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Animal models of asthma: Reprise or reboot?

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ABSTRACT

Animal models of disease represent the pinnacle of hierarchical research efforts to validate targets and compounds for therapeutic intervention. Yet models of asthma, particularly in the mouse, which, for practical reasons, has become the *sine qua non* of asthma research, have been a bone of contention for decades. With barely a nod to their limitations and an extensive history of translational failures, they continue to be used for target identification and to justify the clinical evaluation of new compounds. Recent improvements – including sensitization directly to the airways; use of more relevant allergens; development of a chronic rather than short-term condition; utilization of techniques to measure lung function beyond uninterpretable measures of airway hyperresponsiveness – are laudable but cannot bridge the chasm between the models and the myriad complexities of the human disorder and multiple asthma endophenotypes. While further model developments are necessary, including recognition of key environmental factors beyond allergens, the judicious integration with newer ex vivo and in vitro techniques, including human precision-cut lung slices, reprograming of patient-derived induced pluripotent stem cells and fibroblasts to epithelial and smooth muscle cells, and use of other clinical samples to create a more holistic depiction of activities, might improve their translational success.

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1. Asthma: a complex immune disorder

Asthma is a chronic respiratory disorder where the main characteristic is variable and recurring episodes of airflow obstruction causing impaired breathing, which are reversible [1]. From this common trait, asthma rapidly becomes more complex in its definition. Aside from symptoms associated with narrowing of the airways – shortness of breath, wheezing, and chest tightness – the airways are inflamed and become hyper-responsive to various bronchoconstrictor agents and noxious stimuli (termed *airway hyperreactivity* or AHR). The pattern of features can vary between patients, as can their severity, ranging from mild asthma with occasional symptoms, to severe forms where the symptoms are persistent and impact daily life [2]. However, even patients with mild disease can suffer from severe acute attacks called exacerbations, where there is a rapid deterioration in lung function that is unresponsive to regular treatment. Over the long term airway remodeling with thickening of the epithelium and underlying smooth muscle can also lead to a fixed (non-reversible) obstruction of airflow [1]. Moreover, these different features of asthma, while being inter-related and acting in concert to create the disorder, probably have discrete causal mechanisms since they exhibit differential responses to different medications. For example, β2-adrenoceptor sympathomimetics dilate the airways but do not affect airway inflammation and do not suppress, and indeed may even increase, the incidence of asthma exacerbations [3]. In contrast, the anti-IgE monoclonal antibody omalizumab reduces exacerbations with relatively small effects on airflow [4].

Adding to the complex etiology of asthma is the recognition that there are both genetic and environmental factors that play significant roles in disease causality and occurrence. Twin studies indicate that genetics and environment each contribute about 50% of the risk to develop the disorder [5]. Simplistically, a principal viewpoint in the first half of the 20th century was that a large part of asthma was psychosomatic (even recommending treatment with a few whiffs of chloroform to relax the patient, or, the opposite approach, to take them toward the dentist's home to stress them into recovery). In contrast, the second half of the 20th century promoted the concept that asthma is an allergic disorder, and that the vast majority of asthma patients were atopic. More recently it has been recognized that approximately 50% of the US population in general is atopic, and since ~50% of the asthma population is also atopic it appears that it is not atopy per se that is the problem, but the asthmatic's response to particular allergens [6,7]. While up to half of asthma is still ascribed to atopy, there has been increased recognition of the contribution by other environmental and life-style factors such as pollution, cigarette smoke, chemicals, viruses, and diet. In contrast to the traditional view that the lower airways represent a sterile environment, a disordered airway microbial milieu might also contribute to the disease [8,9]. Changes to the gut microbiome also influence immune function [10]. As stated by Holmes et al. [11] "The realization that factors other than atopy are implicit in asthma has significant implications for disease modeling and drug development", but these have yet to be realized to any substantial measure.

Studies in the ovalbumin-sensitized and challenged mouse (OVA-mouse) model of asthma (see Section 4.1.), iterated with clinical observations, have played a large role in defining the term "asthma" as synonymous with an immune T helper 2 (Th2) cell-mediated disorder [12,13]. According to this paradigm, allergic sensitization and subsequent challenge results in T cell activation and transformation to a Th2 phenotype. The release of the Th2 pattern of cytokines, including interleukin (IL)-4, IL-5, IL-13, and IL-9, promote airway inflammation rich in eosinophils, which are considered responsible for the asthmatic response. The sequence of events and the importance of dendritic cells, Th2 cells and eosinophils to the allergic response, have been mapped out primarily in this mouse model, utilizing a variety of cell, gene and pharmacologic techniques resulting in a detailed model of pathways leading to airway inflammation and AHR, becoming the dominant mechanism of asthma over the last 30 years. While it is now recognized that the original concept of asthma as solely a Th2-mediated disorder is overly simplistic, and other T cell repertoires are also incriminated, including Th1, Th17, regulatory T (Treg) cells, cytotoxic CD8+ T cells, natural killer T cells (NKT) and γδT cells [12], nevertheless, murine asthma models have directed attention to discrete pathways and components of the immunological response deemed critical for the asthmatic response, and continue to be the primary model used to evaluate new therapies.

