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Invited review

Mouse models of severe asthma for evaluation of therapeutic cytokine targeting

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ABSTRACT

Severe asthma is a heterogeneous inflammatory disease of the airways, which requires treatment with high-dose inhaled corticosteroids or their systemic administration, yet often remains uncontrolled despite this therapy. Over the past decades, research efforts into phenotyping of severe asthma and defining the pathological mechanisms of this disease were successful largely due to the development of appropriate animal models. Recent identification of distinct inflammatory patterns of severe asthma endotypes led to novel treatment approaches, including targeting specific cytokines or their receptors with neutralizing antibodies. Here we discuss how different experimental mouse models contributed to generation of clinically relevant findings concerning pathogenesis of severe asthma and to identification of potential targets for biologic therapy.

1. Introduction

Asthma is a chronic inflammatory disease of the airways, triggered by exposure to environmental allergens, chronic respiratory viral infections and pollutants, all of which can be potentially aggravated by genetic predisposition, obesity or smoking. Asthma is currently one of the most common chronic diseases, affecting about 5% of the population [1]. This complex condition is characterized by inflammation and obstruction of the airways, infiltration of immune cells (eosinophils, neutrophils, antigen-presenting cells, Th1, Th2, Th17 $CD4^+$ T cells, $CD8^+$ T cells, B cells, mast cells, etc.), release of proinflammatory factors (such as interleukins, interferons, chemokines, histamines, and leukotrienes), and excessive production of mucus. Clinical symptoms such as wheezing, bronchial hyper-reactivity to non-specific stimuli, chest tightness and shortness of breath are manifested to some extent in almost all asthmatics. However, despite some similarity in the symptoms, the disease severity, molecular mechanisms of pathogenesis, key disease-triggering cytokines, time of onset, and effectiveness of various therapeutic strategies differ among asthma patients suggesting a high heterogeneity.

For decades two major forms of asthma were recognized: allergic (atopic or extrinsic) and non-allergic (non-atopic or intrinsic) [2]. Atopic asthma is the predominant type during childhood, whereas the non-atopic form is more common in older patients and often corresponds to the late onset subtype. Most asthmatics develop allergic asthma after being sensitized with allergens, which drives the formation of allergen-specific IgE antibodies and activation of Th2-mediated immune response. In young patients with allergic asthma, the disease usually begins following sensitization with multiple allergens and can be manifested by allergic rhinitis or eczema. Later, a prolonged allergic reaction can develop into allergic asthma, aggravated by the ingress of the allergen into the respiratory tract. The development of non-allergic asthma is not related to sensitization by allergens and is often characterized by infiltration of Th17 cells as well as cells of the innate immune system. This form of the disease is usually difficult to control and requires long-term corticosteroid treatment.

While the vast majority of asthma patients can achieve good control of their symptoms using standard therapies based on corticosteroids, beta-agonists and oral leukotriene inhibitors recommended by Global Initiative for Asthma (GINA) [3], a smaller fraction of asthmatics with severe allergic or non-allergic steroid-refractory asthma are unable to adequately control the disease with symptomatic treatment. Most severely affected individuals have a poor quality of life and exhibit a high risk of serious morbidity and mortality [4]. Without a doubt, there is a growing need to evaluate novel therapeutic agents, especially for those patients with severe asthma, who do not respond to traditional

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corticosteroid therapy. Anti-cytokine therapy, targeting specific pathogenic cytokines or their receptors, has shown its effectiveness in the management of chronic inflammatory diseases such as rheumatoid arthritis and colitis. Therefore, antibodies blocking one or more pathogenic cytokine should also be considered for patients with severe asthma.

Each individual patient is characterized by complex genetic, environmental and habitual interactions reflecting the extremely high heterogeneity of the human population. Recapitulation of this heterogeneity is difficult, however, powerful technologies like genome editing, including genetic knockouts and CRISPR-Cas technique, provided the possibility to manipulate genes and have led to the development of many useful mouse models. Thus, the vast majority of research and development of new therapeutic approaches for asthma treatment are conducted using mouse models. Indeed, immunological studies in the fields of allergy and asthma have made significant progress due to availability of unique genetically engineered mice. Mice are not only a convenient experimental model to study the disease mechanisms, but they are also widely used in validation studies of novel therapeutic agents, including anti-cytokine antibodies. Recent studies utilizing the existing mouse asthma models have attempted to recapitulate specific aspects of particular types of human asthma [5,6]. This review focuses on the mouse models for severe asthma, including those generated using reverse genetic technologies, and discusses potential targets for anti-cytokine therapy of severe asthma.

2. Severe asthma – a diversified condition

Severe asthma is a complex heterogeneous condition encompassing several underlying pathologies (Fig. 1). Persistent airway inflammation, despite the treatment with corticosteroids, is one of the hallmarks of severe asthma [7]. This form of asthma has a number of consistent characteristics including the presence of severe and frequent exacerbations, low lung functions and resistance to corticosteroid therapy. Other well-recognized clinical symptoms and physiological characteristics are usually related to some, but not all severe asthma subtypes: presence or absence of atopy; eosinophilia or neutrophilia; airway hyperresponsiveness (AHR); and the level of persistent airflow obstruction hyperresponsiveness to specific biological and environmental factors such as gender, age, smog, cigarette smoke, viral infections, fungi, dust and others [8]. However, comprehensive and indisputable classification of severe asthma subtypes has not yet been established. The majority of researchers and clinicians tend to adopt the criteria, which divides a heterogeneous group of patients suffering from severe asthma into more homogeneous subgroups. Both clinical and statistical analyses from the European and North American severe asthma cohorts strongly support the presence of several subgroups or phenotypes. For instance, all studies to date have identified an early onset, mostly allergic phenotype with strong eosinophilia [9,10]. In addition, two late onset phenotypes have also been described: highly eosinophilic and obesity aggravated less atopic [11,12]. Sputum analyses from severe asthmatics have identified a non-granulocytic inflammatory phenotype that does not show increased numbers of sputum eosinophils or neutrophils [13]. Finally, a high neutrophilic phenotype with fixed airway obstruction stands apart from the highly eosinophilic subtypes of severe asthma, since no associations with the age of the disease onset or genetic predisposing factors were found for this subgroup [14,15]. Unfortunately, none of these clinically characterized phenotypes provide much insight into the underlying pathogenic mechanisms. Thus, it becomes evident that to study a complex and diverse syndrome such as severe asthma, appropriate animal model systems are required. Such models should not only allow detailed mechanistic study of the severe asthma pathogenesis, but also provide a platform for preclinical evaluation of various therapeutic drugs. Over the past decades, several mouse models of severe asthma have been generated and validated. Foremost, these are mouse models tailored to selected genes and gene products implicated in the development of allergic asthma. The next section summarizes how different experimental mouse models have helped to generate clinically relevant findings concerning pathogenesis of severe asthma and to identify potential targets for biologic therapy.

