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Review Article

Back to the future: re-establishing guinea pig *in vivo* asthma models

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Research using animal models of asthma is currently dominated by mouse models. This has been driven by the comprehensive knowledge on inflammatory and immune reactions in mice, as well as tools to produce genetically modified mice. Many of the identified therapeutic targets influencing airway hyper-responsiveness and inflammation in mouse models, have however been disappointing when tested clinically in asthma. It is therefore a great need for new animal models that more closely resemble human asthma.

The guinea pig has for decades been used in asthma research and a comprehensive table of different protocols for asthma models is presented. The studies have primarily been focused on the pharmacological aspects of the disease, where the guinea pig undoubtedly is superior to mice. Further reasons are the anatomical and physiological similarities between human and guinea pig airways compared with that of the mouse, especially with respect to airway branching, neurophysiology, pulmonary circulation and smooth muscle distribution, as well as mast cell localization and mediator secretion. Lack of reagents and specific molecular tools to study inflammatory and immunological reactions in the guinea pig has however greatly diminished its use in asthma research.

The aim in this position paper is to review and summarize what we know about different aspects of the use of guinea pig *in vivo* models for asthma research. The associated aim is to highlight the unmet needs that have to be addressed in the future.

Introduction

Respiratory diseases have a complex pathobiology where interplay between inhaled matter, local host defense in the airways, and systemic immunological and endocrine responses lead to different disease phenotypes. Although components in the different disease processes may be investigated in human cell and tissue models, the integrated understanding of the relative importance of implicated disease mechanisms can only be established in the intact body. Experimental medicine approaches in healthy humans or patients with disease provide the highest degree of evidence in the testing of hypotheses for physiological, pharmacological or pathological reactions. Nevertheless, before it is ethical to embark on studies in humans, understanding of mechanisms, pharmacodynamics, pharmacokinetics and safety must be established in valid experimental models.

It goes without saying that the foremost criterion for the use of animal models to investigate questions concerning human physiology or disease pathobiology is that the model is of relevance to the human disease or the specific reaction that is addressed. Over the past few decades, the development of genetically

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modified mice strains has introduced unprecedented opportunities to define mechanisms and pathways by over-expression or deletion of proteins mediating or regulating a variety of processes. There are a number of discoveries in such studies that have been important for the understanding of, in particular, the immune system [1]. In respiratory medicine, and particularly for airway diseases such as asthma and COPD, the mouse models have however been disappointing and led to few, if any, new discoveries with relevance to the modeled disease entities in humans [2,3].

Likewise, animal experiments are often required to follow-up and analyze in greater detail observations in patients. In animal welfare interests (3Rs: for replace, reduce and refine animal use), several alternatives for animal experimentation by using human *in vitro* models have been developed, ranging from very simple models using human cells in mono- or co-culture, whole tissue explants and new tissue engineering approaches [4]. However, when studying such a complex disease as asthma involving multiple genetic and environmental influences whose interactions result in intricate systemic activation and multifactorial alterations of pulmonary function [5], *in vivo* animal experimentation is presently unavoidable. The development of refined asthma models in guinea pigs have great potential to replace irrelevant mouse models and thereby reducing the number of animals needed.

For this reason and relating to the fact that discoveries using guinea pig models already in the early 20th century identified mechanisms that have been directly translated into human physiology or pathology, the Centre for Allergy Research Highlights Asthma Markers of Phenotype (ChAMP) project at Karolinska Institutet held a workshop on guinea pig models in the late spring of 2017. The meeting was followed by a process leading to the compilation of this review article summarizing the state-of-the-art concerning the use of guinea pigs for studies of airway diseases including asthma, COPD, and chronic cough. In this review we have focused on the *in vivo* models, although very interesting *in vitro* findings also have been performed, e.g. using isolated guinea pig airways. We hope that researchers in academia and industry will benefit from this first very comprehensive account of fundamental aspects on the use of guinea pigs for respiratory research.

Guinea pig models of asthma: what have we learned?

Immediate hypersensitivity reaction of the lung was discovered in guinea pigs more than 100 years ago [6]. Preclinical pharmacological studies performed in guinea pigs have proven indispensable to the development of therapies for a wide range of respiratory diseases [7]. The relevance of these therapies to respiratory diseases was often first described in studies performed in guinea pigs. These include β_2-selective adrenoceptor agonists, anticholinergics, anti-IL-5, cysteinyl leukotriene ($CysLT_1$) receptor antagonists and 5-lipoxygenase inhibitors, all of which were first shown efficacious in either *in vitro* studies using isolated guinea pig airway preparations, or *in vivo* in models of allergen-induced inflammatory responses and/or agonist-induced bronchospasm. Long-acting β_2 adrenoceptor agonists and anticholinergics were also first characterized in studies performed in guinea pigs [8–17]. By the emergence of transgenic technology and the development of species-specific immunological tools mouse models have nowadays become the standard for obstructive airways disease, despite some serious physiological and pharmacological shortcomings. As a major drawback, the mediators that regulate airway smooth muscle (ASM) tone and ultimately determine airway responsiveness are appreciably different from those in humans as well as guinea pigs, questioning their usefulness for investigating functional clinical end points in asthma [7,18,19]. For example, early asthmatic responses and airway hyperresponsiveness (AHR) are usually only induced after repeated allergen challenges, whereas late asthmatic reactions are rarely observed [20]. With only few exceptions, these responses have proven to be crucial for demonstrating clinical efficacy of existing and investigational drugs [21]. By contrast, measurements in sensitized guinea pigs have demonstrated allergen-induced dual asthmatic reactions [18,19,22–28], AHR [18,19,25,28–32] and eosinophilic airway inflammation [18,19,22–24,26–28,30–32] similar as in patients upon single allergen challenge [18,19,22–32]. In a comprehensive study, using permanently instrumented, unanesthetized and unrestrained guinea pigs with continuous and prolonged online monitoring of lung function by pleural pressure measurement, relationships among allergen-induced early and late airway obstructions, AHR after both reactions and airway inflammation could be performed within the same animal and were shown to be strikingly similar to those observed in asthmatic patients, both in a qualitative and in a quantitative sense [18,26].

Although inflammation and structural remodeling contribute to asthma pathogenesis, majority of studies focus on the inflammatory reactions [33]. Targeting of inflammation alone has not provided disease modification and thus there is a need for airway remodeling to be addressed for future therapeutic strategies. Likewise, airway remodeling may be induced in guinea pigs. Already in the 1930s it was observed that repeated antigen challenges of guinea pigs over several weeks induced structural changes in the airways, including goblet cell hyperplasia and increased ASM mass ([34], discussed in [35]), observations that have been confirmed and extended in more recent studies [32,36–40].

Due to the vast variety of biological and biophysical processes that may be involved in the regulation of airway responsiveness, the mechanisms underlying AHR in asthma are only partially understood and animal models and *in vitro* model systems are indispensable to unravel these mechanisms at the cellular and molecular level [19]. In this regard *in vivo* and *ex vivo* studies in guinea pig models of allergic asthma, particularly focused on the regulation of ASM function, have shown great translational value and supported the discovery and development of new therapeutic options, some of which are discussed below.

Airway pharmacology

Similarities with human biology

Guinea pigs fulfill the primary and important assumption made when using animal models of human respiratory diseases for drug discovery, namely, that the physiological, immunological and/or signal transduction mechanisms controlling the specific processes contributing to human pathology are recapitulated in the chosen animal system [7]. For example, in studies of the immediate type (allergic) hypersensitivity reaction in the airways, the end organ effects attributed to allergen-induced mast cell activation include ASM contraction, mucus secretion, plasma exudation from airway mucosal postcapillary venules, an eosinophilic cellular infiltrate and an associated increase in AHR. These acute effects induced by allergen in the human airways are mediated by histamine H_1 receptor and leukotriene cysLT$_1$ receptor activation [41–44]. Guinea pig airway responses to acute allergen challenge mimic exactly that seen in humans and similarly involve the activation of H_1 and cysLT$_1$ receptors [45–47]. Subacute effects of the allergic response in human airways and lungs depend in part upon airway defensive reflexes such as parasympathetic, cholinergic reflex bronchospasm resulting from muscarinic M_3 receptor activation on ASM, as well as cough and other respiratory reflexes and sensations. Finally, airway neural control in guinea pigs is very similar to that described in humans [48].

