Asthma (M Jutel, Section Editor)

Non-eosinophilic Asthma Endotypes

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Opinion statement

Several recent studies spanning from induced sputum to gene signature identified non-eosinophilic asthma (NEA) as a distinct asthma phenotype with several relevant clinical features such as increased asthma severity, increased remodelling and lower response to bronchodilator and anti-inflammatory treatment. Two major mechanisms leading to neutrophilic inflammation are postulated: the dysregulated innate immune response, including neutrophil intrinsic abnormalities, and the activation of the IL-17-dependent pathway. Several factors such as age, metabolic or epigenetic factors or the activation of the epithelial-mesenchymal trophic unit have been identified as modulators. The endotyping of NEA is far behind the eosinophilic asthma, and until now, no endotype-driven interventions have been proved to be effective.

Non-eosinophilic asthma as a distinct asthma phenotype

Using induced sputum, four distinct inflammatory subtypes of asthma were described: eosinophilic, neutrophilic, paucigranulocytic and mixed granulocytic [1•]. Studies in large cohorts, such as the SARP (Severe Asthma Research Program) cohort, identified neutrophilic inflammation through induced sputum as an important hallmark of a distinct cluster of patients with moderate to severe asthma [2]. More recent studies evaluating the molecular signature of the Th2 genes [3] or using unsupervised hierarchical clustering of gene expression profiles in asthma [4] reinforce the reality of non-eosinophilic asthma (NEA).

Based on data from Th2 high/low molecular signature studies, the incidence of adult NEA reaches 30–50 % [3]. In the Asthma Clinical Research Network, 47 % subjects with mild to moderate asthma were persistently non-eosinophilic [5]. In addition, almost half of the subjects with mild asthma exacerbations had no sputum eosinophilia [6]. In children, data are scarce and controversial, and the stability of the non-eosinophilic pattern has been challenged [7]. Older studies showed that the pattern of inflammatory phenotypes differs between adults and children, with eosinophilic inflammation being more prevalent in both acute and stable childhood asthma, and neutrophilic inflammation being the dominant pattern of acute asthma in adults [8]. Latter studies report incidence of NEA in 30 % of steroid-naive asthmatic children and in 37.5 % of children with

severe-persistent asthma is [9, 10]. In obese children with asthma the incidence of NEA was significantly higher only amongst females [11].

There are several important clinical features that distinguish eosinophilic from NEA. An observational cross-sectional study on adults with asthma compared lung function, asthma control, exacerbation frequency and the type of co-morbidities between eosinophilic, neutrophilic and paucigranulocytic asthma. Pre- and post-bronchodilator FEV1 percentage predicted was highest in paucigranulocytic asthma and lowest in neutrophilic and eosinophilic asthma, but forced inspiratory flow (FIF50) tended to be lowest in neutrophilic asthma with a significant inverse correlation between sputum neutrophils and FIF50 percentage predicted. Patients with neutrophilic asthma had a significant lower bronchodilator response compared with eosinophilic asthma, while those with paucigranulocytic asthma tended to have less responsive airways. Patients with eosinophilic asthma had the poorest asthma control. The frequencies of severe exacerbations were similar across inflammatory subtypes; however, patients with paucigranulocytic and neutrophilic asthma had more than double the amount of primary care visits for uncontrolled asthma in the past year. There was no difference in smoking histories between patients of different inflammatory subtypes, and no difference in FEV1/FVC ratio; thus, smoking history or the presence of mild or co-existing COPD is not a distinguishing feature of neutrophilic asthma [12•]. Both in children and adults, sputum neutrophils correlated with asthma severity and with the extent of remodelling [10, 13]. In addition, most of the observations describe NEA as less responsive to currently available anti-inflammatory therapy [3, 5, 10, 14].

The concept of asthma complex endotypes

Asthma is a heterogeneous syndrome that covers a broad spectrum of complex genetic, inflammatory, metabolic and remodelling networks that lead to several pathogenetic pathways. Classifying asthma based on underlying pathophysiologic mechanisms, known as endotyping, offers a stratified approach for a better assessment of disease epidemiology, genetic background and environmental risk factors, for the development of new targeted therapies for asthma and for a better selection of responders to targeted treatment [15, 16, 17•]. Single molecular mechanism-linked endotypes can be defined for asthma; however, most asthma endotypes involve concomitantly several pathways. Examples of such complex endotypes are Th2 inflammation or aspirin-intolerant asthma [18••].