3. Mouse models of severe asthma

Severe asthma is a multifaceted syndrome; therefore, mouse models should reproduce as accurately as possible the individual subtypes of severe asthma that may have different symptoms and features. Since the recent identification of the distinct severe asthma phenotypes fostered the concept of specific targeting therapies, choosing the right experimental mouse model is becoming a key step both for research and for drug development. Table 1 summarizes the distinctive features of the currently available mouse models of severe asthma. The terms "steroid-resistant asthma" and "severe asthma" are often used as synonyms. Indeed, severe asthma is fundamentally different from other forms of asthma because patients do not respond to high dose corticosteroid therapy. On the basis of steroid resistance, there is a boundary between common asthma, for which an effective protocol of therapy has been developed, and severe asthma, for which there is no effective treatment. Therefore, for most murine models of severe asthma, it is customary to confirm steroid resistance experimentally (Table 1).

3.1. Viral infection-exacerbated asthma

Respiratory viral infections are the major trigger of acute exacerbations of asthma, which result in increased morbidity and mortality [37]. The fact that asthma symptoms exacerbate after bacterial, viral, or fungal infection was confirmed experimentally in a number of studies using mouse models of severe steroid-resistant asthma. In a mouse model of ovalbumin-(OVA)-induced allergic airway disease, sensitization with OVA followed by *Haemophilis influenza* infection was associated with increased steroid-resistant Th1/Th17-mediated neutrophilic airway inflammation, while OVA-sensitized non-infected mice responded well to steroid treatment [18]. Virus-exacerbated steroid-resistant severe asthma phenotype with Th17-dependent neutrophilia in mice may be experimentally induced by administration of Rhinovirus (RSV) together with OVA sensitization and an adjuvant [19]. Recently, the pivotal role of innate immune response in virus-exacerbated asthma was revealed, using a mouse model of chronic HDM exposure followed by infection with influenza virus A/X31 H3N2 [16].This model also provides the relevant platform for studying the mechanisms of steroid-resistant asthma, since up to 50 percent of asthmatics are sensitized to HDM [38,39]. Another option for virus-exacerbated severe asthma model is the combination of RSV with OVA and an adjuvant in IL-4-deficient mice [17]. In this case, the IL-4-deficient background further shifts the inflammatory response to Th1/Th17-mediated response.

3.2. Aspergillus infection-exacerbated asthma

Aspergillus sp. is ubiquitously present in the environment and represents a genus of potentially dangerous aerobic molds. Aspergillus spores are abundant in the air stream and, due to their small size, are inhaled deep into the lungs of animals and humans with fast colonization and induction of inflammatory response. Some species, such as *A. fumigatus*, can infect humans and provoke severe conditions such as aspergillosis in healthy individuals and uncontrolled exacerbations in asthmatic individuals. While the exact mechanism of Aspergillus-mediated uncontrolled asthma exacerbations remains unclear, a number of studies have reported a strong association between fungal sensitization and increased IL-33 production by ILC2 [40,41]. Moreover, chronic aspergillosis could trigger the development of severe asthma with fungal sensitization (SAFS). It was shown that 39% of children with moderate to severe asthma had evidence of sensitization to one or more fungal species and 76% of those sensitized were