2. Heritability of asthma

The heritability of asthma is estimated to be 40–60% [5]. Genetic linkage studies initiated in 1986 to identify the genetic basis of asthma reported associations with a substantial number of genes that made apparent mechanistic sense based on prevailing wisdom from mouse models and clinical observations, such as IL4, IL13, IL4RA, FCER1B and CD14 [14]. In 2007 genetic linkage studies were superseded by genome-wide association studies (GWAS), resulting in most of the previously reported genetic associations disappearing [15]. However, there is not full agreement even between the major GWAS, while the genes that have been identified account for only a trivial portion of asthma heritability. Even the long recognized association with HLA could not be ascribed to a single genotype. GWAS have differentiated genetic variants associated with asthma from those associated with atopy, indicating the two are not synonymous [16].

A principal finding, since it has been replicated independently in several studies, is that SNPs located on chromosome 17q21 close to the genes for ORMDL3 (orosomucoid-like 3) and GSDML (gasdermin-like) indicate asthma susceptibility, particularly in childhood asthma [16,17]. This region seems to be associated with chronic inflammation since it has also been implicated in ulcerative colitis, Crohn's disease and Type I diabetes by GWAS [18]. ORMDL3 had not been considered as a mediator of asthma previously, and its relevance was not immediately clear. A subsequent investigation found that ORMDL3 expression in airway epithelium is induced in sensitized mice by exposure to ovalbumin or the cytokines IL-4 and IL-13, in a STAT-6-dependent manner [19]. ORMDL3, in turn, regulates activation of the transcription factor ATF6, linked to SERCA-2b and implicated in airway remodeling; while ORMDL3 also activates several other genes encoding certain metalloproteases, chemokines and other factors. Whether it subserves a key role in human asthma remains to be determined.

GWAS have also identified SNPs in regions implicated in Th2 signaling, including the RAD50-IL-13 locus, IL33 and IL1RL1/ST2, while rare variants in the coding exons and flanking regions of candidate genes in the EVE consortium GWAS implicated IL12RB1 [15,17]. Data from the Gabriel GWAS was used to provide evidence for an association between asthma and the gene for the transcription factor TCF3, which binds β-catenin and is a downstream effector of the *Wnt* signaling pathway [15].

Based on the results from a recent GWAS assessing genes associated with lung function in asthmatics it has been proposed that variants in Th2 pathway genes – IL13, IL33, TSLP and IL1RL1 – are associated with asthma susceptibility, while variants in Th1 or IL-12 cytokine family genes (including IL12A, IL12RB, STAT4, and IRF2 among others) are related to lung function and airway remodeling [20].

3. Asthma heterogeneity

It is now well recognized that the term "asthma" represents a heterogenous group of disorders comprised of different phenotypes and endotypes. The Oxford English dictionary defines phenotype as "the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment", while the endotype defines the distinct pathogenetic mechanism underlying the phenotype. While it would seem premature to attempt to differentiate patients into distinct endotypes given the level of mechanistic understanding of asthma, even sorting asthmatics into distinct phenotypes is problematic because of the lack of well-validated, specific markers [21,22]. One early method to differentiate phenotypes was based on sputum cytology to assess inflammatory cell content, resulting in the identification of eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic phenotypes. If specific inflammatory cells are the central component to the different range of asthmatic conditions, such a discriminatory process could be important to help define appropriate treatment and track effectiveness. However, to be a meaningful distinction between phenotypes requires that the cellular profiles remain stable over time. Unfortunately, only one third of moderate to severe asthmatics maintain the same sputum inflammatory cell phenotype over a 12-month period [23].

Several studies have also used cluster analysis to group key variables related to defined clinical aspects and identified novel phenotypes in childhood and adult asthma (reviewed in [21]).

Another approach has been to use molecular phenotyping to segregate patients. Half of the asthmatics appear to express a phenotype consistent with Th2 driven inflammation, while the other half do not – leading to the designations *Th2-high* and *Th2-low*, respectively [24]. The Th2-high phenotype has several distinctive features and immunological markers. The cytokines IL-5 and IL-13 are elevated in bronchial biopsies, and an IL-13 gene signature showing enhanced upregulation and coordinated expression of periostin (POSTN), chloride channel regulator 1 (CLCA1) and serpin peptidase inhibitor, clade B, member 2 (SERPINB2) in airway epithelial brushings and lung biopsies. In addition, Th2-high patients have increased serum IgE levels, blood and airway eosinophilia, and AHR, but not reduced FEV1 or atopy, compared to Th2-low subjects [24]. Significantly, it is the Th2-high and not the Th2-low phenotype that responds to inhaled corticosteroids. Further studies by this group [25] extended the Th2-gene associations, identifying 79 co-expressed genes comprising cytokines, chemokines, growth factors, *Wnt*, and TGF-β family members.