Limitations of mice

In contrast with guinea pigs, mice have limited predictive value for many of the organs that are fundamental to human respiratory disease pathogenesis [7]. For example, while allergen-induced bronchospasm in humans and in guinea pigs depends primarily on histamine H_1 and leukotriene CysLT$_1$ receptor activation in ASM, neither histamine nor the cysLTs induce ASM contraction *in vitro* nor bronchospasm *in vivo* in mice. Rather, bronchospasm evoked by acute allergen challenge in mice and rats depends upon mast cell-derived serotonin release and the activation of airway parasympathetic nerves [49–52]. Human and guinea pig mast cells store or release little if any serotonin, and this biogenic amine is without effect on isolated human ASM [53,54]. Murine airways also lack the bronchial circulation and mucus glands readily identifiable in the airways of humans and guinea pigs [55,56] and, unlike humans and guinea pigs, murine airways have no inhibitory innervation of their ASM to counteract the physiologic effects of acute asthmatic attacks [48]. Even the subtypes of β-adrenoceptors that can be activated therapeutically to reverse acute asthmatic bronchospasm differ in mice compared with either humans or guinea pigs [57].

Agonists and receptors

A second assumption or expectation of animal models of human respiratory disease is that the pharmacological properties of the autacoids and receptors involved closely match those properties in human cells and tissues. This expectation has been established in guinea pigs [7]. Among the autacoid and neurotransmitter receptors that can evoke ASM contraction in human airways when activated, including muscarinic M_3, neurokinin NK_2, leukotriene cysLT$_1$, endothelin ET_B receptors and thromboxane/prostanoid TP receptors, these also evoke ASM contraction *in vitro* in preparations of guinea pig airways, and bronchospasm *in vivo* in guinea pigs. Essentially all drugs developed to target the cysLTs in the airways were developed preclinically in studies performed in guinea pigs [58–65]. Physiologically, stimuli evoking reflex bronchospasm in humans such as bradykinin similarly evoke reflex bronchospasm in guinea pigs [48]. These contractions and parasympathetic reflexes can be fully reversed by agonists for β_2 adrenoceptors, peptide transmitters such as vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) and nitric oxide (and other nitrosylating activators of soluble guanylate cyclase). The relaxant responses evoked by these transmitters and therapeutic agents depend upon formation of the cyclic nucleotides cAMP and cGMP, and in both humans and guinea pigs, inhibitors of cAMP and cGMP specific phosphodiesterases (PDE3, PDE4 and PDE5) expressed by ASM potentiate these relaxant responses by delaying cyclic nucleotide inactivation. Furthermore, for the development of the novel dual PDE3/4 inhibitor RPL-554 [66], the efficacy was evaluated in guinea pigs [67], and confirmed in humans [68], but never tested in a mouse. Thus, the development

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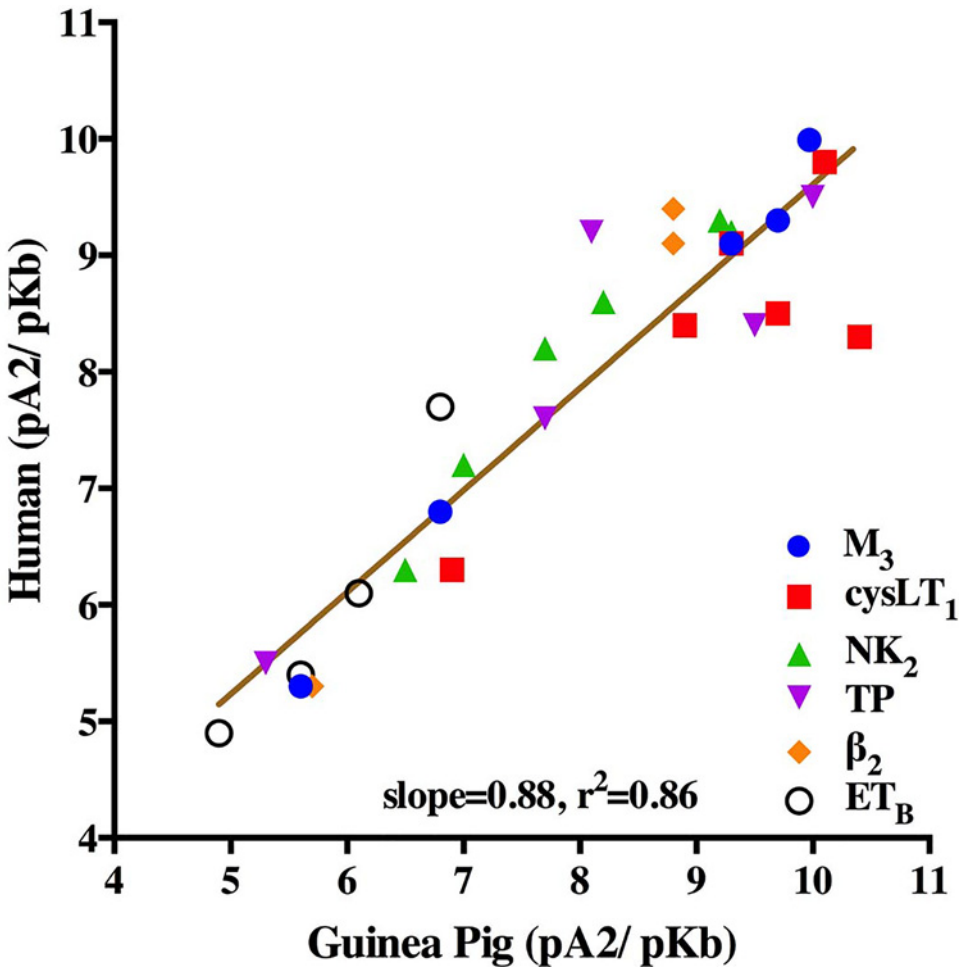

Figure 1. The potency and efficacy of receptor antagonists determined in bioassays performed in guinea pig tissues is highly predictive of their potency and efficacy in human tissues

The pA2/pKb values for antagonists targeting muscarinic M3, leukotriene cysLT1, neurokinin2 (NK2), thromboxane TP, β2 (b2) adrenoceptors and endothelin B (ETB) receptors in guinea pig tissues assays (primarily guinea pig trachea) were highly correlated with their measured pA2/pKb values in human tissue assays (primarily human intrapulmonary bronchi). When multiple pA2/pKb values were reported in the literature, the average responses from these studies are depicted. The figure is constructed using data and the relevant references obtained from [7].

of new drug classes for the treatment of respiratory diseases further support the usefulness of guinea pigs. Moreover, neither histamine nor the cysLTs are able to evoke bronchospasm in mice, and neurokinins surprisingly evoke epithelium-dependent ASM relaxation in murine airways [69].

Also, worth noting is the near perfect correlation between antagonist potencies at receptors relevant to respiratory disease in guinea pigs with their counterparts in preparations of human airways or cells (Figure 1). This latter observation may be attributable to the high identity documented through BLAST sequence comparisons of the relevant guinea pig genes and their human counterparts (85–89% identity for M_3, NK_2, $cysLT_1$, ET_B and β_2 adrenoceptors (mean = 86.8 ± 0.8% identity); https://www.ncbi.nlm.nih.gov/nuccore/). Identity of murine orthologs to these human genes is reasonable but demonstrably lower than that observed in guinea pigs (82–86% identity (mean = 84.2 ± 0.7% identity); $P<0.03$). Moreover, as mentioned, the pattern of expression for these receptors in the airways clearly differs in mice compared with either humans or guinea pigs. Such differences in gene expression also likely account for the unique pharmacology of voltage-gated sodium channel regulation of murine ASM tone, while human and guinea pig airways appear to have comparable patterns of sodium channel expression in airway parasympathetic nerves [70].

Although the predictive value of guinea pigs for preclinical drug discovery in respiratory medicine has been clearly established, there are several notable differences and limitations to using this species for modeling human airway