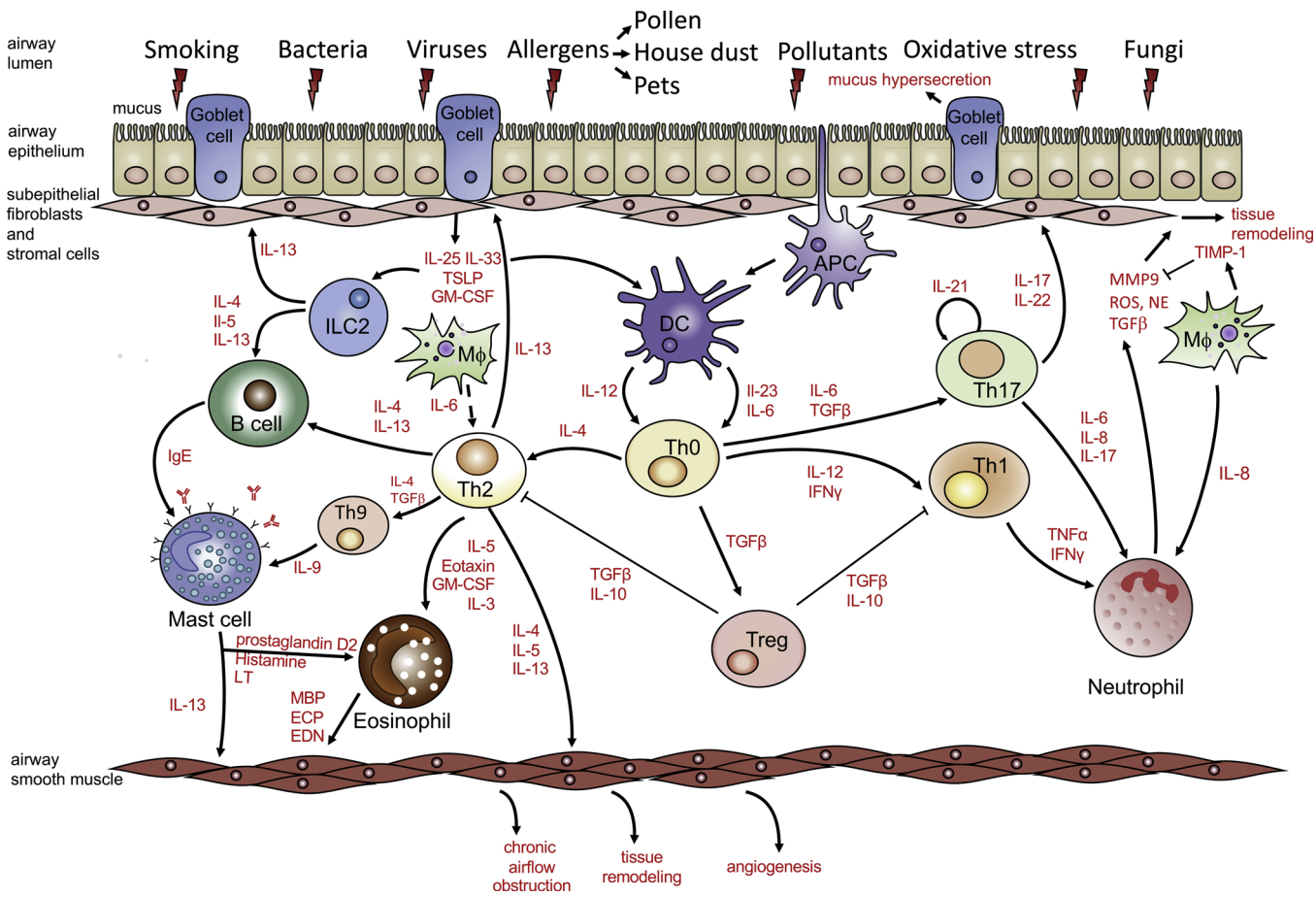

Fig. 1. Pathological mechanisms of severe asthma.

In response to inhaled allergens, resident antigen presenting cells (APC), mainly dendritic cells (DCs) and alveolar macrophages, become activated. Disruption of the epithelial barrier enhances allergen uptake. DCs undergo maturation process; upregulate the expression of co-stimulatory molecules, chemokines and proinflammatory cytokines; and migrate to the draining lymph nodes. In the lymph nodes, DCs present allergen peptides to the naive T cells (Th0) forcing them to differentiate into mature T helper cells. The consequent pathologic mechanisms include activation of Th2/mast cell/eosinophil-mediated pathology, Th1/Th17/neutrophil-mediated pathology, activation of innate immune response and irreversible airflow obstruction.

Th2-mediated pathology. IL-4, produced by T cells, mast cells, eosinophils, and macrophages, is critical for differentiation of naive T cells into IL-4- and IL-13-producing Th2 cells. Th2 cells then drive B cells to produce allergen-specific IgE, both in the lymph nodes and in the airway mucosa. IgE, released from B cells, binds to the high-affinity receptor for IgE on mast cells completing the sensitization process. Macrophages (Mφ) can also contribute to Th2-driven eosinophilic inflammation by producing IL-6 [97]. Additionally, IL-9 producing Th9 cells can further promote mast cell growth and recruitment. In sensitized individuals, re-exposure to the allergen results in the cross-linking of allergen peptide with the allergen-specific IgE on the surface of mast cells. As a result, the presynthesized mast cell mediators are released, including histamine and proteases, as well as newly formed mediators such as cytokines, chemokines, growth factors, prostaglandins, platelet-activating factor and leukotrienes. The release of mast cell mediators leads to the local inflammatory response which enhances the infiltration of T cells and eosinophils to the inflammatory site. As a result, the main asthma features of airway narrowing and obstruction, mucus hypersecretion and airway hyperreactivity develop.

Th1/TH17/neutrophil-mediated pathology. IL-6, IL-23, TGFβ, and IL-12 production during the activation of naive T helper cells by DC shift their differentiation into Th17 or Th1 cells. These cell types are associated with severe neutrophilic steroid-resistant asthma. IL-17a, produced by Th17 cells, is capable of inducing neutrophil influx to the inflammatory site. Neutrophilic inflammation responds poorly to steroid therapy, since corticosteroids can promote neutrophil survival rather than apoptosis. [93]. Neutrophils produce matrix metalloproteinase 9 (MMP9), reactive oxygen species (ROS), and neutrophil elastase (NE), which negatively regulate the levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) thereby promoting tissue remodeling, mucus production and bronchoconstriction. Macrophages can also promote neutrophil migration by producing IL-8 (CXCL1, CXCL2 in mice) [100].

Activation of the innate immune response. In addition to the antigen-dependent immune response, the innate immune response in asthma is represented by lung epithelial and stromal cells producing IL-25, IL-33, TSLP and GM-CSF. IL-25, produced by epithelial cells, amplifies Th2- and innate lymphoid cell 2 (ILC2)-mediated cytokine production and eosinophilia [101,102]. IL-33 stimulates mast cells, eosinophils, basophils, NKT cells to produce Th2 cytokines and is involved in eosinophil survival [103]. TSLP activates mast cells to produce Th2 cytokines, triggering DCs to increase OX40 l expression and therefore enhancing the Th2 inflammatory response [104]. GM-CSF production after allergen exposure decreases the threshold of allergen responsiveness and, hence, increases the susceptibility to develop allergic asthma [105].