The "Holy Grail" of asthma endotyping is to pinpoint the essential nodes of the network that are unique for one endotype and relate to relevant clinical endpoints such as disease severity or response to treatment. For Th2-asthma endotype, three such nodes can be described, each depicting a distinct subendotype: the IL-5 pathway, the IL-4/IL-13 pathway and the IgE pathway [18••].

Animal models for non-eosinophilic asthma

Aligning mouse models of asthma to human endotypes of disease is essential for describing better the underlying networks of various asthma endotypes. In the quest to describe the mechanisms of NEA, several mouse models have been developed that allow the study of airway hyperreactivity (AHR), extensive remodelling, Th17-driven inflammation, association with obesity or corticosteroid unresponsiveness [19–22].

Another animal model for neutrophilic asthma is the recurrent airway obstruction (RAO) or heaves occurring in horses. RAO is a common,

performance-limiting, equine respiratory disease triggered by dust components such as endotoxin with clinical signs and pathophysiological similarities to human asthma, characterised by bronchospasm, neutrophilic infiltration and increased mucus in the airways. Neutrophils accumulate in the airways and their chemotaxis is regulated via MAPK and PI3K pathways [23]. In addition, expression of IL1β, IL8, TLR4, TNFα, TGFβ1 and NFkβ transcripts is significantly up-regulated and is correlated with clinical features [24].

Neutrophilic inflammation in asthma: triggers, mechanisms and potential subendotypes

The mechanisms driving a neutrophilic predominant inflammation in asthma are not fully elucidated.

Potential triggers of neutrophilic inflammation include infection, gastro-esophageal reflux disease (GERD) with aspiration and rhinosinusitis. In the same observational study mentioned above [12•], the frequency of chest infections and the incidence of chronic rhinosinusitis and of GERD were significantly higher in patients with neutrophilic asthma. In another trial including subjects with stable asthma, a significant load of potentially pathogenic bacteria was cultured from the sputum and was associated with higher total cell counts, proportion and number of neutrophils, sputum IL-8 and 8-isoprostane concentrations [25]. Bacterial components in indoor dust, such as nanometre-sized extracellular vesicles, can evoke neutrophilic inflammation as well. In a mouse model of asthma, repeated intranasal application of indoor dust induced neutrophilic pulmonary inflammation accompanied by lung infiltration with both Th1 and Th17 cells [26].

Air pollution may also be a prominent trigger of neutrophilic bronchitis. In a Canadian retrospective study, patients with the highest sputum neutrophils were living within 1000 m of highways [27].

Usually, significantly higher doses of inhaled corticosteroids (ICS) are needed to control neutrophilic asthma. ICS are reported to increase neutrophil survival and therefore may have a direct influence on sputum neutrophil proportions.

Two major pathways seem to be involved in the pathogenesis of neutrophilic asthma: an altered innate immune response and the activation of Th17 specific immune response. Each of these pathways is further modulated by age and by interaction with external triggers such as infections or pollution and by metabolic or epigenetic factors. The input from the resident cells, especially in the remodelled status, is further fine-tuning the neutrophilic inflammatory response. In contrast to Th2 high asthma, the translation into specific disease subendotypes has not been done yet since there are very few targeted interventions for neutrophilic asthma, none of them with a significant effect in large clinical trials.

A. The dysregulated innate immune response in neutrophilic asthma

Growing evidence supports a role for a dysregulated innate immune response in neutrophilic asthma, with altered gene expression of Toll-like receptors (TLR) and increased expression of genes from the IL-1β and TNF-α/nuclear factor-κB

pathways [28], phenotype switch and enhanced neutrophil chemotaxis and survival in the airways, impairment in anti-inflammatory mechanisms and in pathogen recognition and destruction processes and increased protease activity.

A1 The IL-1β and TNF-α/nuclear factor-κB pathway

The recently described six-gene signature that distinguishes between eosinophilic and non-eosinophilic asthma includes three genes involved in innate immune response that all are associated with NEA: IL-1β, alkaline phosphatase, tissue non-specific isozyme (ALPL) and chemokine (C-X-C motif) receptor 2 (CXCR2) [29•].