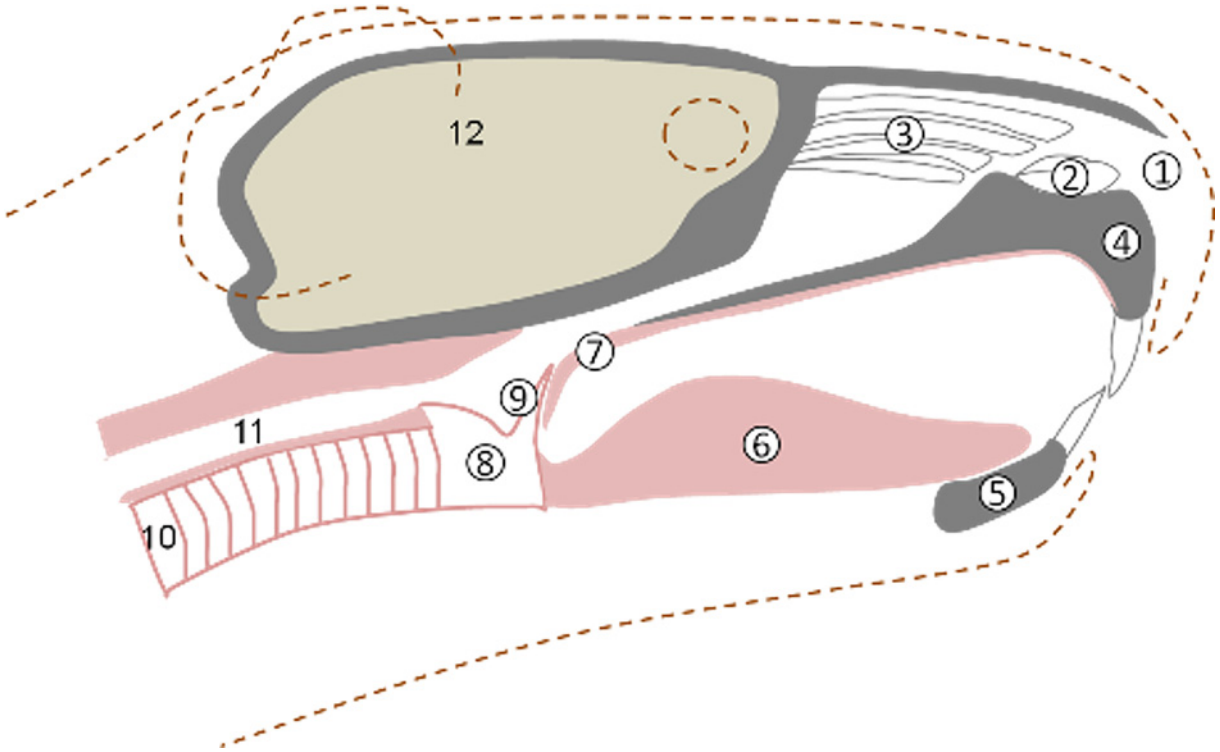

Figure 2. Upper respiratory tract, paramedian section

1: naris, 2: ventral conchae, 3: dorsal conchae (ossa turbinalia I-IV), 4: os incisivum, 5: mandibula, 6: tongue, 7: soft palate, 8: larynx, 9: epiglottis, 10: trachea, 11: esophagus, 12: brain. Picture prepared by Eva Stoffels based on anatomical dissections.

disease [7]. These include the expression of neuropeptides in the peripheral terminals of airway C-fibers (producing axonal reflexes in the airways that are not observed in humans), expression of NK_1 receptors and a $CysLT_2$-like receptor in ASM (in addition to NK_2 and $cysLT_1$ receptors), and the involvement of receptors for IgG_1 in addition to IgE in mast cell activation by allergen. An additional limitation is the minimal capacity for genetic manipulation in guinea pigs, an approach that adds considerable utility to murine models of lung disease.

The respiratory system

Anatomy and physiology

Guinea pigs and humans display similar patterns of AHR [71,72]. Yet there are essential anatomical differences between a guinea pig and a human respiratory tract, which must be taken into account when considering guinea pigs as a model for human respiratory hypersensitivity. A simplified scheme of the upper respiratory tract of a guinea pig is shown in Figure 2. The most striking anatomical feature is the large soft palate flap located rostrally to and overlapping the epiglottis. The palatal ostium is very tight. This makes guinea pigs obligate nasal breathers, which has several consequences for experimental procedures. For example, endotracheal intubation is difficult because of a risk of traumatic injury to the soft tissues. Furthermore, administration of substances acting on the nasopharynx (e.g. acetylcysteine) through the oral route is not efficient. The trachea in an adult animal is approximately 35-mm-long; the bifurcation is located between the second and third ribs. An anatomical view of the ventral surface (facies mediastinalis) is depicted in Figure 3. There are seven lung lobes, three right and two left, and two smaller accessory lobes located ventrally. In contrast with the monopodial branching of the bronchial tree in mice, both the guinea pig and human bronchial tree have dichotomous branching. However, the branching of a guinea pig is different from that of a human. In humans, the two principal bronchi undergo bifurcations into numerous interlobar bronchi that bifurcate further in intralobular bronchi, bronchioles and respiratory bronchioles. In guinea pigs, the two principal bronchi proceed toward the largest lobes (right and left caudal lobes). There are much less bifurcations than in humans – there is only one distinct interlobar bronchus per lobe, and intralobular bronchi depart from each interlobar bronchus laterally at an angle of approximately 90 degrees. The right principal bronchus is positioned more ventrally

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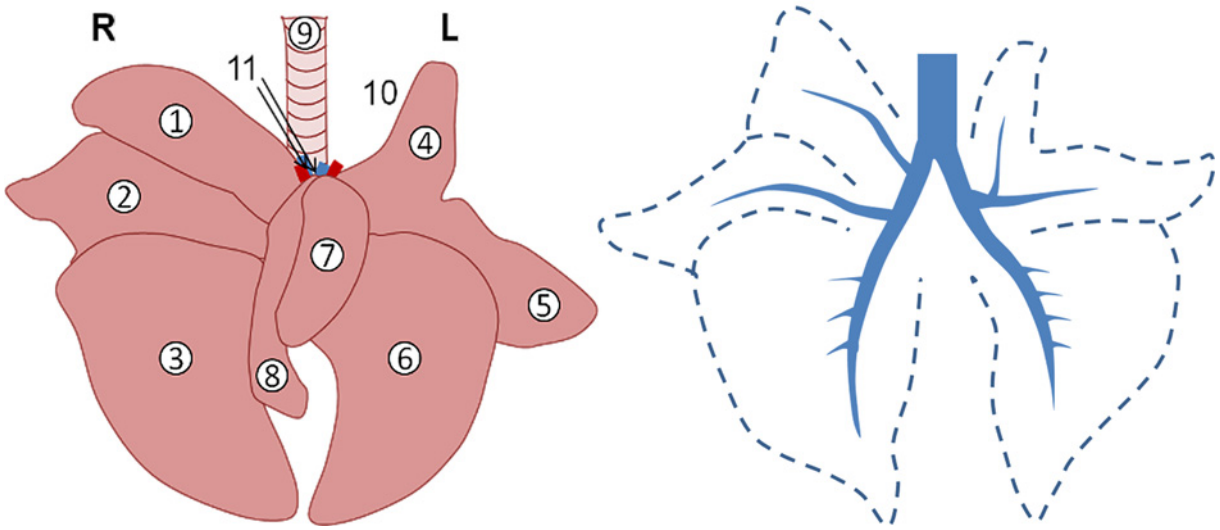

Figure 3. Anatomy of the guinea pig lung (left) and a scheme of the bronchial tree (right)

1–3: Lobus dexter cranialis, medius and caudalis, respectively. 4–5: lobus sinister cranialis, pars cranialis and caudalis. 6: lobus sinister caudalis. 7–8: lobus accessorius sinister and dexter. 9: trachea. 10: impressio cardiaca. 11: aa and vv pulmonales. Pictures prepared by Eva Stoffels based on anatomical dissections.

than the left one (because of *impressio cardiaca*). Therefore, aspiration of bacteria, foreign bodies, allergens etc., is more likely to affect the right caudal lobe.

Guinea pig lung parenchyma is very delicate and lacks connective tissue. For example, there are no intralobular septa. This allows efficient collateral ventilation on one hand, but it also makes the tissue prone to injury during mechanical ventilation or performing bronchoalveolar lavage (BAL) [72]. The cellular composition of guinea pig airways differs from other animals. The bronchi are heavily muscled which is comparable with humans, but markedly differ from with other species, especially mice which lack ASM below the first few bronchial generations [73]. There are strikingly many goblet cells, producing mucous and not serous secretions. In the terminal bronchioles, Club cells are most prominent (approximately 73%). The alveoli are dominated by type I pneumocytes, but in response to stimulation or irritation, type II cells (producing surfactant) increase in numbers. The mast cells are largely located in the smooth muscle layer but also present in the parenchyma. This can be compared with mice where mast cells mainly are present in trachea, with very few located in the lungs. Although the alveoli contain numerous freely migrating macrophages, phagocytosis is primarily performed by neutrophils that extravasate into the alveoli.

Lung physiological measurement

While there are fundamental differences between mice and guinea pigs in terms of airway tree anatomy, it turns out that pretty much all lungs can be fitted with the same mathematical model relating structure to function because lungs obey scaling laws across species [74] (Figure 4). This is good news, not only for the animal scientist but also for the clinician because this means that it is possible to use the broadband forced oscillation technique (FOT) in both animal studies and in human studies and interpret the data in a similar manner across species boundaries allowing for translational studies [75–77]. Used to its fullest power FOT allows the partition of the result into responses in the conducting airways compartment, and in the tissue compartment [78]. This is achieved by fitting a mathematical model of the respiratory system to the impedance of the lung and solving for parameters that have logical and realistic physiological properties, e.g. Newtonian resistance (Rn), tissue resistance (G) and lung elastance (H) [79–83].

