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Chapter

Eosinophilic Phenotype: The Lesson from Research Models to Severe Asthma

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Abstract

Eosinophilic airway inflammation is a hallmark in the pathophysiological and clinical definition of asthma. In the last decades, asthma evolved in the recognition of different phenotypes identified by natural history, clinical and physiological characteristics, and the underlying immune mechanisms. Among these phenotypes, many have been associated with eosinophilic-driven inflammation. This is the case of either early-onset allergic Th2 asthma or late-onset persistent eosinophilic asthma. Both animal models and analysis from human samples have contributed to elucidate the role of eosinophils in the asthmatic inflammatory response and the synergic role of Th2 cytokines. In severe asthma, high numbers of eosinophils can persist despite treatment with inhaled and oral corticosteroids leading to the definition of severe refractory eosinophilic asthma. The combined role of IL-4-, IL-13- and IL-5-associated pathways has focused the view over the T2-type endotypes, wherein a specific biological pathway explains the observable properties of different phenotypes and the identifiable biomarkers can predict response to monoclonal antibodies directed against a selected immune target. In the era of precision medicine and personalized therapy, both the identification of Th2 molecules and eosinophils as targets and biomarkers have become the best clue for treating and monitoring severe asthma.

Keywords: severe asthma, eosinophilic phenotypes, T2-type inflammation, eosinophilic refractory asthma, anti-IL5 treatment

1. Introduction

Asthma and chronic rhinosinusitis (CRS) are chronic inflammatory disorders involving the lower and upper airways. According to the definition by Global Initiative for Asthma (GINA) documents, asthma is a heterogeneous disease characterized by chronic airway inflammation associated with a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough and evidence of variable expiratory airflow limitation [1]. Airway inflammation is usually present and persists even when symptoms are absent or lung function is normal.

In the last decades, the role of chronic airway inflammation has been central in the definition of asthma that was recognized as a chronic inflammatory

disorder in which many cells and cellular mediators play a role and result in the characteristic pathophysiological changes [2]. The inflammation involves all the airways from the main bronchi to the peripheral small airways. A characteristic pattern of inflammation has been described in asthma involving inflammatory cells mainly mast cells, eosinophils, T lymphocytes, dendritic cells, macrophages and neutrophils, which release mediators that induce symptoms. Both animal models and analysis from human samples have contributed to elucidate the type of inflammation involved in asthma [3]. The most common phenotype of asthma is characterized by eosinophilic airway inflammation and the role of eosinophils as a key player in the pathophysiology of asthma is well documented. Eosinophils emerged as leading cells from the first post-mortem studies of asthmatic lungs, passing through the finding of increased in number and activation status of eosinophils in asthmatic airways [4] and of increased eosinophil surrogates as fractional exhaled nitric oxide (FENO) [5]. Nowadays, the focus is on the definition of the forms of uncontrolled or severe eosinophilic asthma in which airways, sputum and blood eosinophils are consistently increased and represent a biomarker of the eosinophilic endotype of asthma and a guide for biologic target therapies [6, 7].

2. Eosinophils and allergic asthma

Allergen challenge models have been conceived to reproduce many features of clinical asthma [8]. Actually, atopy, which is the production of allergen-specific IgE antibodies, is a predisposing factor for asthma development, and birth cohort studies have shown that sensitization to allergens such as house dust mite, cat and dog dander and Aspergillus is independent risk factors for wheezing in children [9]. Moreover, exposure to allergens is one of the most recognized environmental factors that trigger asthma symptoms. The term allergic asthma has been used to define the presence of sensitization to environmental allergens and the clinical correlation between exposure and symptoms, both indoor and outdoor allergens being well-known triggers of asthma exacerbations [10].

Both allergen challenged animal models of asthma and allergic asthma in humans are associated with a T-lymphocyte CD4+ Th2-polarized response as the main feature of airway inflammation. The allergic response is characterized by immediate and late inflammatory responses in which Th2 cells govern the inflammatory cell recruitment and activation by the release of the signature cytokines IL-4, IL-5 and IL-13 as well as IgE antibody synthesis.

2.1 Mouse models of allergic asthma

In acute allergen challenged mouse models of asthma, after the sensitization period (usually 14–21 days), the animal is challenged with the allergen via the airway and this causes many key features of clinical asthma. The analysis of bronchoalveolar lavage (BAL) and bioptic samples of airway walls has supported the hypothesis that asthma is a Th2-mediated disease. A dominating influx of eosinophils has been demonstrated and related to the development of AHR [11]. Moreover, the adoptive transfer of Th2 cells into recipient mice was able to reproduce airway eosinophilia, mucus hypersecretion and AHR after allergen inhalation [12].

However, some of these effects resulted in transient changes and do not involve structural changes. Through chronic allergen exposure in mice, allergen-dependent sensitization, Th2-dependent allergic inflammation, eosinophilic influx into the

airway mucosa, mucus overproduction and AHR have been reproduced [11, 13]. Generally, acute and chronically treated mice had similar early and late asthmatic responses; however, the acute model had higher levels of eosinophilia, whereas the chronic model showed hyperresponsiveness to lower doses of methacholine and had higher total IgE. On the other hand, many of the lesions observed in chronic human asthma, such as chronic inflammation of the airway wall and airway remodeling changes, are absent.