3.1. Validation of molecular phenotyping: IL-13 monoclonal antibodies

In a Phase 2 clinical trial of the IL-13 monoclonal antibody, lebrikizumab [26], the Th2-high group was defined either by the clinical profile of increased serum IgE (>100 IU/ml) plus peripheral blood eosinophilia ($>0.14 \times 10^9$/L), or, at the end of the study, by the serum periostin level (above the median value) chosen as a singular biomarker of IL-13/Th2-high phenotype, as all 3 signature genes were coordinately expressed. Using FEV1 lung function measured at week 12 as the primary endpoint, the Th2-high group responded better to lebrikizumab than the Th2-low group, thereby further validating this group as a distinct phenotype with a different underlying mechanism and hence therapeutic response to particular treatments.

A clinical Phase 2 study in moderate to severe asthmatics of another anti-IL-13 antibody, tralokinumab, gave broadly similar results to those achieved with lebrikizumab [27]. While in this trial the primary endpoint of improvement in an Asthma Control Questionnaire was not achieved, dose-related improvements in a secondary endpoint, FEV1, were observed, reaching statistical significance at the highest dose. A post hoc analysis of a sub-group of patients who provided sputum samples, suggests that patients with sputum IL-13 levels ≥10 pg/ml show the greatest improvement in lung function, whereas patients with lower sputum IL-13 levels do not differ from placebo-treated subjects.

While a detailed discussion of the anti-IL-13 clinical data is beyond the scope of this review, several aspects of the study are relevant to the discussion of appropriate animal models. First, the Th2-high phenotype is not associated with atopy, the principal mechanism of provoking asthma in preclinical models. Whereas they both arrive at a similar cellular profile, the underlying mechanisms might be quite different. Moreover, while mouse studies have implicated IL-13 in AHR development, the identification of IL-13 as a pivotal cytokine in asthma has not been clear-cut since blockade of IL-13 using a variety of approaches, while reducing AHR and mucus production following antigen challenge, has variable effects on the inflammatory component [28,29]. Results from the mouse model alone would be unlikely to allow the selection of IL-13 preferentially over multiple other potential candidates. Moreover, administration of an anti-IL-13 antibody to mild asthmatics challenged with an allergen in a manner analogous to the mouse model had no effect on AHR [30]. The IL-13 biomarker used clinically-periostin - is a secreted matricellular protein implicated in subepithelial fibrosis, tissue infiltration of eosinophils, and increasing TGF-β activity [31], such that it has been considered as a potential mediator in asthma. However, periostin deficient mice show increased, rather than the expected decreased, AHR and serum IgE levels following challenge with *Aspergillus fumigatus* antigen, as compared to wild-type mice [32], thereby obfuscating the picture, and raising potential issues with the use of genetically modified mice.

4. Animal models of asthma

Asthma is predominantly a human disorder and only cats and horses can spontaneously develop similar conditions. About 1% of cats, and an indeterminate number of horses (since it appears to depend on stabling and feeding conditions) develop an allergic airway response analogous to allergic asthma, but do not lend themselves readily to the exploration of disease mechanisms or testing of new therapies in the laboratory setting. Given the complexities of the asthmatic condition described above – involving multiple features, heterogeneity with differing cellular pathologies, underlying genetic factors, poorly identified and controversial environmental components, etc. – it is challenging to create animal models that can capture the full range of components and mirror the human situation.

Several species and methods to induce a human asthma-like condition have been utilized, with most standard laboratory animals being employed. Each model has its proponents – rats [33], guinea pigs [34], rabbits [35], dogs [36], sheep [37] and primates [38,39] – although the standard model adopted almost universally is the OVA-mouse [40]. This is primarily for practical reasons based on cost, short reproductive cycle, size (for housing), convenience, being amenable to the development of transgenic models, and the readily available array of immunologic and molecular tools for analysis of mediators and pathways. Now in addition, an extensive database of putative drugs evaluated in this model allows comparison with any new agents identified. It must be recognized that the OVA-mouse does not develop "asthma", but demonstrates certain cellular and pathophysiological features reminiscent of the asthma condition.

4.1. Ovalbumin mouse model

The OVA-mouse model is created by first sensitizing the mouse with the injection of OVA i.p. together with an adjuvant, usually aluminum hydroxide, to enhance the OVA immunogenicity (for reasons that are not completely clear although it augments the Th2 response). This is followed with a second, booster ip injection 1–2 weeks later. After waiting several more days for the immune response to fully develop, the mouse is then challenged with OVA delivered to the airways, usually either intratracheally or via aerosol. This results in a robust airway inflammatory response developing over the ensuing 24–48 h, and AHR, which represents the principal measure of lung function. There are a multitude of variations to this basic model, only a few of which will be discussed here.

There are striking differences between strains of mice (and rats) in their response to an allergen in both the immunological and functional (AHR) responses [41,42]. Concerns over the need for an artificial adjuvant like aluminum hydroxide, which has a complex and not completely understood mechanism, and questions regarding the relevance of OVA, have prompted the use of alternative allergens considered more appropriate to the human condition, including house dust mite (HDM), cockroach, ragweed and *Aspergillus*. Some of these can sensitize mice when administered by inhalation without the need for an adjuvant, while utilizing multiple antigens simultaneously can also avoid an adjuvant [43].

