot (100) and Ernst Viktor von Leyden (101) more than a century ago in the sputum of an asthmatic and a leukemic patient but remained only an enigmatic biomarker of eosinophilic inflammation until the composition of these crystals was found to be galectin-10 (**Figure 2**) (102). Although this protein was first proposed to be a lysophospholipase (103), currently there is no functional evidence for this idea. In its dimeric form, galectin-10 contains a carbohydrate recognition domain, which shows only weak affinity for glycan ligands (98, 99, 102). Crystal structures of galectin-10 dimer interactions with carbohydrates such as ribose and mannose have been solved (99). Stronger binding to carbohydrate ligands, such as the carbohydrate-rich mucins found in mucus, may be afforded by multiple low-affinity interactions with the crystal structure rather than the dimeric protein of galectin-10, but this is yet to be investigated. Even in the absence of a protein–carbohydrate interaction between the mucins and galectin-10, CLCs may provide an important physical reinforcement to mucus, increasing the tenacity of this substance. Much like steel rebars in concrete, the mere presence of a CLC in the mucus plug of an asthmatic may cause an entanglement of mucin polymers, tethering this plug in the airway.

Despite the description of the role of eosinophils and their proteins in driving asthma in many research papers and review articles, galectin-10 has gone largely ignored, perhaps because there is no expression of this protein in mice. To circumvent this, CLCs were recombinantly produced and administered exogenously to mice, in the context of allergens (99). Many parameters of allergic disease including antibody production and T cell activation by dendritic cells (DCs) were exacerbated by these crystals, but chiefly CLCs in the context of HDM were seen to promote the production of mucus in the lungs of mice (99). Therefore, the potential of this protein as a novel drug target in asthma was also investigated. Antibodies generated against galectin-10 were able to prevent crystal formation and also remarkably dissolved preformed crystals in ex vivo samples of human mucus from patients with CRSwNP (99). Furthermore, in a humanized mouse model of allergic asthma, exogenously administered crystalline, but not soluble, galectin-10 induced *Muc5AC* expression, BHR, and mucus production, which was reversible with antibody treatment.

While mice do not express the gene for galectin-10, it is interesting that they have convergently evolved another protein that is upregulated during type 2 inflammation and extracellularly

crystallizes within mucus plugs. The chitinase-like proteins Ym1 (*Chil3*) and Ym2 (*Chil4*) are also found as extracellular crystals within mucus plugs in mouse models of type 2 disease (104). While these are mostly produced by myeloid and epithelial cells instead of the eosinophil, it is interesting to postulate a role for protein crystals in the airways during type 2 disease, as these are an otherwise uncommon phenomenon. One possibility that needs further exploration is that crystals and altered mucus have a protective role in helminth defense by facilitating the expulsion of mucus-decorated worms from the intestine. MUC5AC has a broad antihelminth role and is required in the gut for expulsion of *Trichuris muris*, *Trichinella spiralis*, and *Nippostrongylus brasiliensis* despite the induction of a potent type 2 immune response in the absence of this mucin (105). The direct administration of MUC5AC, but not MUC2, was able to significantly reduce the viability of the *Trichuris* nematode (105).

The similarity between the components of the type 2 immune response, whether to natural pathogens such as helminths or unnatural antigens such as innocuous allergens, in mice and humans provides new insight into the possible roles of a novel immune mediator. Furthermore, the presence of CLCs in allergic mucin should no longer be viewed as a correlate of hypereosinophilia but as a tangible opportunity for therapy, particularly with respect to mucus plugs (**Figure 2**).

NEUTROPHILS AND MUCUS PLUGGING

As described above, the type 2 endotype of asthma has received the most attention, partly because of the induction of type 2 inflammation in mouse models following administrations of common allergens (HDM, ovalbumin, cockroach allergen, papain, etc.), and equally because of the success of biologicals targeting type 2 inflammation in humans (19). Non-type 2 asthma is significantly more difficult to define and encompasses a range of asthmatic endotypes that only share a commonality of normal eosinophilic levels, although this defines 50% of asthmatic patients (106). However, particularly in the context of airway obstruction, other types of inflammation should be considered. Currently, the role of neutrophils in asthma is not well understood, although they are often associated with a detrimental outcome and sometimes even with mucus plugs.