Moreover, transgenic mice that overexpress the Th2 cytokines—IL-4, IL-5, IL-13 and IL-9—in the airway epithelium exhibit the same inflammatory features. IL5 is a Th2 cytokine essential for differentiation, maturation and survival of eosinophils. A key role in allergen-induced inflammatory responses has been shown in murine IL-5-deficient model chronically challenged with an allergen in which the eosinophilia, lung damage and airway hyperreactivity were abolished. The reconstitution of IL-5 production using recombinant vaccinia virus that expressed IL-5 restored eosinophilia and airway dysfunction [14]. Using a clinically relevant model of chronic allergic asthma in mice, Kumar RK et al. showed that anti–IL-5 inhibited inflammation in terms of accumulation of eosinophils in the tracheal epithelium and inflammatory cells in the lamina propria, but had no effect on airway responsiveness to methacholine [15].

Many studies have demonstrated the significant role of IL/4IL-13 pathway in asthma. Through the agonization of IL-4R, both IL4 and IL13 activate a tyrosine kinase-dependent signal that after phosphorylation of STAT6 regulates the transcription of Th2-involved genes. Models of IL-4−/− mice were protected from the development of AHR and aspects of remodeling, while the administration of soluble IL-4 receptor reduced inflammation and mucus hypersecretion, but had no effect on AHR [8] Similarly, soluble IL-13 suppressed pulmonary inflammation but had a limited effect on AHR [15].

Limitations evidenced in mouse models are that inflammation is not restricted to the conducting airways, but extended to vascular and parenchymal parts of the lung; moreover, some of the clues of asthma inflammation such as the large increases in airway smooth muscle and MC infiltration are not generally observed.

2.2 Human models of allergic asthma

In humans, the role of Th2 cytokines and eosinophils in allergic asthma comes from many experimental data that in part differ from the mice models.

Sensitizations to environmental allergens in allergic subjects are documented by positive skin prick test reactions and elevated allergen-specific IgE serum levels. Activation of FcεRI on mast cells and basophils by allergen-bound IgE induces the release of preformed vasoactive mediators, which rapidly elicit edema of the bronchial mucosa, mucus production and smooth muscle constriction. This mechanism is confirmed by the increased numbers of cells expressing the high-affinity receptor for IgE (FcεRI) in allergic asthmatic tissues [16].

Biopsies from bronchial mucosa show CD4+ cell infiltrates and enhanced expression of Th2-type cytokines and chemokines. IL-4 and IL-5 mRNA were localized in activated T cells (CD3+), mast cells (tryptase +) and activated eosinophils (EG2+) both in BAL and bronchial biopsies from mild atopic asthmatic patients [17], and the number of activated CD4+ T cells and IL-5 mRNA positive cells is increased in asthmatic airways following antigen challenge. This skewed cytokine involvement is reflected by the expression of the transcriptional regulators GATA-3 (GATA binding protein 3) after segmental allergen challenge in asthmatics [18]. GATA-3 is a transcriptor factor that finds its binding site in the IL-5 promoter and induces Th2 cytokine gene expression

by biasing Th1/Th2 balance. The increase in GATA-3 expression in the asthmatic subjects correlated significantly with IL-5 expression and AHR [19]. In summary, CD4+ Th2 cells are believed to initiate and perpetuate the inflammatory response in allergic asthma.

IL-5 expression is increased 18–48 h after allergen challenge in BAL samples in mite-associated bronchial asthma when they were stimulated with Dermatophagoides farinae [20]. The levels of IL-5 mRNA-positive cells and IL-5 correlate with the number of eosinophils infiltrating the bronchial mucosa and BAL of asthmatic subjects, with pulmonary function and symptom severity [21]. Biopsies from the respiratory mucosa of allergic asthmatics show the enhanced expression of other Th2-type cytokines and chemokines such as IL-4, IL-6, IL-9, IL-10 and IL-13. Allergen challenge induces in patients with asthma IL13 and IL4 release in BAL and sputum eosinophils that positively correlate with IL-13 expression in asthmatic bronchial submucosa [22]. IL13 is thus involved in the regulation of allergen-induced late-phase inflammatory responses. IL-13, indeed, can modulate the production of IgE through the isotype class switching of B cells; therefore, it is involved in the early phase of allergic reactions.

2.3 Recruitment of eosinophils in allergic asthma

Eosinophils are recruited from progenitors after allergen exposure. Levels of Eo progenitors arise in the peripheral blood after seasonal allergen exposure, during controlled exacerbations of atopic asthma and after single allergen challenge to the airways in atopic asthmatics and animal models. Trafficking of these cells from the bone marrow, where they are produced, to the airways was also demonstrated. In fact, these CD34+ CD45+ progenitors express the IL-5 receptor alpha and are recruited by IL5 and GM-CSF produced in asthmatic airways, subsequently acquiring an activating form that reaches the inflamed airways [23]. Eosinophilopoiesis develops after 24 h from allergen challenges and is followed by the accumulation of eosinophils in the airways.

2.4 Eosinophils in different phases of allergic asthma

The sensitization phase is supposed to be determined by the differentiation of Th naive cells into Th2 lymphocytes. Dendritic cells (DCs) in response to allergen stimulation drive a Th2-oriented response. DC subsets have been described to respond to various stimuli coming from the inflammatory milieu generated after the allergenic encounter. Myeloid CD1c + DCs respond to thymic stromal lymphopoietin (TSLP) produced by the epithelium after allergen encounter by activating allergen-specific memory CD4+ cells [24]. Eosinophils also contribute to the initiation phase of Th2 response by suppressing the Th1/Th17 pathway.