Asthma is a chronic disorder, while the standard OVA-challenged mouse, like many animal models of human disease states, is an acute model where the response resolves spontaneously over a few days. This limits the suitability of the model addressing questions regarding airway remodeling or if therapeutic interventions can reverse the features reminiscent of clinical asthma once they have been established. This latter point is potentially important since the mechanisms of disease initiation differ to those involved in disease maintenance [43], while the model primarily permits only addressing the former. These concerns have given impetus to the development of more chronic models; an effort which has met with limited success, either due to the development of tolerance with repeated OVA challenges (resulting in diminishing airway inflammation and AHR), or, by using lower doses of allergen to avoid tolerance, the response achieved is relatively mild with a small window to show therapeutic benefits. There have been some successes [43–45] – for example the use of multiple antigens concomitantly seems to avoid the development of tolerance – but a single chronic model has not yet been adopted widely.

4.2. Non-human primate models of asthma

Non-human primate models of asthma have been developed that rely on natural sensitivity to *Ascaris* (or a closely related parasite) observed in the majority of rhesus monkeys in the wild, or by inducing sensitivity in rhesus or cynomolgus monkeys to house dust mite (HDM) or the birch pollen antigens, Bet V1 and V2, which allows for more control over the sensitization process [38,39]. Challenge results in increased IgE and a Th2-biased immune response. Proponents of the primate model point to the striking similarities with the response to allergen challenge in human asthmatics, with an early and a late-phase bronchoconstrictor response, airway eosinophilia and AHR. Moreover, anatomically and structurally the non-human primate resembles the human situation more closely than the mouse, and allows for the study of human protein and monoclonal antibody therapeutics because of the close similarity to the human proteins – a feature often not available in non-primate models. Major limitations to the primate model are the cost and the daunting effort required to create and maintain the model, where HDM-induced sensitization develops over 18 months and requires frequent exposures to the allergen over the whole period [46]. A colony of non-human primates can be re-studied several times if patient-like endpoints are utilized (airway function, BALF sampling) to justify the time and cost investment in this model. While this model offers distinct advantages over murine models, even proponents acknowledge "studies of nonhuman primate models of asthma can complement and supplement studies of mouse models." [46].

5. Suitability of animal models of asthma

Since the OVA-mouse model is the current gold standard, most of the ensuing discussion will focus predominately on this model, coupled with reference to the non-human primate where appropriate. The debate on the merits and limitations of the mouse model has been ongoing for more than two decades, with only some of the publications being cited here [11,40,43,45,47–52]. Some issues are specific to the mouse, while others relate to allergen sensitization and challenge.

The ideals of animal models of asthma would include:

- similar genetic basis to the human disease or can be readily engineered
- similar anatomy and physiology to human
- similar pathological response and underlying mechanisms that recapitulate key features of the human disease
- employs similar endpoints as used in a clinical trial
- responsive to drugs with known clinical efficacy
- predictive of clinical efficacy – translational validity

evaluating the merits of the OVA-mouse model against these criteria is revealing.

5.1. Similar genetic basis to the human disease

Section 2 addressed the results of clinical studies to define asthma susceptibility genes, and those linked to different clinical aspects such as lung function. While the early genetic linkage studies identified genes consistent with the mechanisms defined in the OVA-mouse model, several of these were hypothesis-driven (i.e. searching only for specific genes, and thus self-fulfilling) or screening for familial traits (which might only be relevant to the small group studied), were poorly reproducible and not substantiated by the larger GWAS [14]. The genes identified to date account for only a small part of asthma heritability so more variants are likely to be discovered [17], making it virtually impossible to recapitulate the genetic basis of the disorder in animals. Unfortunately few, if any, of the genome-wide variants identified in patients have come up in corresponding GWAS of the mouse or primate models of asthma [46]. Recent GWAS evidence for the dual importance of certain Th2- and Th1-related genes to the overall asthmatic picture [20] adds a further level of complexity not captured by animal models.

While molecular phenotyping asthmatics has enabled segregation of patients into Th2-high and Th2-low sub-groups [24], the OVA-mouse model is exclusively Th2/eosinophil driven and has some associations with the Th2-high phenotype. Likewise, the Th2-high/IL-13 gene signature has also been observed in lung biopsies in a rhesus monkey asthma model sensitive to HDM [46]. Moreover the extended Th2 gene set used to profile Th2-high asthma patients [25] was also applied to the rhesus monkey, where a number of the genes were modestly upregulated and correlated with the human results [46]. These results were compared to a reanalysis of lung gene expression microarray data from a previously published study in the OVA-mouse model. Certain