Irreversible airflow obstruction. Irreversible airflow obstruction is one of the hallmarks of severe asthma. The narrowing of the airways can become irreversible when the respiratory tract tissues reorganize under control of growth factors released during the development of the inflammatory response. Structural changes seen in asthmatic patients can include thickening of the airway wall reticular basement membrane, formation of abnormal elastic fiber network, alterations in airway cartilage structure, angiogenesis and an increase in airway smooth muscle mass [106]. Tissue collagen deposition is controlled by MMPs, which degrade collagen, and TIMPs, produced by alveolar macrophages. The abrogated balance of MMP-9 to TIMP-1 production plays central role in modifying airway wall, matrix deposition and tissue remodeling [107].

A number of factors can aggravate mild-to-moderate asthma, leading to the development of a severe disease, for example, smoking, chronic allergen exposure, lung colonization by fungi, oxidative stress, pollutants, bacterial or viral infections. T-regulatory cells are able to suppress both Th2 and Th17 immune responses and are thought to play a crucial role in maintaining the lung homeostasis, however, in severe asthma cases their functions can be impaired [108].

Table 1
XXX.

Proposed subtype of SA	Induction protocol	Innate immune response	T cell response	Steroid resistance	Reference
Viral infection-exacerbated asthma	C57BL/6 mice treated with 25 μg HDM for five days a week for five consecutive weeks and then infected with influenza virus A/X31 H3N2	Eos, Neu		Yes	[16]
	IL-4 KO mice treated with OVA, Alum and lysate from RSV infected cells	Neu	Th1	Not tested	[17]
	BALB/c mice infected with *H. influenzae* (day 10), intraperitoneally sensitized (day 0) and intranasally challenged (day 12-15) with OVA and Alum (Rehydrogel)	Neu	Th17	Yes	[18]
	BALB/c mice treated with OVA, Alum (Alhydrogel) and then exposed to RSV	Neu	Th1/Th17	Yes	[19]
Aspergillus sp. Infection- exacerbated asthma	BALB/c mice received intraperitoneal and intranasal injection of *Dermatophagoides farinae* mite extract in aluminum hydroxide and intranasal injection of *Aspergillus sp*. extract	Eos, Neu	Th2/Th17	Yes, for neutrophilia and mucus production	[20]
	BALB/c or C57Bl/6 mice treated with chitin particles and *Aspergillus sp.* extract	Eos, Neu	Th2/Th17	Not tested	[21]
Multiple allergen exposure-exacerbated asthma	BALB/c or C57BL/6 mice treated with b-glucan, 100 ng LPS and 10 μg HDM	Neu	Th17	Yes	[22]
	BALB/c mice treated with HDM, ragweed and *Aspergillus sp*.	Neu, Eos		Yes, for neutrophilia and AHR	[23]
	BALB/c mice treated with HDM, cockroach and OVA	Neu	Th1/Th17	Yes	[24]
Chronic allergen exposure- exacerbated asthma	BALB/c mice received low-dose aerosol OVA for 4 weeks	Neu, Eos	Th17	Yes	[25]
	BALB/c mice sensitized with 2 i.p. injections of 10 μg of OVA in aluminum hydroxide and received low-dose aerosol OVA for 8 weeks	Eos		Not tested	[26]
IFNy- dependent SA	BALB/c mice subjected to adoptive transfer of transgenic $OVA_{323–339}$ peptide-specific IFN-γ-producing Th1 cells and treated with $OVA_{323–339}$ peptide with LPS	Neu	Th1	Yes	[27]
	BALB/c mice were sensitized to 25 μg HDM together with 5 μg cyclic-di-GMP at days 1, 3 and 5 and then challenged with 3 challenge sets consisting of 3 consecutive daily challenges with HDM and cyclic-di-GMP with 4 days of rest before each set	Neu, Eos	Th1	Yes	[28,29]
Non-allergic steroid resistant SA	BALB/c mice treated with IFNγ, IL-27 and LPS	Macrophages		Yes, for AHR	[27]
High neutrophilic Th17-induced SA	BALB/c mice treated with OVA and 10 μg LPS	Neu	Th17	Yes	[30]
	BALB/c SCID mice treated with OVA and subjected to adoptive transfer of Th17 cells	Neu	Th17	Yes	[31]
	BALB/c SCID mice treated with 50 μg of OVA and subjected to adoptive transfer of Th17 cells.	Neu	Th17	Yes	[32]
High eosinophilic (Th2 high) SA	Allergen-naive double transgenic mice on C57BL/6 J background that expresses IL-5 by matyre T cells and eotaxin-2 locally by lung epithelial cell	Eos	Th2	Yes	[33]
	$Lyn^{-/-}$ mice on C57BL/6 or 129Ola backgrounds treated with 20 μg of OVA in aluminum hydroxide	Eos	Th2	Not tested	[34]
IL-13-mediated SA	A/J mice intratracheally treated with recombinant mouse IL-13	Eos, Neu		Yes, for mucous cell hyperplasia and AHR	[35]
	BALB/c mice treated with IL-13 producing adenovirus	Neu		Yes, for Neu, mucus production and AHR	[36]

categorized as asthmatics with severe steroid-resistant asthma [42]. Based on these studies as well as the fact that aspergillosis significantly reduces quality of life and causes development of severe steroid resistant asthma, it is evident that aspergillosis presents a serious problem for asthma patients. Mouse models of severe asthma with fungal sensitization provide a powerful tool for the biomedical research of aspergillosis-exacerbated asthma development, because some species, for example, *A. fumigatus,* effectively infect both humans and mice. *Aspergillus*-exacerbated severe allergic asthma mouse model can be achieved by administration of *Dermatophagoides farinae* mite extract in aluminum hydroxide followed by the intranasal injection of *Aspergillus* sp. [20] or by administration of chitin particles together with Aspergillus sp. extract [21]. There is considerable evidence supporting the role of other fungal species, such as *Aspergillus versicolor* and *Cladosporium cladosporioides,* in exacerbations of asthma. Moreover, different fungal species may use distinct pathways to induce asthma. In mold-induced severe asthma mouse models it was shown that exposure of beta-glucans on the surface of *Aspergillus versicolor* promotes signaling through Dectin-1 and induces a Th17 response, while beta-glucans in *Cladosporium cladosporioides* are not readily available on the surface, thus preventing signaling through Dectin-1 and promoting the use of an alternate pathway to induce a Th2 response [43].