The main role of eosinophils in asthmatic response is yet related to the effector phase of the inflammatory response. After allergen challenge, asthmatics generally develop immediate bronchoconstriction, the so-called early asthmatic response, which is maximized within 30 min and resolves between 1 and 3 h. A proportion of subjects develop a second, delayed bronchoconstrictor response, named the late asthmatic response, which is characterized by prolonged AHR and pronounced airway eosinophilia [25]. So it can be assumed that in isolated early responders a significant or sustained eosinophilic response does not develop. On the other hand, the so-called dual responders develop a sustained IL-5-dependent eosinophilic response in terms of both bone marrow recruitment and sputum accumulation. This response is accompanied by increases in circulating eosinophils, greater

increases of activated eosinophils in the airways, and the development of airway hyperresponsiveness [26].

Recruited eosinophils in the airways release a variety of toxic products, oxygen radicals, granule-associated cytotoxic proteins and membrane-derived proinflammatory mediators that damage the bronchial epithelium and increase AHR.

IL-5 is the most important constituent increasing eosinophil survival, recruitment, degranulation and lung injury following inhalation of antigen, as demonstrated in a segmental antigen lung challenge model [20], and the levels of eosinophils and their cationic proteins in the BAL fluid following allergen challenge correlate with the magnitude of the late phase response. Moreover, a positive correlation between the percentage of BAL eosinophils and the ECP was demonstrated at baseline but not after 4–6 h after allergen inhalation, thus suggesting that eosinophil recruitment and activation seem to follow different temporal kinetics [27].

The effect of IL-5 on eosinophils is demonstrated by the finding of increased expression of the alpha chain of IL-5R mRNA in the bronchial biopsies of atopic and nonatopic asthmatic subjects; the membrane-bound aIL-5R is coexpressed with EG2 in the eosinophils within the bronchial mucosa of asthmatics and inversely correlated with FEV1 [28].

2.5 Eosinophilic chemokines in allergic asthma

IL-5 acts as chemotactic factors for eosinophils, promoting eosinophil-endothelial adhesion by inducing the expression of VCAM-1 on endothelial cells. In turn, VCAM-1 may bind to integrins on the eosinophils leading to the migration of eosinophils to sites of airway inflammation. Blood eosinophils stimulated with IL-5 adhere to VCAM-1 via the integrins α4β1 and αMβ2 that are the major eosinophil integrin-mediating cell adhesion [29]. Eosinophils obtained from BAL after segmental antigen challenge have both β1 and β2 integrins in a high-activity conformation and adhere to VCAM-1 to a higher degree than blood eosinophils [30]. It seems, therefore, that blood eosinophils are primed by IL-5 or P-selectin (expressed by platelets) to an integrin activation status and are consequently arrested in vessels of inflamed bronchi and move into lung tissue. It is remarkable that the administration of anti–IL-5 can lower β2 integrin activation [31]. IL-5 not only has got the ability to prime eosinophils for subsequent activation but also enhances their survival at sites of allergic inflammation.

The role of other chemokines in allergic asthma is sustained by different pieces of evidence. Eotaxin and regulated on activation, normal T-cell expressed and secreted (RANTES) act on eosinophils inducing chemotaxis as well as specifically activation. In human challenges with the HDM allergen, the peak of eosinophils immunopositive for eotaxin, RANTES and IL-5 occurs at 7 h after allergen inhalation, but persisting eosinophilic airway inflammation and AHR remained for 7 days after allergen inhalation [32].

These chemokines are released by several cell types in the lung: endothelial cells, epithelial cells, fibroblasts, DCs and smooth muscle cells. Eotaxin creates a chemotactic gradient so that eosinophils pass the endothelium of the blood vessels and migrate to the site of inflammation [33]. Eotaxin has the potential to mobilize eosinophils and their progenitors from bone marrow and this effect is potentiating with that of IL5. Second, in atopic asthmatic patients, high concentrations of eotaxin in BAL fluid are detected as well as an increased expression of eotaxin mRNA and protein in the epithelium and submucosa of their airways. In the airways of allergic asthmatics, eotaxin is in sufficient concentrations to exert chemotactic activity on eosinophils in vitro and this effect is enhanced by IL-5 [34].

RANTES is also found in high concentrations in the sera in allergic asthma, as well as monocyte chemoattractant protein-1 and -3 (MCP). These chemokines play a role in ongoing lung inflammation, lung leukocyte infiltration, bronchial hyperresponsiveness and the recruitment of eosinophils.

Eotaxins and RANTES bind to the CCR3 receptor expressed on Th2 cells, eosinophils and basophils. Eosinophils in CCR3R knockout mice reach the blood vessels and the endothelium but fail to migrate into lung tissue. Indeed, these mice are protected from AHR after allergen challenges [35]. After antigen challenge, the percentage of CCR3+ eosinophils is downregulated on BAL eosinophils compared with peripheral blood eosinophils, while other chemokine receptors like CCR4, CCR9 and CXCR3 do not, being predominantly involved in activation of eosinophil effector responses [36].

The relationships between the levels of eosinophilic chemokines and AHR or bronchoconstriction are not documented in the same way. Some data suggest that mediators released by cells other than eosinophils, similar to MCs or basophils, can contribute to AHR. In addition, chemokine receptors might be involved in the activation of airway eosinophils for degranulation or prolonged survival. Even if antagonists derived from peptides and small molecules exist to block the chemokine receptor CCR3, the in vivo effect on airway inflammation is not sufficiently proved [33].

Once activated, eosinophils may produce effector molecules like eosinophil major basic protein and eosinophil-derived neurotoxin and degranulate at the site of injury contributing to tissue damage in the asthmatic lung. These molecules have cytotoxic effects on respiratory epithelium, facilitate the entry of other toxic molecules and trigger the degranulation of mast cells and basophils. In asthmatic airways, eosinophils also take part in respiratory-burst–oxidase reactions and generate large amounts of cysteinyl leukotrienes that contribute to increase vascular permeability, mucus secretion and smooth muscle contraction [37].