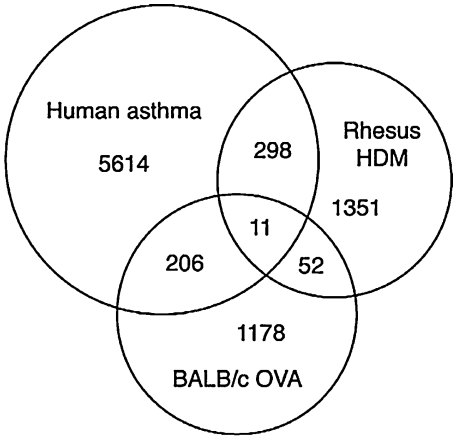


Fig. 1. Venn diagram of asthma genes profiles in Th2-high human patients, rhesus monkeys sensitized with house dust mite (HDM) and BALB/c mice sensitized with ovalbumin (OVA). Numbers within overlapping circles indicate number of genes in common. There are only 11 genes in common between all three species.
Taken from Abbas et al., 2011 [46]; with permission from Elsevier.

select genes associated with Th2-inflammation were identified, including CLCA1 and 15-lipoxygenase. Remarkably, when comparing all the differentially expressed genes associated with Th2-high asthmatics to the rhesus HDM model and the OVA-mouse, only 11 genes were upregulated in common across all 3 species ([46]; see Fig. 1). This should be the human asthma phenotype most closely related to the two animal models. One of the genes in common was periostin, while the significance of the other 10 genes identified is unclear.

5.2. Similar anatomy and physiology to human

Mice have a number of anatomical and physiological differences in their airways and lungs from humans. For example, in mice the primary distal airway is the nonalveolarized bronchiole, whereas in humans (and primates) it is the respiratory bronchiole [11]. Moreover, the type and location of cells can vary – e.g. basal cells are found throughout the tracheobroncial airways of humans, but only in the tracheas of mice, which have a larger Clara cell population in the small airways [46]. Significantly, mice do not have a bronchial circulation [53], which could affect mechanisms of leukocyte recruitment, while mouse airways have substantially less smooth muscle, and do not respond readily to pharmacologic agents affecting bronchial tone [52].

5.3. Similar pathological response and underlying mechanisms that recapitulate key features of the human disease

While the OVA-mouse model mirrors certain key aspects of a Th2/eosinophil driven asthma-like condition, there are some notable differences. While lung inflammation is regarded as the critical feature of the model, paralleling events in asthma, the model focuses on mechanisms of inflammation *development* rather than *maintenance*, or exacerbation of an already inflamed background. Mice have more eosinophils in broncheoalveolar lavage fluid (BALF) – 40–80% as compared to 1–5% in human asthma, and the murine eosinophils do not degranulate [52,54]. In the airways the location of the inflammatory cells also differs [46]. In the mouse leukocyte accumulation is largely perivascular with edema around blood vessels, while the peribronchiolar component is diffuse and variable. In humans immune cell accumulation is in the bronchial wall, largely in the conducting airways. The mast cell hyperplasia and infiltration of airway smooth muscle that occurs in human asthma is not observed in mice [52].

The use of ip OVA and aluminum hydroxide to sensitize mice bypasses innate receptors (e.g. toll-like receptors) and their effectors in the airways, including antigen presenting cells (APCs) and other elements of the epithelial-mesenchyme trophic unit (EMTU) implicated in propagating and amplifying asthma signals [52,55]. This limitation can be overcome with methods to sensitize the airways directly [44]; for example, by using multiple allergens administered intranasally.

Airway remodeling in chronic asthma is attributed primarily to smooth muscle hyperplasia and hypertrophy with angiogenesis occurring in the airway mucosa, whereas the mouse develops fibrosis and no increased vascular component [11].

5.4. Employs similar endpoints as used in a clinical trial

The most common functional endpoint used in the OVA-mouse model is AHR. The measurement of AHR is considered objective and reproducible in clinical asthma and has been used to guide treatment resulting in fewer "mild" exacerbations and improved lung function (FEV1) [56]. Overall, AHR is not considered a replacement for measures of lung function such as FEV1, and also does not appear to be related to asthma symptoms or airway inflammation. However, the ATS task force recommended the inclusion of AHR with other indices of lung function to help ascertain the adequacy of asthma control in patients receiving therapy [57], so this parameter would appear to have some potential clinical relevance.

Clinically AHR is an increased sensitivity and reactivity to a diverse array of bronchoconstrictor agents that include carbachol, methacholine, leukotriene E4, histamine, serotonin, adenosine, PGF2α; in addition to noxious stimuli, e.g. sulfur dioxide. However, it is not a non-specific increase in responsiveness to all spasmogens. The majority of animal asthma models utilize only a cholinergic agonist to define AHR, and do little to assess if the features, such as selective sensitivity to particular agonists, are similar to the clinical condition. The limited relevance of studying just one agonist is demonstrated in a study where in vivo AHR to methacholine was observed in the mouse allergen challenge model, the trachea was then removed and the increased sensitivity to methacholine confirmed in vitro, but no enhanced response to histamine, serotonin or adenosine was found [58]. AHR in mice can be elicited by a number of stimuli in addition to allergen challenge, e.g. LPS, ozone, proteolytic enzymes, cigarette smoke, implying it is a common response to injury that could have different underlying mechanisms and features [13]. AHR is also influenced by airway remodeling in patients with chronic asthma, which involves changes in airway smooth muscle hyperplasia, epithelial function, extracellular matrix composition, mucus secretion, blood flow distribution and airway innervation [45]. Differences in airway and lung anatomy and physiology between humans and animal models (Section 5.2.) also question the extent to which the AHR phenotype is synonymous between the two. Many of these anatomical and physiological differences are minimized in the non-human primate model where AHR is also used as the primary measure of lung function [39,46,59].