In an experimental model of Th1/neutrophil-predominant asthma, TNFα reduced the responsiveness to steroids and neutralising TNFα using etanercept restored glucocorticoid sensitivity [30]. Recently, the tumour necrosis factor superfamily member, LIGHT, has been recognised as a key mediator in severe asthmatic airway inflammation. Human bronchial epithelial cells (BEC) express one of the receptors for LIGHT, the lymphotoxin β receptor (LTβR). Stimulation of BEC with LIGHT induced IL-6, oncostatin M, MCP-1, growth-regulated protein α and IL-8.

Specific siRNA for LTβR attenuated IL-6 and IL-8 production. LIGHT activated intracellular signalling, such as mitogen-activated protein kinase and nuclear factor-κB (NF-κB) signalling [31].

A2 Neutrophil abnormalities

Neutrophils are important in innate immunity, and their numbers are increased in the airways in NEA. The activity of neutrophils differs in relation with the predominant inflammatory asthma phenotype. In NEA, blood neutrophils release significantly higher levels of interleukin-8 at rest. Cytokine gene expression and sputum neutrophil protein production did not differ between asthma subtypes. However, microarrays demonstrated expression profiles for NEA that were significantly distinct from eosinophilic asthma. A total of 317 genes were significantly altered in resting neutrophils from NEA, including genes related to cell motility and regulation of apoptosis supporting further the enhancement of blood neutrophil chemotaxis and survival as a major mechanism of NEA [32]. Persistent elevation of airway neutrophils due to impaired efferocytosis was also described. Galectin-3 (gal-3), a member of the ß-galactoside-binding animal lectins, involved in the recruitment, activation and removal of neutrophils, is significantly reduced in neutrophilic asthma compared with eosinophilic and paucigranulocytic asthma. In addition, patients with neutrophilic asthma have impairment in anti-inflammatory ratio of gal-3/gal-3-binding protein and IL-1RA/IL-1β [33].

Furthermore, neutrophils can switch to an N2 phenotype, contributing to the pool of IL-13, which in addition to IL-4, promotes activation of macrophages limiting further injury and promoting repair [34••].

A3 Other mechanisms

Altered pathogen recognition and destruction processes are present with macrophage efferocytosis impaired in NEA to a similar degree as in COPD [35]. Proteases are important mediators of inflammation, cytokine activation and

tissue remodelling. Subjects with eosinophilic asthma had significantly high levels of active MMP-9 compared with those with neutrophilic asthma. Although, there are high levels of total MMP-9 in neutrophilic asthma, most of it is inactivated and bound to the tissue inhibitor of MMP. On the other hand, in neutrophilic asthma, there is increased activity of neutrophil elastase (NE), in direct correlation with the levels of interleukin-8 and neutrophils [36].

B. The IL-17 pathway

IL-17, through the induction of cytokines and chemokines, is the major driving force for the recruitment and activation of neutrophils. IL-17 is mainly secreted by Th17 cells, a distinct CD4T helper cell subset, characterised by the specific expression of the transcription factor retinoic acid-related orphan receptor-γt (RORγt) [37]. By secreting IL-17, Th17 cells orchestrate the recruitment of neutrophil granulocytes in the lungs and their activation directly through CXCL8 production or indirectly by inducing the production of IL-6, G-CSF and GM-CSF, IL-8, CXCL1 and CXCL5 by airway epithelial cells. Although Th17 cells represent a core source of IL-17, a newly identified population of innate lymphoid cells (ILCs) is also able to produce IL-17 [37, 38]. IL-17-producing ILC3s express CC chemokine receptor 6, produce IL-17 and sometimes IL-22, and are central to the development of asthma in obese mice. Increased numbers of this subset of cells have been found in the bronchoalveolar lavage (BAL) fluid of obese individuals with severe asthma [39].