2.6 Local eosinophilopoiesis

It has been proposed that CD34+ IL-5Ra+ progenitors after mobilization from the BM during allergen challenge are able to undergo in situ differentiation at the site of allergic inflammation. Actually, CD34+45+IL-5Rα+ progenitors are increased in BAL in mouse models after allergen challenge and precede an increase in BAL eosinophils through a local differentiation via an IL-5-dependent mechanism [38]. Moreover, the CD34+ eosinophil committed pool is maintained within the airways via autocrine IL-5 release and IL-5-induced upregulation of IL-5R. CD34+/IL-5Rα mRNA+ cell number is increased in the airways of asthmatic subjects and related to asthma severity [39]. Surprisingly, eosinophilic precursors persist in the sputum of severe asthmatics that are prednisone resistant after anti-IL-5 treatment [40] and it has been documented that anti-CCR3 strategies do not suppress circulating and airway eosinophils in moderate-to-severe asthmatics. Consequently, it can be hypothesized that blocking local differentiation and expansion of CD34+/IL-5Rα+ cells may reduce eosinophilic inflammation in the airway in asthma.

2.7 Other mechanisms of eosinophil activity into allergic asthmatic airways

Allergic inflammation is locally perpetuated in the airway by the cross-talk between eosinophils and other resident cells. MCs are activated by MPB and stem cell factor (SCF), both released by eosinophils, contributing, by their direct effects on mast cells, to the perpetuation of allergic inflammation [41].

Eosinophils can also affect fibroblast properties, modulating the process of tissue remodeling. First, eosinophils are the main source in asthma of transforming

growth factor-beta (TGF-β) that induces proliferation and regulates fibroblast function as well as controls the production of proteins of the extracellular matrix (ECM). In turn, tumor necrosis factor-α (TNF-α) derived from mast cells enhances TGF-β synthesis from eosinophils as well as fibroblasts promote survival of MCs and eosinophils by releasing SCF and granulocyte–macrophage-colony stimulating factor (GM-CSF) [42]. Anti-IL-5 humanized monoclonal antibody has been shown to decrease the deposition of many ECM proteins such as collagen III in the RBM of mild atopic asthmatics as well as the number of eosinophils and the degree of TGF-α in the BAL fluid [43].

In addition, eosinophils express basic fibroblast growth factor (β-FGF) and VEGF in the submucosa of asthmatic subjects and release many pro-angiogenic cytokines such as IL-8, IL-6, TGF-β and GM-CSF.

The effect on T-cell immune modulation of eosinophils is more controversial. Cytokine produced by eosinophils may directly influence T-cell selection by DCs determining T-cell tolerance or activation. One example is the induction by IFN-γ of indoleamine 2,3-dioxygenase (IDO) in eosinophils that in turn converts tryptophan (TRYP) to kynurenine (KYN) inducing apoptosis in Th1 cells, while Th2 cells are spared from KYN-induced apoptosis by IL-4 [44].

3. Eosinophils in nonallergic asthma

The increase of the number of activated Th2 lymphocytes and eosinophils, as well as IL-5 levels, in both BAL fluid and bronchial biopsies from intrinsic asthmatics, has been extensively reported [45]. No difference between atopic and intrinsic asthmatics have been observed in studies examining the expression of high-affinity IgE receptor, IL-5 and IL-4 mRNA and protein expression in bronchial biopsies [16]. Actually, total serum IgE levels have been noted to be increased in the serum of patients with intrinsic asthma. This reflects the increases in Iå and Cå RNA+ cells in the bronchial mucosa and provides evidence for a local IgE synthesis even in the absence of a known antigen or allergen trigger.

Eosinophilic infiltration in nonallergic asthma can be even much more than in allergic asthma and this fact is reflected by the finding of a larger amount of RANTES in the bronchoalveolar lavage fluid of patients with nonallergic asthma compared with patients with allergic asthma [46].

Attempts to differentiate the inflammatory cascade between allergic and nonallergic asthma have proposed a different signal in the Th2 pathway of nonallergic asthma attributed to reduced signal transducer and activator of transcription 6 (STAT6) expression and consequently reduced IL-4R signaling in nonallergic asthma [47]. Another peculiar finding was the increased expression of GM-CSF receptor alpha expression in the macrophages detected in mucosa and BAL. Peripheral blood eosinophilia is present both in allergic and nonallergic asthma, in some studies being higher in the former compared to the latter group [48].

4. The eosinophilic phenotype of asthma

Different attempts have been found in order to identify an eosinophilic phenotype of asthma. Eosinophilic asthma is reported to account for approximately 50–60% of the total asthma population. The definition of eosinophilic asthma implies that eosinophils are the dominant cells responsible for the pathophysiological changes of the disease. The pathogenic role of eosinophils in these patients is

demonstrated by their increased number and status of activation in the airways, and consequently, they are detected in sputum, bronchoalveolar lavage, or bronchial mucosa or submucosa. These findings may be persistent and associated with severe or uncontrolled asthma [7].

Eosinophils may be demonstrated in the airways in the bronchial mucosa or submucosa or in the lumen (in the bronchial wash, BAL, or sputum). Bronchial biopsy is not routinely used as it is an invasive procedure and practicable only far from exacerbations to avoid dangerous complications and the quantification of eosinophils in BAL is not standardized and generally reflects samples of the peripheral airways.

Sputum examination is currently the most comprehensive and noninvasive method for measuring airway inflammation, processing and analysis being standardized and reliability, validity, and responsiveness proven [49].