There are very few published studies in guinea pigs using the full power of broadband FOT, but based on published analyses there is every reason to believe that the respiratory system of the guinea pig will behave in a similar way as in any other laboratory animal [74]. Allergic guinea pigs display characteristic changes in AHR when exposed to methacholine with increases in Rn, G and H, commensurate with elevations in lung lavage eosinophils and neutrophils [83] supporting previous studies where glucocorticoids, xanthines, anti-histamines and β_2 adrenoceptor agonists have been shown to have positive effects on the guinea pig lung phenotype [84–88]. It is interesting to note that younger guinea pigs appear to be more sensitive to the sensitization allergen than older animals [89], suggesting that guinea

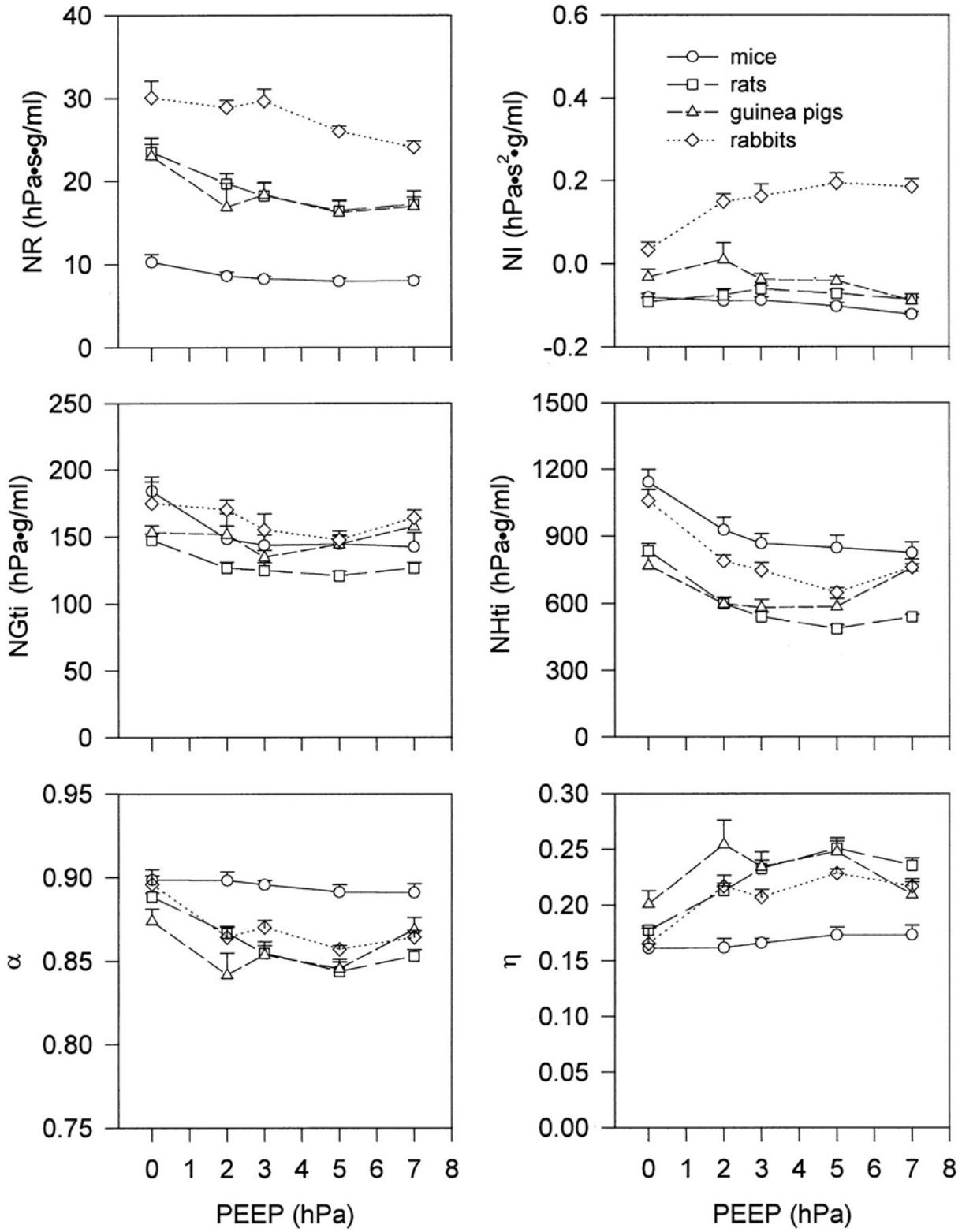

Figure 4. Parameters of the constant-phase model fitted to respiratory system impedance data of mice (*n*=11), rats (*n*=8), guinea pigs (*n*=5) and rabbits (*n*=6) as a function of PEEP

Values are normalized to BW and represent means ± SE. NR, normalized Newtonian resistance; NI, normalized inertance; NGti, normalized tissue damping; NHti, normalized tissue elastance; $\alpha = (2/\pi)\arctan(\mathrm{Hti}/\mathrm{Gti})$; η, hysteresivity. (Reproduced from [74] with permission from the publisher).

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pigs might be a model for age-dependent longitudinal studies. Sensitized guinea pigs have a strong response to allergen that can be modified to various degrees by inhibiting histamine, cyclooxygenases and CysLTs, demonstrating mast cell involvement in the response [90]. Mast cell-dependent responses in mice have rarely been demonstrated and they do not appear in the lung unless a vigorous sensitization protocol is employed [80].

Guinea pig lungs, on the other hand, have a significant number of mast cells [91] and will respond with a rapid bronchoconstriction that is antagonized with either anti-histamines [92,93] or sodium cromoglycate [84] suggesting that mast cell degranulation is responsible for the response. Sensitized guinea pigs have also been shown to have a late response, hours after an allergen challenge, similar to what patients with asthma can experience [18,22–28].

FOT is quickly becoming an important tool in assessing lung function in patients, both in research and clinical practice, and like animal FOT it can elucidate effects in the smaller airways thus making it a better tool for early diagnosis of lung disease [94]. Using broadband FOT allows for detailed examination of the respiratory system, separating effects in the central vs. smaller airways, and using the same technique in both animals and humans will allow us to extrapolate physiological findings from one species to the other and likely improve outcome in drug research and eventually in patient care.

Unrestrained plethysmography has been used as a technique implying to measure lung function in guinea pigs [95]. However, it has been demonstrated in the mouse, and theoretically, that only when the airways are severely constricted does the airway resistance contribute to the signal of the main parameter, the enhanced pause (Penh). As most small animals are obligate nose breathers, any swelling or other obstruction of the nasal passage is very likely to have an impact on the Penh. In addition, stimulation of irritant receptors like the J-receptor can have profound effects on the ventilatory pattern [96]. Thus, neither nasal obstruction nor CNS effects necessarily reflect on physiological changes in the lung airways or lung parenchyma [97].

Model development

Immunological responses in different protocols

An experimental asthma model in the guinea pig was introduced in 1937 by Kallós and Pagel ([34], discussed in [35]), since then, several protocols have been established. The field has a wide spectrum with respect to age of animals, allergens, doses, route and frequency of allergen sensitization and challenges, which altogether influence the 'asthmatic' response. Table 1 summarizes some methods described in the literature, as well as the pathological features found in those experimental models. Asthma severity and heterogeneity depends on many factors that drive the immune response, including genetics, age and gender. In guinea pig asthma models, albino outbred animals are the most common laboratory strain, although other, including inbred strains, have been used in early studies [72,98,99]. Despite that the outbred nature of guinea pigs is a disadvantage in terms of genetic influence in the development of experimental asthma, this is a suitable factor that contributes to modeling the heterogeneity of asthma in the guinea pig.

The allergic response

OVA is the major antigen that has been used for experimental asthma models in guinea pigs. Single or repeated doses of systemic OVA challenges mimic many asthma-related features (Table 1). It has been established that guinea pig sensitization with low dose of OVA, i.e., 10 μg, promotes EAR with the induction of IgG_1 and IgE synthesis [100,101], whereas large dose of OVA, i.e., 100 μg, induces both EAR and LAR together with IgG_1 production [40,100–102]. Nevertheless, it has also been described that following challenge with aerosolized OVA for one hour, guinea pigs develop both EAR and LAR although the sensitization was performed with a low dose of OVA [28,103]. Notably, those sensitization protocols were performed through intraperitoneal OVA injections. Instead, aerosol sensitization with a low dose of OVA induces EAR and LAR with IgG_1 and IgE production [104], and large dose generates EAR, AHR, and eosinophilia [105,106]. Smith et al. demonstrated that the addition of a booster sensitization extends LAR and increases the AHR, and eosinophil influx in guinea pigs sensitized [101]. Moreover, the combination of intraperitoneal and subcutaneous injections together with aerosol boosters cause EAR and OVA-specific IgG_1 and IgE, besides AHR and eosinophilia, but no LAR [39,107]. Several other compounds have been used as allergens in guinea pig models, such as HDM extracts [108,109], purified proteins from mites [109], cockroach extracts [110–112], and trimellitic anhydride dust [113,114]. However, there have been very few models developed and AHR was not established.