Certainly standardization of techniques and improved rigor in how AHR is measured in mouse models can only improve the value of the information obtained [60]. Valuable critiques of different methodologies and pitfalls to interpretation have been published [51,60,61]. Changes in baseline levels of bronchomotor tone are frequently not defined but can impact bronchoconstrictor-induced changes. AHR reflects both an increased sensitivity and an increased reactivity, requiring judicious use of dose-response analyses to define both the leftward shift (denoting increased sensitivity) and an increased maxima or slope (to define reactivity).

Whole body unrestrained plethysmography and the measurement of "enhanced pause" or Penh as an artificial parameter of airway function has become a popular non-invasive method applied to small animals such as mice to measure AHR. However, there are significant concerns regarding what is actually measured and how it is interpreted [51,60]. For example, comparing lung function changes to methacholine measured by Penh to the more direct forced oscillation technique in 29 different strains of mice led to the conclusion that Penh was regulated by underlying genetic factors rather than mirroring changes in airway resistance and lung compliance [62].

There are marked differences between mouse strains in the development of AHR. Comparing 31 mouse strains showed a 4.2 fold difference in AHR slope (the slope of dose-related increases in logarithmically transformed resistance induced by methacholine) across the different strains [63]. Using available genotype data for the 31 strains, in silico association mapping of SNPs with AHR was performed and compared with GWAS data in asthma patients to identify a subset of homologous genes associated with both asthma and AHR. From this effort the Kv channel interacting protein 4 (*KCNIP4*) gene was identified [63], suggesting some commonality, but this finding requires validation.

Using computer simulations to define AHR in the OVA-mouse model based on measures of resistance, intrinsic dissipative and elastic properties of the lung, led to the conclusion that an abnormality of the airway smooth muscle was not involved, but instead was linked to physical changes occurring in the periphery of the lung itself [51]. This suggests that AHR in mice is measuring something that is fundamentally different to the response in human asthma, and thus requires further investigation. Since lung volume affects the bronchoconstrictor response, lung volume status should be measured concomitantly when determining AHR [60], but this is rarely performed.

Regardless of whether or not clinical AHR and OVA-mouse AHR are both measuring airway smooth muscle contractile function, and despite the potential for AHR to add another dimension to patient profiling for risk analysis and treatment options, currently AHR is not an endpoint for clinical trials seeking approval by drug regulatory authorities, since it is not clear what it defines or how critical it is as an independent outcome measure. Consequently, AHR is not a relevant endpoint that is readily translatable to the clinical evaluation of new therapies.

To date asthma drugs have been approved based on measures of lung function such as FEV1, and the ability to reduce the incidence of severe exacerbations. These endpoints are not provided by most of the animal models resulting in a significant gap between measures of efficacy in animals and humans.

5.5. Responsive to drugs with known clinical efficacy

There are currently 5 classes of drugs used in the treatment of asthma [64]. These can be divided into 3 groups based on their activity profiles:

- bronchodilators that include β2-adrenoceptor agonists including salbutamol, salmeterol and formoterol, and M1/M3 selective muscarinic cholinergic antagonists, e.g. tiotropium and ipratropium
- mixed anti-inflammatory/bronchodilators that include inhaled corticosteroids like fluticasone and budesonide, and leukotriene antagonists, in particular montelukast
- the anti-IgE monoclonal antibody omalizumab.

5.5.1. Bronchodilators

As noted, the primary measure of lung function commonly used in the mouse (and non-human primate) model of asthma is AHR. Consequently bronchodilation per se is not evaluated, but rather an ability to blunt AHR. Acutely the β2-adrenoceptor agonist salbutamol diminishes AHR to methacholine in the antigen-challenged mouse [65], behaving as it would clinically as a short-term "reliever" medication to treat asthma. The situation becomes more complicated when attempting to assess the benefits of long-term β2-adrenoceptor stimulation in the murine asthma model. Salbutamol treatment for 28 days produces no reduction in AHR, in contrast to its acute effects [65]. Moreover, β2-adrenoceptor deficient mice show a dramatically reduced AHR, rather than the enhanced reactivity expected when intrinsic functional antagonism is removed; while AHR is enhanced in mice overexpressing β2-adrenoceptors [66]. More recently it has been shown that activation of β2-adrenoceptors paradoxically is actually *required* to develop the asthma phenotype in the mouse, including the features of airway inflammation and AHR [67].

Cholinergic antagonists such as tiotropium attenuate 5-HT-induced AHR in a chronic OVA-mouse model (although only assessed using a single dose of 5-HT), and also inhibit airway inflammation and Th2 cytokine release from cells [68], making it difficult to separate actions on the different events.