The definition of "eosinophilic asthma" implies the existence of noneosinophilic asthma. A large cohort of patients with mild-to-moderate asthma in longitudinal studies resulted in approximately 50% of them with the absence of eosinophilic airway inflammation. The cellular pattern in noneosinophilic asthma may result in either predominant neutrophilic inflammation or normal sputum cell count. Within eosinophilic asthma, eosinophilia may result in persistent (22%) or on at least 1 occasion (intermittent eosinophilia, 31%) under multiple sputum examinations [50]. Sputum inflammatory granulocytes may identify phenotypic subgroups of differing pathology and clinical characteristics within asthmatics. Within the Severe Asthma Research Program (SARP), which included a population of severe and nonsevere patients with and without corticosteroid treatment, the stratification in four groups by granulocyte % in sputum showed significant clinical differences. Patients were divided combined for stratification by granulocytes in <2%Eos + <40%Neu, <2%Eos + ≥ 40%Neu, ≥2%Eos + <40% Neu, and ≥ 2%Eos + ≥40%Neu. In this study, eosinophilic asthma, indicated by ≥2%Eos, accounted for 31% of patients, those being with combined increased sputum eosinophils and neutrophils the most severe patients in terms of lowest lung function measures, worse asthma control, greatest symptoms and use of healthcare resources [51]. In another retrospective series of 508 asthmatics, the proportion of patients with raised sputum eosinophil counts ≥3% was 42% independent of the exclusion of steroid-treated patients. Eosinophilic phenotype exhibited higher atopy, levels of IgE, bronchial hyperresponsiveness to methacholine, FENO levels and lower asthma control, while the mixed granulocytic phenotype, with both eosinophilic and neutrophilic inflammation, had the lowest lung function and the highest degree of bronchial hyperresponsiveness to methacholine [52].

In most mild-to-moderate asthma patients untreated with steroids, sputum eosinophilia >2% was significantly and inversely associated with PC20 methacholine identifying 69% of the asthma group [53]. Sputum eosinophils correlate, in addition, with symptom score and FEV% and, as previously reported, are increased by exposure to common allergens. The association between asthma exacerbations and sputum eosinophilia is suggested by different pieces of evidence. First, sputum eosinophil count is able to predict asthma deterioration after cessation of ICSs treatment in mild-to-moderate asthma, while it is decreased by treatment with corticosteroids [54]. Sputum eosinophilia may be a good additional predictor of FEV1, PC20 methacholine or quality of life of response to inhaled steroids [55].

Consequently, a clinical strategy, based on re-administration of ICSs when a change in sputum eosinophil percentage by using the 0.8% threshold was reached, could lower the rates of asthma deteriorations and the number of individuals treated with ICSs by 48%. In addition, an increase in sputum eosinophils is detected up to 3 months before the development of a clinical exacerbation [56]. The usefulness of sputum cell count to improve treatment has been shown by Green et al. that

showed the efficacy of reducing exacerbations when treatment was guided according to the sputum eosinophils (to achieve a sputum eosinophil count of less than 3%) [54]. A different study used sputum cell counts to guide corticosteroid therapy to keep eosinophils <2% in moderate-to-severe asthma resulting in the sputum strategy group lower number and milder exacerbations (overall risk of exacerbations by 49%, it reduced the number of severe exacerbations) that were prevalently noneosinophilic [57].

Management of asthma-inhaled corticosteroid treatment based on sputum eosinophil levels has been the object of a Cochrane review that concluded that actually the risk of exacerbations is significantly reduced compared to that based on clinical symptoms with or without lung function, as well as the rate and severity of exacerbations defined by requirement for use of oral corticosteroids and hospitalizations [58]. Sputum eosinophilia may, therefore, be considered a modifiable risk factor to reduce exacerbations. Small studies in selected populations have suggested increasing ICS dose independent of the level of symptom control.

In this contest, the eosinophilic subtype of asthma may be defined as symptomatic asthma in the presence of airway eosinophilia and that is characterized generally by a good response to glucocorticosteroids.

5. Eosinophilic refractory severe asthma

When eosinophilic inflammation in asthma leads to uncontrolled disease, the patient is at risk of exacerbations. In a proportion of patients, asthma becomes difficult to be treated despite the adequate use of high-dose corticosteroid treatment. Once the management of modifiable factors such as incorrect inhaler technique, poor adherence, smoking or comorbidities is optimized but asthma remains still uncontrolled, the diagnosis of severe asthma can be formulated [59].

In a subgroup of patients with severe asthma, eosinophilic inflammation is still active despite the high-dose ICS treatment or oral corticosteroid intake. The use of sputum cell counts was thus defined as a marker allowing the identification of a subgroup of subjects with severe eosinophilic asthma who were at risk of more frequent asthma exacerbations [60].

In patients with severe eosinophilic asthma, sputum eosinophils may be suppressed by using increasing doses of inhaled steroids reducing the number of subsequent exacerbations [54, 57]. Yet, the persistence of airway eosinophilia in these subjects reflects a failure of usually adequate doses of corticosteroid to suppress inflammation [61]. Corticosteroid insensitivity is therefore intrinsic in the definition of severe asthma resulting in persistent lack of control despite corticosteroid therapy or worsening of asthma control on reduction or discontinuation of corticosteroid therapy [62]. A majority of severe asthmatics are corticosteroid dependent, refractory or insensitive and require oral corticosteroids (OCS) in addition to ICS to maintain some degree of asthma control. Only a small portion of severe asthmatics can be considered completely "corticosteroid unresponsive" or resistant [63]. The proportion of asthmatics with corticosteroid insensitivity is confirmed from the fact that one-third of the current SARP cohort were on regular OCS, with over half needing more than three bursts of OCS in the previous year [64].