Strategies for induction of asthma models

The most common regimen for allergen exposure consists of a sensitization phase and a challenge phase (Table 1). The sensitization phase is usually achieved by a single or repeated allergen dose; nonetheless, some protocols include

Table 1 Protocols for guinea pig asthma models

Guinea pigs	Sensitization phase		Challenge phase	Key features	References
	Initial dose	Booster dose			
Both sexes, Dunkin–Hartley, 250–300 g	Two sensitization procedures: A) 0.1 ml of 5 mg OVA in saline, i.p. or B) 0.5 ml of 0.1, 1, 10, or 100 μg OVA containing 100 mg $Al(OH)_3$ in saline, i.p.	Day 2 A) 0.1 ml of 10 mg OVA in saline, i.p.	Day 14 1, 5, 10, 20, 40, 80, 160, or 320 μg/kg OVA, i.v	Acute bronchoconstriction with low OVA doses (1–10 μg) plus $Al(OH)_3$ and IgG and IgE production, high OVA doses induced only IgG production (A) Less response than (B) method, higher doses of OVA (10–320 μg/kg) to produce bronchospasm (B) 0.1 μg OVA sensitization no response to the provocation doses, 10 μg OVA induce an optimal bronchospasm	[100,122]
Male, Dunkin–Hartley, 450–500 g	1% OVA aerosol for 3 min	Day 7 Same dose as initial day	Day 14 2% OVA aerosol for 5 min *Mepyramine maleate (10 mg/kg, i.p.)	Allergen-induced bronchoconstriction: EAR (5 min–2 h) and LAR (17 h) Eosinophil and neutrophil increase in BALF	[115]
Male, 500–600 g	0.35 ml of 5% OVA into each hind limb, intramuscular injection	Day 6 Same dose as initial day	Week 5 1% OVA aerosols for 1 min *Pyrilamine maleate (2 mg/kg, i.p.)	EAR and LAR Eosinophil infiltration in lung and BALF	[171]
Female, Perl Bright-white, inbred and specific pathogen-free, 300–350 g	Days 1–5 0.1 ppm SO_2 for 8 h Day 3 0.1% OVA aerosol for 45 min	Day 4 to 5 Same OVA dose as on day 3	Day 7 1% OVA aerosol for 5 min	Allergen-induced bronchoconstriction: EAR (30 min) Eosinophils in BALF Inflammatory cells in lung and epithelial damage OVA-specific IgG1	[105,134]
Female, Cam–Hartley, 250–300 g or 500–550 g	1% OVA aerosol with 4% heat-killed *Bordetella pertussis* vaccine in saline, for 10 min		Day 8 0.5% OVA aerosol for 5 min Chronic model: OVA twice a week for 4–6 weeks *Diphenhydramine (40 mg/ml, i.p.)	Allergen-induced bronchoconstriction Tissue eosinophilia AHR to acetylcholine IgE and IgG1 production	[131–133]
Male, Hartley strain, specified pathogen-free, 500–700 g	100 μg OVA and 100 mg $Al(OH)_3$ per ml saline, 0.5 ml i.p and 0.5 ml divided over seven s.c. injections in the paws, lumbar regions and neck		Weeks 4–8 0.1, 0.3 and 0.5% OVA aerosols for 3 min with 10-min intervals until obstruction Or 0.2% OVA aerosol for 15 min	EAR and LAR AHR to histamine Inflammatory cells influx	[26]
Male, Hartley strain, 200–250 g	1–2 mg OVA and 100 mg $Al(OH)_3$, i.p. *Day 3 cyclophosphamide (30 mg/kg, i.p)	Week 4 0.01 mg OVA and 100 mg $Al(OH)_3$, i.p.	Week 7 10 mg/ml OVA aerosol for 60–90 s *Diphenhydramine (20 mg/kg, i.p.) and procaterol (0.1 mg/kg i.p.)	Allergen-induced bronchoconstriction Cough AHR to methacholine Inflammatory cells in BALF and tissue IL-4, IL-5, and IL-13 SP, PGE2, histamine, LTB4 and TXB2 in BALF	[127–130,172,173]
Male, Hartley strain, 250 g	0.5 ml of 0. OVA and 2 mg $Al(OH)_3$ in saline, i.p.	Week 2–4 Same dose as initial day, once a week	Week 5 1 mg/kg OVA, i.v. or 3 mg/ml OVA aerosol for 3min *Mepyramine maleate (1 mg/kg, i.v.)	Allergen-induced bronchoconstriction: EAR (4 min) Plasma leakage in the airways	[174,175]
Male, Dunkin–Hartley, specific pathogen-free male, 300–550 g	0.5 ml of 2.5 mg OVA and 50 mg alum in saline, i.p.	Day 10 Same dose as initial day	Days 20 and 22 0.15 ml of 2% OVA intratracheal *Pyrilamine maleate (10 mg/kg, i.p.)	Allergen-induced goblet cell degranulation Neutrophil recruitment Elastase release	[176]
Both sexes, C2BB/R-(*inactivates the C3aR)* and C2BB/R+ (wild-type C3aR), inbred, 600-650 g	Days 0 and 7 0.5 ml of 10 μg OVA and 2 mg $Al(OH)_3$ in saline, i.p.	Day 21 1% OVA aerosol in saline	Day 35 1% OVA aerosol for 8 min *Pyrilamine (5 mg/kg, i.p.)	Reduced allergen-induced bronchoconstriction and AHR in defective strain Eosinophils infiltration in lung	[99]

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Table 1 Protocols for guinea pig asthma models (Continued)

Guinea pigs	Sensitization phase		Challenge phase	Key features	References
	Initial dose	Booster dose			
Male, Hartley strain	10 μg OVA and 100 mg $Al(OH)_3$ in saline, i.p.	Day 14 Same dose as initial day	Day 18 0.3, 1, 3, 10 mg/ml OVA aerosols for 3 min	Allergen-induced bronchoconstriction AHR to histamine and methacholine Inflammatory cells in BALF, blood, and tissue Increase EPO activity	[117]
Male, Hartley strain, 300-400g	0.5 ml of 5% OVA, s.c. plus 0.5 ml OVA, i.p.	Day 7 Same dose as initial day	Day 15, 22 and 29 1% OVA aerosol for 3 min *Mepyramine (10 mg/kg, i.p.) **The animals fasted for 1 day before all three inhalations	Allergen-induced bronchoconstriction: EAR and LAR (8 h) AHR to methacholine Eosinophilia in BALF	[138–140,177]
Male, Dunkin-Hartley, 200-250g	100μg OVA and 100 mg $Al(OH)_3$, i.p.	Day 5 Same dose as initial day	From day 15 Eight aerosols applied every 48 h for 1 h 0.01% OVA *Mepyramine (30 mg/kg, i.p.)	Allergen-induced bronchoconstriction: EAR (0–6 h) and LAR (6–24 h) AHR to histamine Inflammatory cells in lung tissue and BALF Collagen deposition and mucus production	[40,101]
Male Dunkin–Hartley 200–300 g, male, specified pathogen-free	100 μg OVA and 100 mg $Al(OH)_3$ per ml saline, 0.5 ml i.p. and 0.5 ml divided over seven s.c. injections in the paws, lumbar regions and neck	Day 5 Same dose as initial day	0.05, 0.1, 0.3, 0.5 and 0.7% OVA aerosols for 3 min with 7-min intervals until obstruction, once weekly for 12 consecutive weeks, starting 4 weeks after sensitization	Enhanced ASM mass and contractility Subepithelial fibrosis Goblet cell hyperplasia Mucus gland hypertrophy Airway eosinophilia Enhanced IL-13 in lung homogenate AHR to methacholine	[37,178]
Male, Hartley strain, 4–7 weeks old	1% OVA aerosol for 10min	Days 2–7 Same dose as initial day	One week after final sensitization 2% OVA aerosol for 5 min *Metyrapone (10 mg/kg, i.v.) *Pyrilamine (10 mg/kg, i.p.)	Allergen-induced bronchoconstriction: EAR (1 min) and LAR (4–8 h) AHR to acetylcholine Inflammatory cells in BALF	[106]
Male, Dunkin–Hartley, 350–400 g	0.5 ml of 60 μg/ml OVA and 1 mg/ml $Al(OH)_3$ in saline, i.p. and 0.5 ml s.c.	Day 8 3 mg/ml OVA aerosol for 5 min	From day 15 Twelve OVA aerosols applied every 10 days for 1 min 1 mg/ml OVA in the first and 0.5 mg/ml in the subsequent challenges	Allergen-induced bronchoconstriction: EAR (20 min) AHR to histamine OVA-specific IgG1 and IgE Inflammatory cells in lung tissue and BALF Collagen deposit in airway TNF-α, histamine, TXA2, and leukotrienes in BALF	[39,107,179]
Male, TRIK tribe strain, 150–350 g	1 ml of 5 mg OVA and 1 mg ($AlOH_3$), i.p. and 1 ml s.c.	Days 4–21 0.1 ml of 5 mg OVA, i.p. every 3 days	Days 16–21 1% OVA aerosol	Allergen-induced bronchoconstriction Cough Tracheal smooth muscle reactivity *in vitro* AHR to histamine and acetylcholine IL-4, IL-5, IL-13, and TNF-α in BALF Lung infiltrated by eosinophils and mast cells	[137]
Male, Dunkin–Hartley, specific pathogen-free, 300–500 g	100 μg OVA and 100 mg of $Al(OH)_3$ in saline, i.p.	Day 14 Same dose as initial day	Days 18–29 0.5% OVA aerosol daily for 10 min	Histamine and acetylcholine-induced bronchospasm Inflammatory cells in BALF, blood, and tissue TNFα, IL-4, IL-5, and IL-6 in serum and BALF	[180]
Male, TRIK tribe strain, 150–350 g	1% OVA, 0.5 ml i.p. and 0.5 ml s.c.	Day 3 1% OVA, 1 ml i.p.	Days 14 and 21 1% OVA aerosol for 30 s	Allergen-induced bronchoconstriction AHR to histamine Inflammatory cells in BALF	[181]
Male, Dunkin–Hartley, specific pathogen-free male, 350–500 g			Days 1, 4, 7, 10, 13 (1 mg/ml) Days 16,19, 22 (2.5 mg/ml) Days 25, 28 (5 mg/ml) Days 31, 34 (10 mg/ml) OVA aerosols for 15min	Allergen-induced bronchoconstriction AHR to methacholine IL-13 and TNF-α mRNA expression in lung Increase in ASM, subepithelial collagen deposition and mucus production Inflammatory cells infiltration in lung Eosinophils in BALF	[143,182]