B1. IL-17 and neutrophilic asthma

A likely role of IL-17 in neutrophilic asthma is suggested by the significant association between IL-17 and other diseases with underlying neutrophilic inflammation, such as psoriasis, and by the efficiency of IL-17-targeted therapies in these diseases [40]. The link between neutrophilic inflammation and Th17 immunity is well established in mouse models of asthma, where it has been linked to the development of AHR and remodelling [41, 42]. Human data are also accumulating; however, a cause and effect relationship between IL-17 production and neutrophilic asthma has not been demonstrated. A strong correlation between IL-17 and neutrophils was established in induced sputum and blood [43, 44]. IL-17 has been linked to remodelling, AHR asthma severity and inflammation [44–47]. In the Clinical Asthma Research Association cohort study including steroid-naive asthmatic children and healthy controls, patients with non-allergic asthma showed very distinctly IL-17-shifted proinflammatory immunity, promoting neutrophil inflammation and less functional Treg cells. In parallel, expressions of anti-inflammatory IL-37, proline-serine-threonine phosphatase-interacting protein 2, the neutrophil-associated genes CD93, triggering receptor expressed on myeloid cells 1, and regulator of G-protein signalling 13 were increased in patients [48]. The IL-17 pathway was also related to decreased corticosteroid sensitivity, also a characteristic of neutrophilic asthma [49].

B2. Co-factors for IL-17 induced neutrophilic inflammation

A mixed Th17/Th2 activation is frequently observed in asthma. The relationship between IL-17 and Th2-type immunity is highly complex. Th2 cells can differentiate into dual-positive Th2/Th17 cells [50] and these cells were identified in

the BAL fluid of asthmatic patients [51]. The subgroup of asthmatic patients with dual-positive Th2/Th17 cells had glucocorticoid resistance in vitro and had the greatest airway obstruction and hyperreactivity [51•]. An interesting model suggests that IL-17 produced by γδ T cells and by Th17 cells in response to injured epithelium enhances the production of IL-4 and IL-13 from ILC2 and Th2 cells, which acts as a negative feedback loop shutting down further IL-17 production and neutrophilia [52]. Conversely, IL-4 and Il-13 may amplify Th17 responses by up-regulating CD209a expression on dendritic cells [53]. When the type-2 response fails to fully suppress the IL-17 response, or further promotes it, excessive tissue damage can occur. Understanding what regulates the IL-17–IL-4/13 feedback loops will provide invaluable insight into situations in which IL-17 exacerbates type-2 inflammation. Chitinases and chitinase-like proteins (CLPs) may be an important link between type-2 immunity and IL-17. Acting as danger-associated molecular patterns, CLPs release IL-1 with IL-17 production by innate γδ T cells, which in turn drives a type-2 response. Subsequently, type-2 cytokines induce even more CLP expression, which likely further contribute to repair. Additionally, CLPs can promote Th2 differentiation and suppress IFNγ, further promoting type-2 immunity [54].

IL-33 is another co-factor that cooperates with IL-17A to exacerbate AHR by enhancing neutrophilic inflammation via CXCR2 signalling. Furthermore, CXCR2 signalling derived Th2 responses are enhanced [55].

C. Modulators of the non-eosinophilic asthma endotypes

C1. Immune factors

The innate immune response has a critical role in the development of a specific immune response. For example, TLR4 expression by haematopoietic cells is critical for neutrophilic airway inflammation following LPS exposure and for Th17-driven neutrophilic responses to the house dust mite lysates and ovalbumin, while TLR4 expression by airway epithelial cells was found to be important for robust eosinophilic airway inflammation following sensitization and challenge with these same allergens [56].

An imbalance in Th17/Treg in peripheral blood was reported in NEA adult patients undergoing ICS treatment [57] in association with increased severity of the disease. The same observation was reported for paediatric asthma [48].

A subgroup NEA responds to inhaled steroids. An experimental model suggested that non-eosinophilic asthma associated predominantly with Th1/Tc1 cells is susceptible to glucocorticoid treatment and has no goblet cell metaplasia [58].

C2. Epigenetic factors

MicroRNAs (miRs) are small noncoding RNAs that regulate the function of immune cells by controlling mRNA stability and translation. MiR-9 expression is increased in sputum of patients with neutrophilic but not those with eosinophilic asthma. Exposure of pulmonary macrophages to IFN-γ/LPS synergistically induced miR-9 expression and reduced levels of its target transcript, protein phosphatase 2 regulatory subunit B (B56) δ isoform. Increased miR-9 attenuated PP2A activity and inhibited the nuclear translocation of the glucocorticoid receptor (GR). Inhibition of miR-9 increased both PP2A activity and

GR nuclear translocation in macrophages and restored steroid sensitivity in multiple models of steroid-resistant AHR [59].