A dose-response relationship between the use of OCS in asthmatic patients and the risk of many adverse events has been documented. Long-term exposure to OCS leads to increased risk of osteoporosis, arterial hypertension, diabetes, metabolic syndrome, cataracts, gastrointestinal bleeding and neuropsychiatric diseases such as depression [65]. The negative effect of systemic corticosteroid is associated not only to its maintenance or use but also to cumulative prescriptions of OCS burst [66].

In the global strategy for asthma management and prevention (GINA) 2019 update, sputum eosinophilia ≥2% is presented as a criteria to identify patients with severe asthma with refractory type 2 inflammation despite high-dose ICS or daily OCS treatment [1, 59].

Type 2 high asthma was initially used to identify the eosinophilic phenotype of asthma. The current concept of type 2 asthma includes a phenotype, characterized by the release of signature cytokines like interleukin IL-4, IL-5 and IL-13 from cells of both the innate and the adaptive immune systems. Th2 cells and type 2 innate lymphoid cells (ILC2s) are primarily responsible for the production of high levels of T2 cytokines in the airways. The demonstration of this cytokine pathway from cellular to molecular and transcriptomic levels represents the signature for type 2 (T2) asthma [67]. The importance of identifying different phenotypes of asthma has been addressed by hypothesis-based and unbiased analyses that showed different characteristics of asthma phenotype in terms of severity, functional and clinical features, comorbidities, prognosis and response to treatment [68].

5.1 Severe eosinophilic asthma in cluster analysis

Asthma phenotyping has involved biased and unbiased approaches with the aim of grouping clinical, physiological and genetic characteristics.

In the TENOR study (the epidemiological and natural history of asthma: outcome and treatment regiments), a severe allergic asthma phenotype emerged as a high-risk group of patients for severe exacerbations with early-onset, IgE and allergen sensitization [69]. The existence of this population was confirmed in the cluster analysis by Haldar P and coworkers who found an early-onset atopic asthma cluster in which a concordance between symptom expression and eosinophilic airway inflammation is present and a symptom-based approach to therapy titration may be sufficient. On the other side, a marked discordant cluster with late-onset active predominant eosinophilic inflammation emerged as a refractory phenotype of severe asthma [70].

The predominance of sputum eosinophilia in the inflammatory patterns of severe asthmatic subphenotypes is confirmed in the unsupervised hierarchical cluster analysis of the Severe Asthma Research Program cohort where cluster 4 of severe asthmatics was associated to atopic disease and reversible severe reductions in pulmonary function, while cluster 5 was characterized mainly by later-onset disease and airflow limitations that remain with a FEV1 < 80% predicted [71].

The expansion of this analysis using a supervised learning approach that included blood, bronchoscopic, exhaled nitric oxide and clinical data gave a further focus on severe asthma phenotypes. Therefore, while cluster 4 resembled that previously described with early-onset allergic asthma with low lung function and eosinophilic inflammation, the eosinophilic refractory asthma could be further split into cluster 5, characterized by late-onset severe asthma with nasal polyps and eosinophilia and cluster 6 with persistent inflammation in blood and bronchoalveolar lavage fluid, increased FENO levels and exacerbations despite high systemic corticosteroid use and side effects. Consequently, cluster 5 was characterized as more prone to respond to corticosteroid treatment, even if rapidly deteriorated after discontinuation (corticosteroid dependent), while cluster 6 was characterized to be corticosteroid complete insensitivity [72].

5.2 Blood eosinophilia as a biomarker of severe eosinophilic asthma

The question of whether blood eosinophilia may be considered, in this contest, a surrogate marker of airway eosinophilia, is debated. The measurement of

eosinophil counts in blood is inexpensive and widely available. However, blood eosinophilia is nonspecific for asthma and often asthmatic patients have normal levels of eosinophils. In asthmatics with increased blood eosinophilia, there exists a direct correlation with symptom scores and an inverse correlation with FEV1 in both children and adults, independently of atopy [73].

Blood eosinophilic counts have been reported to exhibit a moderate-to-good correlation with sputum eosinophils in asthma in large cohorts of asthmatics. A high blood eosinophil count >220/mm^3 resulted in good predictors of sputum eosinophilia ≥3% as revealed by an AUC of ROC curves of 79% that yielded 77% sensitivity and 70% specificity and an independent factors associated with the presence of sputum eosinophilic inflammation in multiple logistic regression models [52]. Other studies confirmed that blood eosinophils are an accurate biomarker of eosinophilic airway inflammation comparing two independent cohorts, mild-to-moderate asthma versus moderate-to-severe asthma. The authors used a cut-off point of ≥0.27 × 10^9/L blood eosinophils that were able to differentiate eosinophilic inflammation of ≥3% with a sensitivity of 78% and specificity of 91% [74].