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Table 1 Protocols for guinea pig asthma models (Continued)

Guinea pigs	Sensitization phase		Challenge phase	Key features	References
	Initial dose	Booster dose			
Female, Hartley strain, specific pathogen-free, 1 month of age	10 mg/kg OVA, i.p.	Day 3 Same dose as initial day	Day 21 parainfluenza virus I, i.n. Day 45: 0.5% OVA aerosol for 20 s Day 70: re-exposed to parainfluenza virus I, i.n.	AHR to histamine Eosinophilic airway inflammation	[183]
Male, Hartley strain, 200–300 g	1 ml of 150 mg OVA and 100 mg $Al(OH)_3$ in saline, i.p.	Day 3 and 7 Same dose as initial day	Day 15 histamine inhalation Days 16–21 vehicle or steroid Days 19 and 21 LPS (30 mg/ml) inhalation Day 21 OVA aerosol (300 mg/ml) 1 Day 22 Histamine inhalation	Allergen-induced bronchoconstriction: EAR (0–6 h) and LAR (6–12 h) OVA plus LPS increased the duration of EAR AHR to histamine Inflammatory cells in BALF IL-13 and IL-17 in BALF	[102]
Both sexes, 300–350 g	100 μl of 2 mg OVA and 10 mg ($AlOH_3$) in 10 ml saline, i.p.	Day 14 100 μl of 1 mg OVA in saline, i.p.	Days 21–30 1% OVA aerosol daily for 10min	Eosinophilia in blood OVA-specific IgE Oxidative stress markers in BALF Collagen deposition	[184]
Male, Hartley strain, at 6 weeks of age	Weeks 0 and 5 1 mg of nDer f 1, 10 mg of rDer f 2, or 10 mg of mite extract plus 4.5 mg ($AlOH_3$), and 10^{10} killed *Bordetella pertussis* s.c.	Weeks 3 and 7 1 mg of nDer f 1, 10 mg of rDer f 2, or 10 mg of mite extract plus 4.5 mg ($AlOH_3$), s.c.	Week 9 0.05 ml of either 10 mg/ml rDer f 2 or mite extract solution, i.n. * Mite extract-sensitized animals were rechallenged with 0.05 ml of 30 mg/ml mite extract 15 min after the first challenge	Early and late-phase cutaneous reaction Allergen-induced bronchoconstriction, EAR (10–25 min) Eosinophils in BALF Plasma anti-mite IgE, IgG_1, and IgG_2	[109]
Male, Dunking-Hartley strain	0.25% HDM aerosol for five consecutive days for 5min	0.5% HDM aerosol for 5 consecutive days for 5min	Day 14 skin prick test with 15 μl of 0.5% HDM; i.d.	Early (weaker, in 4/10 guinea pigs) and late (stronger 10/10 guinea pigs)-phase cutaneous reaction Cough, sneezes and nasal symptom during the EAR No AHR to histamine or methacholine	[108]
Male, Hartley strain, 200–250 g			0.05, 0.5 or 2.5 mg/ml of cockroach extract aerosols for 45 min, twice a day, 5 days a week, for a period of 4 weeks	Allergen-induced bronchoconstriction Allergen-induced contractile response of tracheal rings Inflammatory cells in BALF Anaphylactic antibodies (IgE, IgG1) Acetylcholine-induced contractile response of tracheal rings. No AHR *in vivo*	[110–112]
Female, Hartley strain, 200–300 g	Day 1 100 μ1 of 0.3 or 30% TMA in corn oil, i.d.	Days 3 and 5 Same dose as initial day	Three weeks after sensitization 4 mg of TMA-GPSA, i.t. instillation *Pyrilamine (6.1 mg/kg, i.p.)	Allergen-induced bronchoconstriction: EAR (10 min) Inflammatory cells in BALF Allergen-specific IgG1 and IgG2 Total protein and red blood cells in BALF Complement activation product C3a in the BALF and plasma EPO and MPO activity in BALF and lung tissue	[113,114,185,186]
Male, Dunkin–Hartley, 200–250 g	Day 1 100 μ1 of 3 mg TMA in corn oil, i.d.	Days 10 and 20 100 μ1 of 1 and 0.1 mg TMA in corn oil, i.d.	Days 30–34 0.15 or 0.03% of TMA-GPSA, aerosols for 15min Day 35 250 μg TMA-GPSA in 50 μl PBS, i.t. *Pyrilamine (10 mg/kg, i.p.)	EAR is reduced in repeated low dose of allergen Inflammatory cells in BALF AHR to acetylcholine at low allergen dose Reduction in cysLTs, and TXB2 levels in BALF but enhanced capacity to produce cysLTs, and TXB2 *in vitro* at low dose allergen	[187]

Dunkin–Hartley guinea pigs correspond to the HsdDhl: DH colony, whereas Hartley guinea pigs correspond to the Crl:HA colony. Abbreviations: BALF, bronchoalveolar lavage; EAR, early asthmatic response; HDM, house dust mite; i.d., intradermal; i.n., intranasal; i.p., intraperitoneal injection; i.t., intratracheal; LAR, late asthmatic response; LPS, lipopolysaccharide; OVA, ovalbumin; s.c., subcutaneous injection; TMA, trimellitic anhydride; TMA-GPSA; TMA conjugated to guinea pig serum albumin.
*Denotes pharmacological treatment before challenge.

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intermittent allergen aerosols without a clear sensitization phase. The sensitization phase primes the immune system to initiate an allergic inflammation with no visible reactions or symptoms. The challenge phase elicits an acute reaction manifested by a temporal bronchospasm or EAR. One goal has been to create a model that expresses LAR, which also has been reported in certain studies [26,102,106,115] (the latter discussed in [116]). However, several studies have also described that allergic guinea pigs have no noticeable LAR [39,106,117]. Indeed, bronchoconstriction induced by allergen provocation is variable in guinea pigs [118,119], similar to asthmatic humans [120,121]. It is possible that the outbred nature of the guinea pigs influence reproducibility and that seasonal oscillation occur, but to our knowledge this has not been verified. In guinea pig models, the heterogeneity of physiological and immunological responses has been primarily associated with the sensitization method and the allergen dose [100,101,122]. In addition, other factors such as anesthesia and use of antihistamines to prevent anaphylaxis may similarly play a role [18]. Accordingly, guinea pig elicits different patterns of allergic reactions. For instance, Yasue et al. found that the allergic airway reaction provoked with rDer f 2 was higher than with whole mite extract, nevertheless, a further mite extract-challenge increased the airway resistance in a dose-dependent manner [109]. Interestingly, in the aforementioned study, only 50% of the guinea pigs showed an immediate airway reaction, whereas 87% revealed an immediate cutaneous reaction with erythema towards both allergens [109].