5.5.2. Mixed anti-inflammatory/bronchodilator agents

Inhaled corticosteroids (ICS) are the mainstay of asthma treatment, decreasing airway inflammation, improving measures of lung function and blunting AHR [2]. These effects are mirrored in the OVA-mouse model treated with systemic corticosteroids, where both lung inflammation and AHR are decreased [69]. Cys-LT1 receptor antagonists, e.g. montelukast, show a similar profile in the OVA-mouse in reducing BALF inflammatory cell and AHR [70,71], although in the clinic montelukast does not affect AHR [72]. Switching the allergen from OVA to respiratory syncytial virus (RSV) reaffirms the actions of montelukast on airway inflammation and AHR, but only if the drug is given one day before the initial infection [73].

5.5.3. Anti-IgE monoclonal antibody

IgG1 rather than IgE mediates the allergic response in the standard OVA-mouse model [52], although antigen-challenged mice can be manipulated to create a major role for IgE. For example, combining RSV infection with HDM inhalation to sensitize mice results in an exacerbated eosinophilic inflammation and airway mucosal thickening accompanied by elevated serum IgE levels [74]. Treatment with a rat anti-mouse IgE monoclonal antibody results in the amelioration of the airway inflammation and epithelial pathology. An alternative approach has been to use OVA in mice specially selected for their production of large amounts of IgE [75]; a model associated with large numbers of basophils in the airways together with eosinophils. In this setting an anti-IgE monoclonal antibody decreases the recruitment of both cell-types to the airways, and reduces AHR.

The wealth of data defining the importance of IgE to allergic responses in general fueled the search for a therapeutic anti-IgE antibody regardless of results in animal models of asthma. Omalizumab, the only approved anti-IgE monoclonal antibody, reduces the incidence of exacerbations and emergency room visits in patients with more severe forms of asthma, and improves scores on the asthma quality of life questionnaire, when added onto a background of ICS and β2-adrenoceptor agonist treatment [4]. In a review of the clinical trial experience with omalizumab it was concluded that effects on lung function were conflicting. Although small improvements in lung function are likely, there is "no

definitive benefit evident" [4]. Similarly only small effects on AHR have been reported in asthmatics subjected to allergen challenge [76], while omalizumab does reduce airway eosinophils, T-cells and mast cells [77]. However, the clinical response to omalizumab is variable with no reliable predictors of the magnitude of efficacy.

5.6. Predictive of clinical efficacy–translational validity

The literature is replete with studies demonstrating the benefits of a range of therapeutics in the OVA-mouse model. An analysis [13] of the clinical translational success of compounds targeting the Th2 asthma archetype, based on target validation and murine model testing, showed that 38 agents acting on 22 separate targets within this paradigm only 3 or 4 of the targets translated successfully. These include the monoclonal antibodies to IL-5 and IL-13; pitrakinra, an antagonist of the common α-chain in the IL-4 and IL-13 receptors; while the jury is still out on the mixed oligonucleotides in ASM8 targeting both the common β-chain to the receptors for IL-5, IL-13 and GM-CSF, together with the chemokine receptor CCR3, since the successful clinical allergen challenge study was not replicated. Even in the examples of successful translation, the profile and utility of the compound bore little resemblance to the predictions of the animal models. For example, to date all drugs targeting IL-5, IL-13 or the IL-4/IL-13 receptor do not work in mild to moderate asthmatics (90%+ of the asthma population), and only on select measures of the disorder such as rate of exacerbations rather than symptoms or AHR (referenced in [13]).

The poor translational success to date for drugs targeting the Th2 pathway is only a part of the story of the poor predictability of animal models of asthma. The list of failures is extensive and has continued for decades, and includes antagonists to platelet-activating factor (PAF), substance P and other neurokinins, thromboxane A_2, prostaglandin D_2, bradykinin, as well as inhibitors of inducible nitric oxide synthase (iNOS), cathepsins, and a range of natural product analogs. Despite this poor translational track record, publications continue to conclude that mouse efficacy data can indicate the potential value of a compound to treat clinical asthma.

6. Does this infer that animal models of asthma have no utility?

It is reasonable to question whether the animal model or the overly zealous investigator is the primary cause of the current poor translational success of asthma models. Mouse models have utility "up to a point, but it is important to recognize the associated problems and limitations" [45]. Unfortunately, few investigators define the parameters to the conclusions of their studies, but promulgate often meaningless interpretations, where a mediator is deemed "obligatory" or "critical" or "crucial" before it becomes marginalized by an expensive clinical trial failure. Awareness of the limits of the animal models, and insights into appropriate interpretations of datasets would improve their value.