In a multiple clinical variable analysis within the SARP cohort, the sensitivity and specificity of blood eosinophil counts of greater than 300/mL to detect an "eosinophil phenotype" based on sputum eosinophil counts of greater than 2% were 59% and 65%, respectively. This means that a blood eosinophil count of less than 300/L yields a 41% false-negative that has yet a sputum eosinophil percentage of greater than 2%, and likewise, many subjects with sputum eosinophil count of less than 2% would also be misclassified with a false-positive rate of 35%. Therefore, although statistically significant, the AUC of the ROC curve for predicting sputum eosinophil percentages of less than 2% or 2% and greater shows fair-to-poor accuracy and positive predictive values. These results are not improved when the cut-off of sputum eosinophil counts is more than 3% or whether the analysis is restricted to subjects with severe asthma only [51]. The stratification of SARP subjects based on blood eosinophil counts of less than 300 or 300/mL and greater showed significant differences only in methacholine bronchial hyperresponsiveness (log PC20), FEV1 percent predicted and FEV1/FVC ratio, neither in any variable related to overall asthma health care use or frequency and severity of exacerbations. This not-enthusiastic result has been confirmed both in patients with mild-to-moderate or in those with severe asthma who entered a clinical trial for mepolizumab for severe eosinophilic asthma [6].

In a study that evaluated 75 uncontrolled asthmatic patients, a significant positive relationship between the percentage of sputum eosinophils and the percentage of blood eosinophils ($r = 0.3647$; $p = 0.0013$) was demonstrated. An important limits of this study were the cut-off point of blood eosinophils of 2% of WBC and again the low accuracy of ROC curves [75].

Increasing the peripheral blood eosinophil cut-off percentage (2.7% or 0.26 × 10^9/L) yielded a significant higher sensitivity and specificity and AUC as a diagnostic biomarker of sputum eosinophilia (≥3%) in a population of uncontrolled asthmatics [76] suggesting that blood eosinophils can be used in the clinic for detecting airway eosinophilia in uncontrolled asthma. These data are confirmed when looking at the population selected for treatment with reslizumab, another anti-IL-5 mAb, in which blood eosinophil counts of greater than 400/mL might be able to improve the prediction of sputum eosinophil counts of greater than 3% [77].

A systematic review and meta-analysis estimated the diagnostic accuracy of markers for airway eosinophilia in patients with asthma. Looking at the 14 studies that investigated blood eosinophils as a predictor marker, an overall modest ability to distinguish between patients with or without airway eosinophilia was reported

with a summary estimate of AUC of 0.78 [78]. To be noticed that among the different studies, five different definitions of airway eosinophilia had been used, but either eosinophils ≥2% or 3% was used and this did not affect the accuracy of the test. Moreover, a subanalysis of the study showed the forest plots for blood eosinophilia in detecting sputum eosinophilia in subgroup populations of asthmatics. Smoking habit, steroid-treated or untreated and asthma severity revealed a considerable variability of positive thresholds of the marker. In severe asthma, only groups with the cut-point between 275 and 315 μL gave the highest sensibility and specificity [79]. As the most robust clinical value of sputum eosinophilia is tailoring inhaled corticosteroids and reducing the frequency of asthma exacerbations, it is expected that blood eosinophilia to replace induced sputum in this context should yield a sensitivity and specificity of at least 90%, so that only a small portion of patients will be misclassified. One of the most evident limits in the role of blood eosinophilia as a biomarker comes for the cross-sectional nature of the study populations. Significant variability of blood eosinophil count in the same patient over time and according to treatment status must be taken into account.

5.3 Treatment of severe eosinophilic asthma

Asthma guidelines are recommending the use of sputum eosinophil count in severe asthma. The international ERS/ATS guidelines on the definition, evaluation and treatment of severe asthma addressed the phenotypic management of severe asthma and evaluated the utility use of sputum eosinophilia to guide treatment. The document suggested that treatment guided by clinical criteria and sputum eosinophil counts should be performed in centers with experience in this procedure and in selected patients, allowing avoidance of inappropriate escalation of treatment and waste of resources [62]. In the global strategy for asthma management and prevention (GINA) 2019 update, this concept is reinforced by claiming that treatment guided by sputum eosinophil count has the best benefits in patients with moderate-to-severe asthma requiring secondary care [1]. Within step 5 of treatment scale, adults with persistent symptoms or exacerbations despite high-dose ICS or ICS/LABA are advised to adjust treatment based on sputum eosinophilia >3%.

When a refractory or underline type 2 inflammation is proven in severe asthma, add-on biologic type 2 target treatment must be considered for patients with exacerbations or poor symptom control [1, 59]. Actually, sputum eosinophil count also provides an effective method to identify patients who will benefit from targeted therapy with anti–IL-5 monoclonal antibodies (mAbs). In patients with refractory eosinophilic asthma that had a sputum eosinophilia >3% DCC, despite high dose of inhaled corticosteroids, and at least 2 exacerbations in the last 2 years, with the need to make a short course of systemic corticosteroids, mepolizumab therapy reduces exacerbations and improves AQLQ scores [80]. Other studies confirmed the efficacy of anti-IL-5 mAb therapy in patients with asthma who had consistently increased eosinophil counts in sputum of greater than 2.5–3% on at least two occasions [81] .

Yet, the measurement of eosinophils in sputum or airway fluids may not truly reflect the contributions of airway tissue eosinophils. Actually, a study was assessed to understand whether induced sputum has the ability to distinguish the eosinophilic and noneosinophilic phenotypes compared to bronchial biopsies in moderate and severe asthma. This study showed that among patients with severe asthma could identify a BrEos+ group with high mucosal eosinophils and a BrEos– group. Even if there was no a correlation between sputum eosinophil count and eosinophils found in the bronchial mucosa, there was a significant correlation between

the number of asthma exacerbations reported by the subjects with severe asthma during the year preceding the study and the percentage of sputum eosinophils [82]. This result is reflected by the fact that on one hand mepolizumab depleted <50% of bronchial tissue and bone marrow eosinophils in spite of its effect in reducing blood, BAL fluid and sputum eosinophils abolishing established airway eosinophil infiltration [83]. Among the explanation to this phenomenon, it can be supposed that eosinophils in the airway lumen may be in a different state of activation than in the bronchial mucosa or may reflect greater concentrations of intraluminal chemokines such as eotaxin and RANTES or epithelial activation.