Guinea pigs unresponsive to allergen challenges are frequently not included in the studies [26,109,123], even though these subjects might outline a similar phenotype to that observed in humans. A recent study showed that guinea pigs with no allergen-induced bronchoconstriction do develop AHR and specific IgG_1 responses [107]. Thus far, the exact mechanisms of AHR in this particular model remain unclear; however, asymptomatic AHR in humans has been described as a condition that may lead to asthma [124–126].

Avoidance of tolerance

A fine balance between allergen response and the induction of tolerance is a crucial phase in developing models of allergic inflammation. To increase antigen immunogenicity, allergens are often adsorbed to adjuvants. For instance, sensitization protocols with OVA often include aluminum compounds (Table 1) to evoke a T_H2-dominant bronchial response [127–130]. Likewise, *Bordetella pertussis* as adjuvant enhances the IgE production and rises a long-lasting AHR [109,131–133]. Sulfur dioxide (SO_2) enlarges OVA-induced EAR, eosinophilia, and antigen specific-IgG_1 [105,134]. This is an important finding since SO_2 is a major air pollutant. In fact, other pollutants suspended in the atmosphere may exacerbate the AHR or can act as adjuvants [135,136].

Developing experimental asthma protocols involves a complex interplay between immune tolerance and exacerbated responses. Therefore, the allergen dose is carefully considered in the protocols due to guinea pig sensitivity to develop fatal anaphylaxis. Pre-treatment with antihistamines or bronchodilators is widely used to prevent an exacerbated bronchoconstriction [40,137–140], but may have effects on allergen-induced obstructive responses, inflammation and AHR [18]. Conversely, antigen administration might also induce allergen unresponsive guinea pigs. To avoid lacking response, increasing doses of allergen are administrated for long periods. This, in turn, results in thickening of the airway wall with a residual and permanent airflow limitation, both hallmark features of chronic asthma [141–143]. In this regard, continuous monitoring of lung parameters upon allergen challenge is an important approach to validate whether the experimental model does show the asthma-related pathophysiologic features, i.e. EAR or LAR.

Exacerbation models

Infections are the most common cause of exacerbation in asthma. The dominating viruses are a specific picornavirus, rhinovirus, followed by influenza/para influenza and respiratory syncytical virus [144,145]. Unlike guinea pigs, where there are relatively few examples where asthma models have been exacerbated, there is a relatively large body of work describing exacerbation of murine models of asthma (see reviews: [146,147]). In one study, sensitized guinea pigs are inoculated with human parainfluenza-3 (hPIV-3) virus 5 days before ovalbumin challenge [148]. The presence of hPIV-3 changed the time-course of airway obstruction induced by ovalbumin challenge. Instead of two relatively transient phases, airway obstruction was much longer lasting with no discernible late phase. Airway obstruction, AHR to histamine and elevated level of eosinophils in the BAL were all resistant to corticosteroid treatment. Compared with challenge with ovalbumin alone, the presence of hPIV-3 elevated the numbers of neutrophils in the BAL.

As viral exacerbation of animal asthma models is difficult to replicate across different research groups, exacerbation models using lipopolysaccharide (LPS) to mimic gram-negative bacterial stimulation (e.g. *Haemophilus influenza*) have been developed [149]. The duration of the early phase of airway obstruction induced by allergen challenge was increased by treatment with LPS. Although LPS had no significant effect on the magnitude or duration of late-phase

airway obstruction, it did confer resistance to treatment with fluticasone propionate. LPS also made AHR to histamine, and influx of leukocytes in the BAL resistant to fluticasone propionate. However, protein exudation into BAL (an indirect measure of edema) was sensitive to fluticasone propionate indicating the lack of efficacy against other parameters was not due to failure in delivery.

Modeling the cough reflex

In healthy subjects, coughing is part of a protective, airway sensory nerve-driven reflex designed to expel foreign material from the lung. However, when this reflex becomes excessive and inappropriate, it develops into a clinical problem, having a huge impact on people's lives [150].

The guinea pig is an appropriate and necessary species for investigation into the mechanisms involved in normal and chronic cough, and the search for novel, effective and safe therapies. While working with guinea pigs has currently some disadvantages compared with using rats and mice, e.g. the range of assays currently available is less and genetic manipulation is challenging, they are solvable. Whereas, altering the sensory airway structure and function in a mouse/rat to be more like man is not.

Modeling airway sensory nerve activity

In the search for new antitussive therapies, one approach is to work toward understanding the mechanisms by which cough is initiated and propagated in healthy subjects, and then how this can change in respiratory diseases. To do this one requires appropriate systems for the investigations. Typically, preclinical investigations are performed in guinea pigs, as unlike mice and rats, they possess the cough reflex and their airways sensory nerves share similar structural and functional features to humans [151]. Like healthy subjects, naïve guinea pigs do not cough spontaneously. They can, however, be induced to cough by exposing them to an aerosol of tussive solutions like the extract from chilli peppers (capsaicin), or citric acid. Indeed, these are the same solutions that are used to trigger coughing in man [152]. Furthermore, it is possible to model respiratory diseases in the guinea pig and then demonstrate they have an altered sensitivity to inhaled tussive agents. This modification in cough responses parallels the change in phenotype observed in patients with respiratory disease, for example it has be shown that patients with the smoking related disease, COPD, have an increased cough response to capsaicin challenge. In a parallel cigarette smoke-driven model, the guinea pigs cough more to the capsaicin challenge compared with the sham controls [152]. These *in vivo* systems allow one to study cough sensitivity and the actions of potential antitussive therapies. While work is ongoing, one issue with these model systems is that even with respiratory disease modeled in the guinea pig, they do not spontaneously cough. Thus, replicating the clinical condition exactly is proving difficult and selecting an appropriate tussive stimuli to profile new therapies can be problematic.

Studies of single airway sensory nerves *in vivo*

Other issues with using the whole animal cough system include: the large number of animals required (often need *n* of 8–12 to obtain statistical significance with a 50% change); the need for appropriate tools for *in vivo* work and the issues with being able to investigate details of the mechanisms, e.g. the type of sensory nerve fibre involved. To address the latter issue, an *in vivo* system to record single airway sensory nerve firing has been developed [153,154]. While this system is a challenge to master and takes a great deal of time, it is essential for preclinical research. To circumvent some of the other issues, parallel systems have been employed (reviewed recently by Bonvini et al, [154]). Briefly, one system used is to record tussive stimuli trigged depolarization of the isolated sensory nerve (housed in the vagus) tissue. The vagal tissue can be divided into multiple pieces and repeatedly challenged with tussive stimuli (with and without test therapy, if required). This reduces the numbers of animals used and does not require *in vivo* ready tools. Another advantage of the isolated vagal system is it is also possible to do parallel investigations using human vagal tissue. The data produced has a very similar profile to the guinea pig vagus nerve and thus enables the generation of translational data for targets of interest [152].

Like in humans, airway sensory nerve cell bodies are contained in jugular and nodose ganglia; rats and mice do not have separate ganglia. It is possible to harvest and isolate airway neurones from these ganglia for investigations [155]. As with the vagal systems, it is possible to examine the function of these cells (for example by tracking Ca^{2+} flux and changes in membrane potential, 8). In parallel, target expression patterns can be established at the mRNA and protein level using techniques such as RT-PCR and immunohistochemistry [156]. A big advantage of using these primary neurones, as with the vagal tissue, over configured cell-based systems is the fact that one is using innate receptors/ion channels that are appropriately coupled to native, *in vivo* relevant functional machinery/proteins. Furthermore, the growing body of data shows that the neurons (and the vagal tissue) harvested from respiratory disease

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models, maintains the change in phenotype observed *in vivo* (and in the case of human vagus nerve, in the patient group). For example, the vagus nerve and neurones harvested from the guinea pig model of COPD, exhibit a larger *ex vivo* response to capsaicin challenge compared with the sham controls – paralleling the cough observation). The fact that the phenotypic change is maintained *ex vivo* should aid the investigation into the mechanisms.

Opportunities and limitations

The guinea pig as a model species has great potential, however, the strength of an experimental model is not only defined by its translatability to the human physiology and pathology, even more crucial is the ability to analyze such a model. As comprehensively described above, the guinea pig can be used to study many physiological (e.g. cough, AHR, EAR and LAR), histological (e.g. eosinophilia and airway remodeling,) pharmacological (drug discovery and validation), immunological (e.g. immune cell infiltration and differentiation, and cytokine expression) processes. However, guinea pig research is still restricted by the lack of guinea pig-specific reagents and chemicals. Especially compared with human and more conventional laboratory animal species, such as mouse and rat, the availability of reagents and specific tools suitable for guinea pig research is limited. Consequently, the interest to develop more advanced models using natural allergens, that also affect for example the mucosal immunity, has not much been applied in the guinea pig.