3.3. Chronic allergen exposure asthma models

A single exposure to allergen can exacerbate asthma symptoms, while prolonged exposure to allergen in the absence of corticosteroid control can lead to the development of steroid-resistant asthma [44,45]. This was demonstrated in the mouse model of chronic asthma induced by prolonged exposure to aerosol OVA following sensitization with OVA and an adjuvant [25]. Such exposure induced disease exacerbation with increased tissue inflammation and steroid-resistant AHR. The same study also showed the important role of histone diacetylases in the development of resistance to corticosteroids. It was found that exposure to allergen caused an irreversible decrease in the concentration of histone deacetylase 2 (HDAC2), which is capable of deacetylating glucocorticoid receptor, leading to a reduction of the expression of proinflammatory genes. Moreover, synthetic corticosteroids were not able to reverse the allergen-induced decrease in histone deacetylase protein or increase in proinflammatory gene expression activity. This finding implicated the impaired recruitment of histone deacetylase as a potential mechanism for steroid-resistant severe asthma development. The same phenotype can be achieved in a mouse model with more prolonged allergen exposure [26]. In this model, mice were sensitized by two intraperitoneal injections of OVA, followed by exposure to low concentrations of aerosolized OVA for up to eight weeks. After sensitization and challenge procedures, mice developed progressive steroid-refractory inflammatory response in the airways with increased eosinophilia and mucus production along with evident subepithelial fibrosis and epithelial thickening. Experimental models with chronic allergen exposure following acute sensitization with an adjuvant, like those described above, replicate many of the features of human asthma and may be useful in studies of the pathogenesis of chronic long-term progressive human disease.

3.4. Multiple allergen exposure-exacerbated asthma

A study of the allergic characteristics of children with asthma in seven U.S. urban communities revealed that more than 50% of asthmatic children had positive skin tests to three or more allergen groups, indicating that sensitization to multiple allergens may be associated with increased severity of asthma [46]. Studies using mouse models confirmed the observation that exposure to several allergens can lead to the development of severe asthma. For example, mice treated with β-glucan, LPS and house dust mite extract (HDM) display robust neutrophilic steroid-resistant inflammation, indicating that sensitization with a combination of commonly encountered microbial components may have a synergistic effect and promote the inflammatory response [22]. Steroid-resistant neutrophilic airway inflammation also develops in response to a challenge with the pooled extracts of dust mite, ragweed, and Aspergillus species (DRA) [47], or with a mix of HDM, cockroach extract and OVA [24].

3.5. Highly eosinophilic asthma models

Another group of experimental mouse models of asthma mimics the phenotype of highly eosinophilic severe asthma in humans. For example, OVA-challenged Lyn (non-receptor tyrosine kinase)-deficient mice display a phenotype of severe eosinophilic airway inflammation, suggesting that Lyn tyrosine kinase may be a negative regulator of Th2 polarized allergic response [34]. Furthermore, some models implicate the link between specific cytokines and asthma manifestations. For example, allergen-naive double transgenic mice with expression of IL-5 by mature T cells and expression of eotaxin-2 by the lung epithelial cells spontaneously develop airway remodeling and eosinophil degranulation in the airways, suggesting that IL-5 and eotaxin-2 may contribute to non-allergic steroid-resistant asthma [33].

3.6. IFNγ - dependent asthma models

Clinical studies showed a strong association between AHR in response to inhaled histamine and increased IFNγ production by peripheral mononuclear cells. This data provided evidence for the role of IFNγ-producing Th1 cells in the pathogenesis of severe asthma [48]. Moreover, adoptive transfer of antigen-specific IFNγ-producing Th1 cells into naïve mice followed by OVA and LPS challenges induced strong steroid-resistant AHR and neutrophilia, reaffirming the critical role of Th1 cells and TLR4-MyD88 signaling pathways in the development of steroid-resistant asthma [27]. Additional evidence suggesting the involvement of IFNγ in the pathogenesis of severe steroid-resistant asthma in humans was reported by Ray et al. who showed that severe asthma subjects harbor more $IFN\gamma^{+}CD4^{+}$ T cells in their airways compared to patients with moderate asthma [28]. This analysis of immune responses in human subjects helped establish a relevant mouse model of IFNγ-dependent severe asthma. In this model, mice were sensitized to 25 μg HDM together with 5 μg cyclic-di-GMP at days 1, 3 and 5 and then challenged with 3 sets, consisting of 3 consecutive daily challenges with HDM and cyclic-di-GMP, followed with 4 days of rest before the next set [29]. The combination of HDM and cyclic-di-GMP generated a high type 1 signature with increased airway resistance and poor response to corticosteroids. Moreover, the model is also suitable for studying mixed granulocytic severe asthma pathogenesis.

3.7. Non-allergic steroid-resistant asthma model

All of the models, described in this section, refer to allergen-induced forms of severe steroid-resistant asthma. However, it should be considered that some patients display non-allergic steroid-irresponsive AHR. Though pathogenesis of this asthma endotype is not fully understood, some mouse models could mimic this allergen-independent steroid-resistant AHR condition. For example, administration of both IFNγ and LPS to murine airways resulted in the influx of activated $CD11b^{+}$ pulmonary macrophages into the airways and in increased expression of IL-27 in the lungs, leading to steroid-resistant AHR without neutrophilia or eosinophilia. Moreover, IL-27 and pulmonary macrophages played a central role in the regulation of steroid-resistant AHR [27]. Interestingly, IL-27 neutralization may completely inhibit the development of IFNγ/LPS–induced AHR, suggesting that IL-27 may be a potential target for the therapy of steroid resistant AHR in severe asthma.