Another possible consequence of the supposed partial effect of mepolizumab over all the aspects of eosinophilic inflammation is that FEV1, symptoms and FENO levels were not affected [80]. On one hand, this means that these therapeutic strategies may not be sufficient to reverse remodeling changes of severe asthma even if mepolizumab has been shown to decrease the deposition of tenascin, lumican and collagen III in the basal membrane of mild atopic asthmatics as well as the degree of TGF-β in the bronchoalveolar lavage fluid [43]. Accordingly, lung function was not expected to be positively modified by anti-IL-5 treatments in severe asthma and a meta-analysis of nine randomized, placebo-controlled, clinical trials including mepolizumab or reslizumab reported a mild absolute difference of FEV1 in favor of the anti-IL-5 treatment compared to placebo [84].

On the other hand, the persistently high level of FENO can guess, in a proportion of eosinophilic refractory severe asthmatics, that the IL-5 pathway is not in these patients the predominant. This fact can explain why targeting the type 2 phenotype on the IL4/IL13 pathway with dupilumab, a humanized MoAb to IL4-Ra, gave partially different results. When type 2 severe asthmatics with sputum eosinophilia >3% had been enrolled to be treated with dupilumab, the endpoints consisting of improvement of control (ACQ), symptoms and FEV1 were reached. These clinical and functional results were coupled with decreasing FENO, eotaxin 3 and IgE levels [85].

Another question is whether blood eosinophils are a good predictor of response to mepolizumab in patients with severe eosinophilic asthma. The DREAM study identified a blood eosinophil count of 300/mL or greater as a high predictive biomarker of response to mepolizumab [86].

In systemic corticosteroid severe asthma with persistent blood eosinophilia, at least 150 cells, the goal of reducing >75% of oral corticosteroid dose was reached in more than 40% of patients, confirming the role of persistent blood eosinophilia as predictor marker [87].

Benralizumab binds with high affinity to the alpha-chain of human IL-5R, blocking its activation and transduction and determining a neutralizing activity. Moreover, it is able to induce antibody-dependent cell-mediated cytotoxicity (ADCC) on NK cells that release cytotoxic mediators and cause eosinophil apoptosis [88]. A significant clinical efficacy in terms of reduction of annual exacerbations, improvement of FEV1 and steroid-sparing effect was demonstrated in the clinical trials [89]. A threshold of >300 cells per mcl represents a useful marker for quantifying the advantage of this treatment in patients with steroid-dependent asthma. It has been supposed that benralizumab results in a complete depletion of eosinophils in the airway lumen and this can in part explain why in the registrative studies pre-bronchodilator FEV1 improved in the treatment groups. Actually, benralizumab is highly selective on eosinophil and basophil protein and gene-related immune signaling pathways [90] and not only reaches almost complete eosinophil eliminations at plasma levels but also determines the reduction of blood eosinophil precursors [91].

6. Conclusions

The definition of eosinophilic asthma engages different models according to different contests [92]. However, a single common thread can be glimpsed in the ability of eosinophils to catch biological and clinical features that are crucial in each contest. Mouse and human allergic asthma models teach us that as the eosinophilic cascade can be dominant after acute exposition to triggers but only within the chronic stimulation, it contributes to deeper structural changes of the airways [11, 17]. The role of eosinophils in different phases of allergic asthma as well as the involving of Th2 cells, cytokines including IL5, IL4 and IL13 and chemokines has been smartly showed in the majority of the experimental studies. In addition, the mechanisms leading to AHR or persistent inflammation imply the need of sharing of different pathways of the Th2 cascade and the cross-talk between eosinophils and other immune cells [41]. The contribution of either IL-5–independent ways or the regulation of local or systemic eosinophilopoiesis has been addressed [40]. In real life, these phenomena can explain the ability of eosinophilic inflammation to be controlled by corticosteroid treatments, and, under certain circumstances, it becomes insensitive to this treatment.

Accordingly, in the context of severe asthma, eosinophilic airway inflammation becomes exceptionally deregulated and needs a biological approach to be controlled. The eosinophilic phenotype of asthma is currently defined by sputum examination that reveals eosinophilic airway inflammation. Generally, eosinophilic subtype of asthma may be defined as symptomatic asthma in the presence of airway eosinophilia and that is characterized by a good response to glucocorticosteroids. The efficacy of reducing exacerbations when corticosteroid treatment was guided according to the sputum eosinophils has addressed the point of eosinophilic target therapy in a subgroup of patients who encounter worse asthma control, higher use of healthcare resources, higher risk of exacerbations and the need of high-dose ICS or systemic corticosteroid treatment to be controlled. Continuous or bust oral corticosteroid exposure is associated to significant adverse effects that significantly impact on the patients' outcome [66], highlighting the urgent need of sparing corticosteroid approaches. Even if limits in accuracy have been evidenced, blood eosinophils can be used in the clinic for detecting airway eosinophilia in uncontrolled severe asthma [78] and as eligibility criteria for anti–IL-5 target therapy. Therefore, new add-on therapies for severe asthma have showed to reduce both asthma exacerbation rate compared to standard of care and daily OCS use. Five biologicals have been now approved for severe eosinophilic asthma and can be applied depending on asthma phenotype and endotype [93]. As a consequence, the precision medicine and personalized therapy have become the best clue for treatment and monitoring the response by identification of suitable biomarkers in patients with more severe and refractory forms of asthma [94].

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