The range of neutrophilic asthma can vary across countries and age groups, with India reporting one of the highest rates of neutrophilic asthma in the world (107). However, many studies show the prevalence of neutrophils in severe asthma (108), often associated with poor asthma control, with the majority of these patients self-reporting high sputum production (109, 110). Adult asthmatics with high neutrophilia (>60%) in their sputum tend to be older, late onset, and male, with more severe lung disease and a greater risk of hospitalization and exacerbations (111). Neutrophilia is also associated with severe corticosteroid-resistant asthmatics, and indeed this therapeutic is sometimes hypothesized to skew the immune response toward neutrophilia. However, regardless of the reason for their recruitment, once they are present, the role of these cells should not be discounted, as these granulocytes have multiple mechanisms by which they contribute to and exacerbate airway inflammation and mucus plugging.

Indeed, high numbers of sputum neutrophils, but not eosinophils, are correlated with an accelerated decline in FEV1 over time (110). In a cohort of severe asthmatics, patients could be stratified by IL-13 level in the bronchoalveolar lavage (BAL). Patients with high IL-13 were associated with significantly lower lung function, increased production of other type 2 cytokines (IL-5, IL-4) in the lavage fluid, and a higher number of neutrophils but not eosinophils (108). These results were seen to positively correlate with the abundance of pathogenic bacteria (*Haemophilus* spp., *Streptococcus* sp., *Moraxella catarrhalis*) (108). Therefore, the recruitment and activation of neutrophils may occur in the context of bacterial infection—but they may act to exacerbate disease further.

In one study of ~400 severe asthmatics, patients with high extracellular DNA (eDNA) were found to have significantly worse asthma control and chronic bronchitis compared with patients

with low eDNA (109, 112). This neutrophilic extracellular DNA originates from NETs—a form of suicidal cell death initiated by stimuli such as parasites, viruses, bacteria, and fungi. Indeed, the defense mechanism of these web-like extrusions appears to be to physically trap pathogens and bring them into contact with the numerous antimicrobial granular proteins of neutrophils that decorate the extruded DNA, including myeloperoxidase, neutrophil elastase, and cathelicidins (113). However, in the context of an airway, these can also have physicochemical consequences on the mucus. NETs from phorbol myristate acetate-stimulated neutrophils increase the viscoelasticity of human mucus collected from endotracheal tubes, an effect that was reversed by treatment with dornase alfa (to degrade DNA) or dimethylthiourea (to prevent oxidative damage) (83).

NETs can further potentiate inflammation by the inducing IL-8 and IL-6 from the airway epithelium (109). NETs have been shown to directly exacerbate both the development and exacerbation of asthmatic inflammation. Prior induction of a distinct population of CXCR4^{hi} neutrophils can promote allergic asthma in a mouse model (114). These neutrophils, induced by a low dose of endotoxin, influenza, or ozone are prone to NETosis, providing an inflammatory environment in which HDM-induced type 2 inflammation is exacerbated. As well as contributing to the development of allergic inflammation, neutrophils are well characterized in asthmatic exacerbations. In a study of 126 exacerbations in 63 asthmatic patients, the majority were found to be neutrophilic rather than eosinophilic (115), confirming earlier studies of sputum cell numbers in exacerbations of severe asthmatics (116). Experimental infection of asthmatic patients and healthy controls with rhinoviruses demonstrated higher levels of NETs in asthmatic patients, an effect that also was unexpectedly associated with an aggravated type 2 response marked by higher levels of IL-4, IL-5, and IL-13 (117). This outcome was reversed by administration of DNase or NET inhibitors, demonstrating an important link between NETs and heightened type 2 disease (**Figure 2**). Patients with high bacterial load in their sputum were also more likely to have increased neutrophils, associated with higher levels of IL-1 β , IL-8, IL-12, IL-17A, and tumor necrosis factor alpha (TNF α) (118).

The presence of CLCs in mucus plugs is also a likely candidate to drive neutrophilic inflammation, as in mouse models it has been shown that the instillation of CLCs results in the recruitment of neutrophils and Ly6C^{hi} monocytes (**Figure 2**) (99). In human samples, stimulation of nasal epithelial cells with CLCs resulted in the translocation of neutrophils *ex vivo*, accompanied by the production of IL-1 β , TNF α , IL-6, and granulocyte-macrophage colony-stimulating factor (119). NETosis was also observed in human neutrophils following exposure to CLCs (119).