3.8. High neutrophilic Th17-induced asthma model

Th17 responses during asthma exacerbations were reported long ago [49], however, the detailed mechanisms of Th17 involvement in the inflammatory response in asthma have yet to be established. Several studies have implicated Th17 associated cytokine, IL-17a in neutrophilic asthma [50,51]. Th17 cytokines help to recruit neutrophils to the airway by increasing secretion of epithelium-derived neutrophilic chemokines [52]. Despite the fact that the role of IL-17 in neutrophil recruitment is well established, the involvement of Th17 cells in asthma is an area of intense current investigation [53]. To this end, Th17-mediated neutrophilic steroid-resistant asthma can be induced by transtracheal administration of a high dose of LPS (10 μg) in combination with OVA sensitization and challenge [30]. OVA challenge followed by adoptive transfer of OVA-specific Th17 cells can induce steroid-resistant AHR with severe neutrophilic airway inflammatory response. At the same time, adoptive transfer of OVA-specific Th2 cells induces steroid-responsive inflammation, suggesting that antigen-specific Th17 cells may play a crucial role in some subtypes of severe steroid-refractory asthma [31,54].

It should be noted that the genetic background of mice may result in different sensitivity to asthma [55]. Moreover, the frequency and dosage of asthma-provoking substances should be optimized for individual animal facilities and titrated individually for each mouse strain.

4. Therapeutic targeting of cytokines in severe asthma

Severe refractory asthma represents an unsolved clinical problem. Treatment options for most severe asthma cases involve a combination of inhaled or systemic corticosteroids together with long acting β2-adrenergic agonists as a symptomatic treatment during exacerbations. In 2003 the anti-immunoglobulin E (IgE) biologic, Omalizumab, was approved for the most severe cases of asthma. Omalizumab inhibits binding of IgE to high-affinity IgE receptors on the surface of mast cells and basophils, which in turn limits the release of inflammatory mediators. Omalizumab therapy is effective in improving asthma control in patients with severe allergic asthma with high sputum IgE levels [56]. However, a number of undesirable side effects were reported in association with Omalizumab, including injection site reactions, as well as increased viral and upper respiratory tract infections [57], underscoring the need for more advanced therapeutic strategies for severe asthma. Recent identification of distinct inflammatory patterns of severe asthma endotypes led to the development of novel treatment approaches [58,59], including specific targeting of cytokines or their receptors (Table 2).

The immunogenicity of murine-derived domains in chimeric antibodies diminishes their efficiency in immunotherapy. Since humanized antibodies can overcome these problems [60], the majority of recently approved therapeutic antibodies are fully human or humanized. They typically contain murine sequence-derived CDR regions that were engrafted into human sequence-derived V regions. Fully human antibodies that contain no murine sequences can be produced in two ways: by phage display technologies [61] (that were recognized in 2018 by the Nobel prize in Chemistry) and in specialized transgenic mice [62]. Recently, fully human antibodies isolated from mice carrying functional human immunoglobulin loci and inactivated murine immunoglobulin genes were approved for therapeutic use [63]. Such antibodies undergo affinity maturation in vivo and may represent naturally occurring sequences. In the following section, we provide a brief summary of the current state in the field of severe asthma anti-cytokine therapy.

Therapeutic (usually humanized) monoclonal antibodies represent most successful and effective drugs as they demonstrate very high specificity for their molecular targets and may confer effector functions such as receptor-ligand blockade, target cell cytotoxicity and receptor antagonism. Neutralizing antibodies bind to cytokines and prevent their association with the receptors on the cell surface leading to inhibition of cytokine-mediated signaling cascades and downstream effects, such as cell proliferation or programmed cell death [64].

The distribution of therapeutic antibodies inside the organism depends on the rate of their extravasation into the tissues. The main mechanism of antibody extravasation is convective transport [65], determined by the fluid flow from the vascular space into the tissue, due to the hydrostatic gradient and the presence of paracellular pores in the vascular epithelium [66]. However, in tissues where extravasation through convective transport is limited, there is another important mechanism – transcytosis – that is mediated through the neonatal Fc receptor.

Elimination of therapeutic antibodies from the system is usually carried out by intracellular catabolism in lysosomes after pinocytosis or receptor-mediated endocytosis. Uptake through pinocytosis is non-specific. During endocytosis cell surface receptors interacting with the antibody Fc domain or with one of the Fab-binding domains trigger antibody internalization into the vesicle, followed by lysosomal degradation [65]. However, there is an intrinsic mechanism that helps to maintain the appropriate concentration of therapeutic antibodies. This recycling mechanism is provided by the FcRn receptor and functions similarly for both natural antibodies and artificial therapeutic antibodies [67]. After endocytosis and endosome formation, endosome acidification causes an increase in FcRn affinity allowing antibodies to bind to the Fc domain. Finally, the FcRn-antibody complex returns to the cell surface and the antibody is released to the intracellular space [65].