Airway smooth muscle (ASM) cells isolated from asthmatic individuals secrete more CXCL8 than cells from non-asthmatic individuals. CXCL8 is a CXC chemokine that drives steroid-resistant neutrophilic airway inflammation. CXCL8 increased secretion is due to increased histone H3 acetylation and increased binding of histone acetyltransferase to the CXCL8 promoter complex. The epigenetic defect can be modulated by Brd inhibitors that may provide a new therapeutic strategy for steroid-resistant inflammation [60].

C3. Ageing and non-eosinophilic asthma

A significant high incidence of asthma severity was reported for elderly patients [61]. Airway neutrophilia is related to age in adults, with a neutrophilic asthma phenotype present in older adults [62•].

Therapeutic implications

Correct identification and correction of co-morbidities such as infections, CRS and GERD might prove successful as a first step to control non-eosinophilic asthma. Reversal of steroid resistance is also a reasonable approach for severe cases. Targeting remodelling or switching-off the secretory phenotype of ASM is still experimental.

In the AZISAST study azithromycin did not reduce the rate of severe exacerbations and lower respiratory tract infections in all patients with severe asthma but had a significant effect in subjects with non-eosinophilic severe asthma defined by blood eosinophilia ≤200/μl [63]. A recent meta-analysis of 18 trials and 1306 asthmatics treated with macrolides showed differential effects according to race: macrolides increased FEV1 and PEF in Caucasians and Asians, decreased AHR in Caucasian, and improved cell counts of sputum amongst Asians. There was no effect on symptom scores or quality of life [64]. The longitudinal effect of macrolides on lung microbioma was evaluated in adult patients with moderate/severe asthma before and after 6 weeks of daily 250 mg azithromycin therapy. Azithromycin decreased bacterial richness in the airways and altered the airway microbiota leading to *Anaerococcus* becoming dominant within the bacterial community, while *Pseudomonas, Haemophilus* and *Staphylococcus* (three pathogenic genera associated with airway disease) were all reduced. In addition, azithromycin decreased mucus secretion and airway neutrophil accumulation as well as specific antibiotic and antipseudomonal activity [65•]. Macrolide antibiotics have been reported to be effective in the treatment of the neutrophilic asthma for reasons unrelated to their antibacterial action [66]. This has resulted in research activities aimed at gaining a better understanding of the immunomodulatory actions of macrolides and the synthesis of various novel anti-inflammatory macrolides without antimicrobial activity. Further work, including proteomic, genomic and microbiome studies, will advance our knowledge on the macrolide-responsive non-eosinophilic asthma endotype.

None of the targeted interventions for non-eosinophilic asthma proved successful in large clinical trials. One might argue that patient selections was not ideal and certainly did not follow the endotype-driven classification like it

was done for Th2 asthma.

There is only one trial in asthma investigating the anti IL-17 pathway inhibition with brodalumab in subjects with inadequately controlled moderate to severe asthma taking regular inhaled corticosteroids. Inhibition of IL-17 receptor A did not produce a treatment effect in the over-all group. In the high-reversibility subgroup (post-bronchodilator FEV1 improvement ≥20 %) there was a significant ACQ improvement only in the low-dose group, but the effect was not observed in the higher-dose group, so the effect is of uncertain clinical significance [67•].

Strategies using small molecule antagonists against the interleukin-8 receptor, CXCR2, are able to reduce airway neutrophilia, and proved clinical efficacy in small proof-of-concept trials [68] but failed in large phase III clinical trials. A new series of monocyclic CXCR2 antagonists with improved solubility and good pharmacokinetic profiles is under investigation [69].

Conclusion

The progress in the endotyping of non-eosinophilic asthma is well behind the eosinophilic asthma. Several small steps have been taken but no clear subendotypes can be pinpointed. Until results from the large clinical trials with targeted endotype-driven interventions become available, the reliable identification and reduction of potential triggers such as infections, CRS, GERD or pollution exposure remains the recommended approach for non-eosinophilic asthma.

Compliance with Ethics Guidelines

Conflict of Interest
Ioana Agache declares that she has no conflict of interest.

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by the author.

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