Therefore, the presence and role of neutrophil traps may have slipped through the net in type 2 diseases, particularly in exacerbations or in the less-sampled, more-peripheral regions of the lung. In diseases such as cystic fibrosis, where NETs are a well-established feature of sputum, treatments such as dornase alfa show excellent efficacy in improving lung function and reducing exacerbation frequency (120). However, there is a paucity of evidence for the role of this drug in asthma, and clinical studies are lacking (121, 122). However, in certain stratified patients, perhaps with frequent exacerbations, this treatment may be beneficial. It can be seen by high-resolution computed tomography (HRCT) scans that asthmatic patients with high levels of neutrophils in the sputum have increased bronchial wall thickening and mucus plugs relative to CF patients with high levels of neutrophils in the sputum (123).

At the site of mucus plugging, it is relevant to note that we may be underestimating the role of neutrophils by analysis of these cells in the sputum alone. Several studies have used bronchial biopsies to demonstrate that sputum neutrophilia underestimates the number of neutrophils present in the lower airways (124–126). While sputum eosinophilia and neutrophilia are seen to positively correlate in samplings of BAL or biopsies from asthmatic patients, this becomes a negative correlation in sputum of the same patients (125). By mapping the respiratory tracts of 20 fatal

cases of asthma, de Magalhães Simões et al. (126) showed that neutrophils only become evident in the lower airways, from the peribronchiolar parenchyma and distal alveolar parenchyma outward. Furthermore, in a comparison of nocturnal asthma (in which symptoms are worse at night) versus nonnocturnal asthmatics, neutrophils could be detected only in the alveolar but not in epithelial tissue, concurrently with eosinophils, indicating their recruitment in deep lung tissue (127). Indeed, half of the sudden-onset fatal asthma cases from this study showed a predominance of neutrophils over eosinophils in the lower airways (126). Furthermore, if neutrophils undergo NETosis or cell death in the airways, their influence becomes even harder to detect.

Moreover, there is evidence that the local and systemic inflammatory environment of asthma primes neutrophils for a more potent response. Neutrophils exposed to the BAL of asthmatic children with neutrophil-high asthma were seen to have a higher phagocytic index and to be more prone to NETosis but produce a reduced oxidative burst (128). These neutrophils also showed increased surface expression of CD11b and CD16. Neutrophils derived from the blood of severe asthmatics, but not healthy controls, induced transforming growth factor beta in a culture of normal bronchial epithelial cells, indicating a profibrotic phenotype for these cells (129).

CONSEQUENCES OF MUCUS PLUGGING ON THE CHRONICITY AND NATURAL HISTORY OF ASTHMA

While we have thus far considered the detrimental contribution of mucus following allergen or immune stimulation, an established mucus plug has the potential to be immunogenic even in the absence of stimulus, perpetuating disease even if the initial trigger for inflammation and mucus production has long disappeared. Static mucus or a plugged airway provides a perfect environment for bacterial colonization and infection, rendering the host more susceptible to infections and exacerbations, which would result in further inflammation and mucus production, perpetuating the cycle. Severe asthmatics stratified by IL-13 production have been shown to have lower FEV1, which correlates with the presence of pathogenic bacteria, indicating a link between mucus induction, bacterial infection, and poor lung function (108). HRCT scans have demonstrated that, relative to their nonexacerbated state, asthmatics have increased airway narrowing and mucus occlusions during exacerbations (130). This is particularly evident in the bronchi at lower airway generations (130). It is thus possible that mucus plugs grow with every exacerbation and that the presence of a plug, with its accumulation of crystals, extracellular DNA, dead cells, fibrin debris, and tenacious mucus, represents a hot spot for exacerbation-induced inflammation, although more research is needed to substantiate this idea.

Even the physical nature of a plug could promote development of type 2 inflammation. The beta-epithelial sodium channel (β -ENaC) transgenic mouse was originally developed to model the overproduction of mucus in diseases such as cystic fibrosis, as overexpression of the β subunit of ENaC results in an increase in airway Na^+ absorption and reduced mucus clearance. Indeed, this alteration results in early mortality in young (1-week-old) mice, associated with more than 75% obstruction of the larynx by mucus, local hypoxia, epithelial necrosis, and an inflammatory environment associated with neutrophil influx. Interestingly, in surviving mice, type 2 inflammation was seen in the lungs from 2 weeks onward, with strong eosinophil recruitment and production of IL-13. Importantly, this work conceptually demonstrates the immunological consequences of mucus overproduction and the ability of luminal obstruction to drive type 2 inflammation (131).