Airway eosinophilia is characteristic of the late-onset eosinophilic, early-onset eosinophilic and mixed granulocytic endotypes of severe asthma. Since anti-cytokine therapeutic antibodies affect the biological activity of a specific cytokine, they can be used for the treatment of only a few of the severe asthma endotypes such as those associated with eosinophilia. Th2 inflammation is associated with the overproduction of IL-4, IL-5 and IL-13, involved in the recruitment of eosinophils and in the activation of B-cell receptor isotype switching from IgM to IgE. IL-4 deficient mice display reduced eosinophilic inflammation and IgE production in the OVA-induced asthma model [68]. IL-13 is involved in mucus hyperproduction, epithelial metaplasia and airway remodeling [69]. Moreover, IL-13 neutralization ameliorated an eosinophilic inflammation in an OVA-induced asthma model [70]. Finally, IL-5 receptor-deficient mice show attenuated eosinophilia and Th2 cytokine levels in a house dust mite antigen-induced mouse model of asthma [71]. Taken together, these findings indicate, that IL-4, IL-5 and IL-13 are key mediators of eosinophilic asthma development and may represent the most valuable targets for anti-cytokine therapy [72].

IL-5 is a Th2 cytokine responsible for maturation of eosinophils in the bone marrow and for their survival and activation at the sites of allergic inflammation [84]. However, complete asthma remission does not occur following anti-IL-5 antibody administration, confirming that multiple pathological processes and mechanisms may be implicated in high-eosinophilic severe asthma [86]. To date, two anti-IL-5 antibodies and one anti-IL-5R antibody have been approved for severe eosinophilic asthma therapy. Mepolizumab is a humanized monoclonal antibody that blocks IL-5 and allows gradual reduction of systemic corticosteroids dosage. Reslizumab is a second humanized monoclonal antibody against IL-5 approved by the FDA. Benralizumab is a monoclonal antibody directed against alpha-chain of IL-5 receptor that inhibits heterodimerization of IL-5 receptor a/b subunits and the downstream signal transduction cascades. Benralizumab is also capable of binding through its constant Fc region to the FcγIIIa receptor, expressed on the surface of natural killer cells, macrophages and neutrophils [85]. Mepolizumab, Reslizumab and Benralizumab therapies could be beneficial in high-eosinophilic severe asthma cases because they prevent allergen-induced exacerbations and eosinophil recruitment to the airways. Interestingly, recently reported indirect treatment comparison (ITC) of Mepolizumab, Reslizumab and Benralizumab implicated an improvement of the

Table 2
XXX.

Target cytokine or receptor	Drug name	Antibody type	Target patient group	Positive therapy outcomes	Stage of clinical trials	Reference
FDA approved						
IL-5	Mepolizumab	Humanized monoclonal antibody	Severe asthma > 150 eosinophils in 1 μl of blood	Steroid synergism, improved lung function	Approved since 2015	[73,74]
	Reslizumab	Humanized monoclonal antibody	Uncontrolled moderate to severe asthma > 400 eosinophils in 1 μl of blood > 1 exacerbation in the past year	Uncontrolled moderate to severe asthma. Reduced exacerbations and need for oral steroids	Approved since 2016	[75]
IL-5Ra	Benralizumab	Humanized monoclonal antibody	Severe asthma > 300 eosinophils in 1 μl of blood	Reduced exacerbations	Approved since 2017	[76,77]
Ongoing trials						
IL-4Ra	Dupilumab	Human monoclonal antibody	Uncontrolled moderate to severe asthma > 300 eosinophils in 1 μl of blood	Improved lung function, reduced exacerbations, reduced eosinophils at the site of allergic inflammation	III	[78]
IL-13	Tralokinumab	Human monoclonal antibody	Severe uncontrolled asthma with 2-6 exacerbations per year	Little or no effect	III	[79]
	Lebrikizumab	Humanized monoclonal antibody	> 300 eosinophils in 1 μl of blood	Little of no effect	III	https://clinicaltrials.gov/ct2/show/NCT01868061
IL-17RA	Brodalumab	Human monoclonal antibody	Moderate to severe asthma	No effect	II	[80]
TNF	Etanercept	Human dimeric p75 receptors fused with human Fc	Mild to severe asthma	Improved quality of life, improved lung function, reduced AHR. At the same time, no positive effect was shown in another study	II	[81,82]
	Golimumab	Human monoclonal antibody	Severe asthma		Discontinued due to severe side effects	[83]
IL-6	Sirukumab	Human monoclonal antibody	Severe uncontrolled asthma		Withdrawn before participants were enrolled	ClinicalTrials.gov Identifier: NCT02794519

exacerbation with all three treatments versus placebo. However, this ITC also demonstrated that, when compared with Benralizumab and Reslizumab, Mepolizumab dampened exacerbations more effectively [86].

Dupilumab is a human monoclonal antibody to IL-4 receptor alpha (IL-4RA) subunit. Since IL-4RA is also a subunit of the IL-13 receptor, Dupilumab can prevent signaling mediated by both cytokines. This therapeutic antibody was originally developed for atopic dermatitis followed by clinical trials for eosinophilic severe asthma. Preliminary data suggest that Dupilumab treatment can improve lung function and reduce exacerbations in severe asthma patients with high eosinophil levels in the peripheral blood. Dupilumab clinical trials are still in progress and the drug is not yet approved for severe asthma therapy. However, meta-analysis of several randomized double-blinded placebo-controlled trials in severe asthma patients revealed that treatment with Dupilumab is associated with a reduced rate of severe asthma exacerbations with improved forced expiratory volume (FEV1) when compared to the placebo group. These promising results suggested that Dupilumab may become an effective drug for treating severe eosinophilic asthma [87].

IL-13 is a Th2 cytokine involved in B cell activation, airway hyperresponsiveness, mucus production and subepithelial fibrosis [88]. Tralokinumab is a humanized antibody that neutralizes IL-13 and may be beneficial for patients with severe eosinophilic asthma with high IgE levels. In phase IIb clinical trials, with Tralokinumab administration every two weeks, the drug was able to slightly improve FEV1 without any effect on exacerbation rates [79]. Phase III Tralokinumab trials are currently in progress. Another anti-IL-13 antibody, Lebrikizumab, failed to provide patients with mild-to-moderate asthma with any significant improvement of lung functions when administered without corticosteroids in phase III clinical trials [89]. In summary, only anti-IL-5-directed biologics are currently approved as an anti-cytokine therapy for high-eosinophilic severe asthma.