Asthma is a complex, heterogenous disorder and it would be a mistake to believe that it can be reproduced in any single animal model. Just looking at the common genetic basis between the human disorder and mouse and primate models indicates how far apart they are in reproducing similar underlying predispositions (Fig. 1; [46]). There are a number of disconnects. Laboratory animals that live in an artificial, controlled, pathogen-free environment are unlikely to model the environmental factors that contribute approximately 50% to the asthma susceptibility risk. Similarly, atopy and asthma are not synonymous, yet almost all asthma models are allergen-based. It is sobering to recognize that not just new mediators, but also new major cell-types continue to be implicated in the mouse model of asthma on a regular basis. These include Th17, regulatory T cells, natural killer T cells, γδ T cells and type 2 myelid cells [12,78], despite the cellular make-up of airway inflammation having been a primary focus of asthma research for over 30 years, indicating that our level of knowledge remains rudimentary.

Depending on the questions being asked, a particular model may help *guide* the investigator, but one should not be deluded into thinking they are studying asthma per se or even the mechanisms defining the underlying pathogenesis. The recognition of distinct asthma phenotypes in humans has yet to be replicated in animal models of asthma, and probably requires the creation and validation of asthma models outside of the allergenic type.

While significant improvements to historical models have been made – e.g. sensitization with more relevant antigens and without the need for an adjuvant [43]; sensitization via the airways rather than ip [43,44,51]; chronic rather than acute models to look at experimental compounds administered therapeutically after the pathology has been established [44,45]; improved methods to measure lung function rather than AHR [51,60,61] – their practical benefits have yet to be established with regard to target validation and prediction of clinical drug activity.

A combination of animal models used judiciously can aid in defining the profile of a molecule when the target has been validated by other means (see for example [59]). They can also help define the relationships between routes of administration, levels of systemic and lung exposure, duration of compound levels, and relate them to their pharmacodynamic activities providing relevant information for planning clinical trials.

7. Alternatives to animal models

While a discussion of alternatives to animal models is beyond the scope of this article, it is relevant from the viewpoint that decisions regarding target validation and drug activity are based on data from multiple sources in addition to animal models, many of which have been reviewed recently [11,54]. Aside from cell culture, patient sputum and BALF samples, newer in vitro techniques are being developed including human tissue explants and precision-cut lung slices [61], although these are handicapped by their short lifetime for investigation and limited supply. A more promising source of human cells is the use of patient-derived induced pluripotent stem cells, redirected to form epithelial or smooth muscle cells for example, or by direct reprogramming of patient fibroblasts [79]. These cells retain the genetic components of the disorder that underlay development of a particular phenotype. Genetic pathways can be probed to recreate the phenotype using techniques involving zinc-finger endonucleases or TALENs (transcription activator-like effector nucleases), preferably on an isogenic background to improve delineation of the endotype [80]. While asthma is a disorder involving multiple cell-types acting in concert, there is merit to defining how particular cells are affected and contribute to the overall pathology, as well as validating targets and evaluating new therapeutics.

Various in silico techniques are also being explored [54]. Computer models of airways use mathematical descriptors to determine mechanisms underlying changes in lung function. Attempts to create a "virtual patient" by integrating structural and functional information with genomics, proteomics and cell biology are ongoing. However, such models are only as accurate or as useful as the relevance and quality of the data upon which they are based. Many investigators supplement clinical information with animal model data to define important pathways, targets for drug discovery, and anticipated clinical profiles of new drugs. This can hinder their value dramatically.

Validation of animal model data using a human allergen challenge setting has been proposed as a bridge between

preclinical studies and large clinical trials in a broader asthma setting [81]. This human model takes advantage of known sensitivities to particular allergens in mild asthmatics to provoke short-term changes in lung inflammation, airway function and AHR, so has a number of features analogous to the animal models. Indeed, for a while activity in a human allergen challenge setting was almost obligatory for a compound to advance in development. However, this setting is not predictive of clinical asthma, and provides only limited insights into the therapeutic profile of any new agent, so does not represent an early clinical go/no go decision point. While it can still provide useful information and be used to determine appropriate routes of administration for example [82], it is not appropriate to be on the "critical path" of clinical evaluation.

8. Conclusions

Asthma is prevalent and growing. In the US approximately 18.7 million people (8.2% of the population) have asthma, with the total health care burden being estimated at $20.7B per year, 80% of which is taken up by emergency room visits and urgent care [83]. Given the magnitude and scope of the problem it is disconcerting that few new drugs have entered the market in the last 30 years, since there is a clear and urgent need. Animal models have been used to map the detailed immunological pathways that result in an asthma-like syndrome following antigen challenge; to demonstrate efficacy of a large number of research compounds active at key sites in the pathways; and been used to justify advancement to clinical trials where they have either failed to show efficacy, or are effective only in a small sub-population and with an activity profile not predicted by the animal studies. Clearly, animal models of allergen sensitization have limited value, a fact that needs to be acknowledged and addressed. Thus, while there are substantive concerns that the mechanistic complexity of asthma can never be reduced to a definitive animal model for the reasons outlined in this article, what is needed is the development of a range of clinically-relevant (e.g. validated), targeted models in vitro, ex vivo and in vivo designed to address different aspects of the disorder, titrated to the specifics of a particular approach or pathway, which when combined provide a comprehensive overview of activities [11].

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