In the lungs of these juvenile mice, where the airways are already polarized toward a type 2 environment with excessive mucus production (similar to the lungs of human asthmatics), allergen challenge (in the form of *A. fumigatus* or HDM) aggravates this phenotype, and increased uptake of allergen is seen by conventional DCs resulting in more pronounced allergic inflammation, as

measured by eosinophil influx and BHR. Importantly, these effects were reversed by inhibition of STAT6 and rehydration of the airway surface by use of amiloride (132).

Furthermore, as long as static mucus persists in the lungs of humans, it will act as a trap for other inhaled stimuli, such as pollutants, cigarette smoke, viruses, and bacteria, which in turn can trigger local inflammation in the airways (**Figure 2**). The activation and phagocytic capacity of macrophages have also been shown to be altered in an elastic mucin hydrogel system compared with standard tissue culture conditions (133), becoming more proinflammatory with increasing gel stiffness (134). Neutrophils are also more prone to activation and NETosis on stiffer surfaces (135, 136). Mucins themselves may also have direct immunomodulatory effects. The acute upregulation of *MUC5AC* is seen in viral exacerbations of COPD, and in a study by Singanayagam et al. (137) it was shown that exogenous administration of *MUC5AC* in the context of a murine RSV infection exacerbated neutrophil recruitment and inflammatory cytokine production. The presence of CLC crystals inside a mucus plug might be an intense, chronic stimulus for epithelial cells that lays down the basis for a hyper-type 2 niche around the airways. In this peribronchial niche, we predict that DCs communicate with resident memory Th2 cells, ILC2 cells, and antigen-specific B cells. Until we find ways to intervene with mucus plugging in patients, the existence of such a niche will remain speculative.

CONCLUSIONS AND OUTLOOKS

While there are clearly many challenges in retrieving and understanding the biology of mucus plugs in type 2 diseases, it is time that we faced these challenges and devoted attention to understanding this unique facet of the asthma syndrome, so that we can make progress toward developing treatments. With our clinical colleagues, we need to develop clinically applicable scoring systems on the basis of dynamic, high-resolution CT scans and other advanced imaging modalities that can pinpoint ventilation heterogeneity, perhaps supplemented with invasive bronchoscopic procedures allowing us to access mucus plugs. Sputum biomarkers are not easy to implement in daily clinical practice, so the development of biomarkers that are strongly associated with the presence of plugs would be very helpful. Serum galectin-10 is one potentially valuable marker that could point to the presence of a crystal-rich plug. Additionally, with the knowledge of airway narrowing and occlusion in even mild asthma, we should endeavor to develop a more effective delivery of therapeutics to diseased areas of the lung, which may in fact be harder to reach and assess.

While biologicals are clearly an improvement on previous therapies for asthma, there are still many improvements that can be made, and we are still learning their strengths and limitations. It has not been investigated, through prolonged use of biologicals, whether the endotypes of asthma will change and whether treating eosinophilic inflammation will result in a shift to neutrophilic inflammation. We also argue that as long as mucus plugs remain in the airways, they will perpetuate exacerbations and local inflammation in the lung, interfering with the eradication of asthmatic symptoms.

Perhaps this situation is unsurprising when we consider the multifactorial, heterogeneous nature of a disease such as asthma. While mucolytics alone may not be a wholly effective form of treatment for diseases such as asthma, they should be considered in combination with current biologics to deliver the most effective, all-encompassing solution to the clinical features of airway diseases. The current focus on biologics as a treatment in asthma arises from the great deal that is known about the role of immune cells and cytokines in this disease, and to develop new treatments directed toward mucus plugs, a great deal of research needs to be focused on the consequences of chronic mucus plugs in patients. While inflammation is clearly a very important facet

of allergic inflammation, an applicable phrase to the state of current asthma research is “When the only tool you have is a hammer, everything starts to look like a nail.” We should consider expanding our toolbox to consider not only the inflammatory aspect of asthma but also the important contribution of mucus plugs—but not to the degree that we throw a spanner in the works.

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