It should be noted that comparative efficacy of anti-IL-4, IL-5 and IL-13 drugs (Benralizumab, Dupilumab, Lebrikizumab, Mepolizumab, Reslizumab, Tralokinumab) for treatment of severe eosinophilic asthma indicated that all these drugs, except for Tralokinumab, can significantly improve FEV1 and patient quality of life as compared to the placebo group [90]. Additionally, Dupilumab and Reslizumab were associated with significantly fewer asthma exacerbations as compared to other drugs.

Neutrophilic asthma is predominant in patients with severe refractory asthma [50,91,92]. Corticosteroids are known to inhibit neutrophil apoptosis, which may further enhance airway neutrophilia [93]. Neutrophilic inflammation in asthma is related to systemic inflammatory response involving both the adaptive and the innate immune pathways with increased production of TNF, IL-6 and C-reactive protein [94].

Since neutrophilic inflammation in asthma requires Th1/Th17 responses with the release of IL-6, IL-17, IL-8 and IFNγ, studies of potential molecular targets in neutrophilic asthma therapy are focused on these cytokines. Brodalumab is a human monoclonal antibody against IL-17a, designed for the treatment of various Th17-mediated inflammatory diseases. In 2017, the FDA approved Brodalumab as a drug for uncontrolled psoriasis treatment. This therapeutic agent appeared promising for the treatment of steroid-resistant neutrophilic asthma, however, randomized studies of Brodalumab did not show significant therapeutic efficacy for severe asthma treatment [80].

TNF is a keystone cytokine for immune regulation and cell activation that plays a pivotal role in many inflammatory processes, including eosinophilic and neutrophilic airway inflammation. TNF produced by Th1 lymphocytes, macrophages and mast cells upregulates endothelial adhesion molecules [95]. Because TNF is overexpressed in the airways of patients with severe asthma, anti-TNF therapeutic agents were tested for efficacy in the severe asthma treatment with somewhat contradictory results. In an earlier study Etanercept was found to significantly improve lung functions in asthmatic patients with high levels of TNF in the sputum [81]. However, in a more recent study Etanercept failed to show efficacy in improving lung functions of moderate to severe asthma patients [82]. Furthermore, another study in patients with severe persistent asthma treated with Golimumab, another anti-TNF antibody, failed to provide any positive outcomes, but rather provoked serious adverse infections and malignancies [83].

The exact molecular mechanism of IL-6-dependent neutrophilic inflammation in asthma is not yet established, nevertheless, in vitro studies of human airway smooth muscle suggest that trans-signaling through IL-6 receptor may contribute to airway remodeling in asthma [96]. Moreover, in the mouse model of house dust mite-induced asthma, loss-of-function of IL-6 signaling – specifically in macrophages or dendritic cells – differentially abrogated elevations of eosinophil- and neutrophil-recruiting cytokines as well as allergen-induced airway inflammation [97]. Unfortunately, clinical trial of Sirukumab, a fully human monoclonal antibody against IL-6, in severe asthma was withdrawn (NCT02794519) after FDA disapproved Sirukumab for the treatment of moderately to severely active rheumatoid arthritis in the US.

All these aforementioned cytokines play an important and well-documented role in the pathogenesis of severe asthma. However, the reasons why some of the anti-cytokine therapeutics did not show efficacy in clinical studies are puzzling. The lack of a therapeutic effect could potentially be explained by the fact that systemic administration of anti-cytokine antibodies does not allow them to reach the necessary drug concentrations at the site of asthma-mediated inflammation: lung tissue and mucous membranes [98]. Recent study using non-human primates revealed that administration of monoclonal anti-IL-13 antibody Fab fragment via nebulizer, which transforms antibody solution into the respirable fraction capable of reaching terminal bronchioles, can effectively lower airway eosinophilia and Th2 cytokine production [99]. Moreover, this route of anti-IL-13 administration could enhance the effects of the biologic on the airway smooth muscle cells, airway epithelium and mucus-producing cells [98]. Taken together, this promising result warrants further investigations into the potential of therapeutic drug delivery directly into the airways during severe asthma therapy.

5. Concluding remarks

Evaluation of the molecular mechanisms underlying the pathogenesis of asthma and the nature of various environmental triggers has been performed primarily in mouse models of severe asthma. In addition, availability of unique genetically engineered mice has allowed to establish the role of individual components of innate and adaptive immunity, including distinct cell populations, cytokines and chemokines involved in the progression of each specific asthma subtype. Furthermore, these mice provide an opportunity to develop new therapies targeting specific molecules, cytokines or their receptors. Although mouse models do not replicate exactly human asthma, the mechanistic insights relevant to pathogenesis of this disease are generally applicable to humans. The main obstacle in studying severe steroid-resistant asthma in mice is the lack of a consensus disease model for pre-clinical drug evaluation or exploring novel therapeutic strategies.

Due to a better understanding of the pathophysiology of asthma together with the genome-wide expression studies in patients, the previously accepted practice of dividing asthmatics into two groups is currently perceived as oversimplification. Moreover, the concept of severe asthma management is rapidly evolving from the one-drug-fits-all paradigm to the endotype-specific therapy. Clinicians have now come to terms with the understanding that it is impossible to effectively manage different forms of asthma with a single drug and that susceptibility to therapy will depend on the severity of the symptoms as well as risk factors such as genetic predisposition and age of disease onset.

Over the past decades, several relevant mouse models for severe asthma were generated, which should help in evaluating novel therapeutic approaches including administration of neutralizing antibodies against pathogenic cytokines and their receptors. Additionally, studies using genetically engineered mice may help to identify much needed biomarkers for patient cohorts that are likely to respond to specific anti-cytokine therapies.

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