For decades, researchers' willingness to spend time, effort and money has been the key driver in establishing suitable reagents for guinea pig research, not only by adapting human, mouse or rat specific reagents, but even by developing guinea pig-specific arrays [157–159]. Although the costs for guinea pigs usually are five times more than for mice or rats, and with additional higher costs for housing, this needs to be offset by the greater relevace of the experimental data. as outlined in this review. Researchers' actions have led to an increase in commercially available guinea pig specific reagents, and undertakings such as The Guinea Pig Genome Project by The Broad Institute greatly improve guinea pig research. In the past efforts have been made to gather the information spread throughout the field of guinea pig research, as greatly exampled by Lasco et al. paper on translatable guinea pig markers [160] and Schäfer and Burger's overview of the development of guinea pig monoclonal antibodies [161]. In this paper, a similar effort has been made to compose a repository of guinea pig tools that can be used in asthma research, for ELISA (Supplementary Table S1), for flow cytometry (Supplementary Table S2), for immunohistochemistry (Supplementary Table S3) and for PCR (Supplementary Table S4).

Gene-editing of the guinea pig genome

In a complex disease such as asthma many genes and their products interact for the manifestation of the disease. Certain genes can be crucial for the etiology of the disease whereas others are important for the cardinal characteristics of airway inflammation, bronchial reactivity and tissue remodeling. The introduction of genetically modified mice at the end of the 1980s revolutionized medical research. With the development of the techniques during the following decades it has become possible to 'knock-out' or 'knock-in' genes in a tissue/cell and time-specific manner. However, gene-modified guinea pigs have yet not entered the scene. The main reason has been a lack of embryonic stem cells preventing homologous recombination-based knockout approaches. Moreover, detailed knowledge of the guinea pig genome came quite late, in the beginning of this century, and it was not, until this achievement, possible to identify genes of interest in greater detail. With the rapid developments in genomics and CRISPR/Cas9 technology for gene editing, it is now time to take the opportunity to use gene-editing of the guinea pig genome to generate animals that can be used in experimental models to decipher the pathophysiology of asthma.

The guinea pig genome was sequenced as part of the Mammalian Genome Project funded by the National Institutes of Health and performed at the Broad Institute, Cambridge MA. It was originally sequenced at a low coverage, 2×, but was in 2008 sequenced to 7× coverage with 95.55% of the bases assembled. The guinea pig genome is provided by the UCSC genome browser (http://hgdownload.cse.ucsc.edu/downloads.html#guinea_pig) or through Ensembl (http://www.ensembl.org/Cavia_porcellus/Info/Index?db=core). Although the high-coverage, 7×, ensure increased accuracy for genome analysis it might be necessary to increase the coverage further, e.g., by PacBio assembly to reach 30× and >99% accuracy, especially for the analysis of immunoregulatory genes and other complex regions of interest for asthma research. In addition to the original sequence of *Cavia porcellus* the genome of *Cavia aperea* is now also sequenced and available. Furthermore, the Guinea Pig Genome Project at Broad is planning to obtain low coverage of additional guinea pig strains (https://www.broadinstitute.org/guinea-pig/guinea-pig-genome-project).

With the sequence of the guinea pig genome available, it is possible to identify orthologs to human genes of interest for asthma research. In addition to investigating the expression of genes by PCR or RNA sequencing, we also have the opportunity to generate genetically modified guinea pigs for *in vivo* experiments. The CRISPR/Cas9 genome

editing technique, which is adapted from an innate immune defense system that bacteria use against viruses, was first proposed in 2012 [162] and has in a few years changed the possibility to edit genomes. The clustered regularly interspersed short palindromic repeats (CRISPR) associated protein 9 (CRISPR/Cas9) technique is much quicker and less expensive than traditional approaches. It also allows the generation of complex models with large deletions, inversions, duplications or mutations in multiple genes. Already several publications have reported the use of this technique to introduce mutations in domestic animals, including pigs, sheep, cattle and goats, but also in ferrets, fish and other animals [163]. Considering the cost-benefit aspects for generating gene-edited guinea pigs, there are no other reasons to not explore how experimental models with modified guinea pigs can contribute to our understanding of asthma.

Drug development aspects

Despite large investments in drug development, drug attrition remains a problem across all therapeutic areas including respiratory. The average number of new drugs approved by the U.S. Food and Drug Administration (FDA) per year has declined since the 1990s, and only approximately 1 in 10 drugs that enter clinical phases makes it through to FDA approval. The biggest attrition is in Phase II, where the success rate is 29% for respiratory indications within the 2006–2015 timeframe [164]. Whereas safety issues constitute the major reason for project closure preclinically and in Phase I, lack of efficacy is by far the most dominating factor in Phase IIa and IIb failures [165]. One contributing explanation is that the current use of animal models in preclinical research fail to bridge the translational gap to the clinic.

Animal models have been and remain a cornerstone in drug discovery and are primarily used to [1] build confidence in the target and its link to disease (predict clinical efficacy), [2] provide a clear understanding of the pharmacokinetics/pharmacodynamics (PK/PD) relationship including demonstration of target engagement (predict dose to man) and [3] establish clear safety margins (predict clinical safety). However, whereas the use of animal models for establishing PK/PD relationships and safety margins is standard procedure, the contribution of current animal models to target validation and prediction of therapeutic effect in patients is far more debatable in light of the high clinical failure rate due to lack of efficacy.

When modeling aspects of human diseases, it becomes essential to select test species that are relevant for the research questions at hand in relation to human disease biology. The guinea pig offers a number of unique characteristics that make it suitable as a small animal species for preclinical studies related to asthma and COPD. Receptor and mediator pharmacology are comparable with the human situation, the anatomy and physiology of the guinea pig lung are similar to that of humans and the airway innervation of guinea pig airways is also similar to that of humans [7,152]. Furthermore, the airway response to allergen challenge in guinea pigs involves both early and late allergic responses, which makes it similar to that in humans and very different from that in mice [26]. Mice are easily bread, highly amenable to genomic manipulation [166] with a wide array of specific reagents available for analysis of the cellular and mediator response. For these reasons mice have become the preferred test system also for preclinical respiratory research, which is unfortunate as for most endpoints, guinea pig models of asthma and COPD are superior [167]. To narrow the translational gap between preclinical models of asthma and COPD and human disease biology, the guinea pig needs to be resurrected as an important test species.

For this to happen though, the apparent disadvantages of using guinea pigs need to be addressed and resolved. One important area will be to enable efficient generation of gene-modified guinea pigs (see above). The ease by which genes can be knocked out or knocked in in mice has been a major driver of the explosive increase in the use of mice in preclinical research. Guinea pig genetics is certainly more challenging than mouse genetics due to their significantly lower reproduction rate, but the prospects for transgenic animals are improving with the introduction and implementation of new technology. Another blocker to using guinea pigs for preclinical research has been the limited availability of reagents including antibodies for immunoassays and immunohistochemistry. A renewed interest in guinea pigs as a test species would drive a demand for tools, which in turn would automatically start build a commercial supply of such reagents. In addition, implementation of new technologies such as RNA sequencing and RNAscope *in situ* hybridization offers new analytical opportunities.

Future perspectives

The reason to develop a model of asthma is to identify the mechanisms with relevance to the disease and to define pharmacological treatments. As the knowledge of the mechanisms are not fully defined, the model development aims

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to mimic specific features and criteria typical for asthma of which the main are airway obstruction, chronic inflammation and the remodeling. However, this is complicated because these criteria may have different requirements for distinct asthma phenotypes.

Allergic asthma is a common phenotype and the development of models has therefore focused on different strategies to mimic this. In the earlier models, OVA was the dominant allergen of choice. The exchange from OVA to clinically relevant allergens in murine models has demonstrated more composite immunological reactions compared with OVA [168]. The airway reactions to natural allergens are complex and starts at the site of the airway epithelium. The natural allergens are not only composed of the epitopes that are detected by IgE, but also molecules that in different manners trigger or damage the epithelium that makes them to serve as adjuvants for allergic reaction. The adjuvant reaction has shown to be induced by specific activation of the innate immunological defense system. In parallel with the innate response, the adaptive reaction through dendritic cells to engulf the molecules that subsequently will end up being presented as antigen will take place. Thus, the model development in guinea pigs may be much improved using clinically relevant allergens and relevant routes of exposure.

The switch to natural allergen, has in many mouse models indicated that the sensitization phase should also be performed through airway exposure. As aerosol inhalations can be expensive when using natural allergens, it would be attractive to use intranasal instillation that is commonly performed in mice during a short isoflurane anesthesia. Intranasal instillation can be performed in guinea pigs but is technically challenging without anesthesia and complicated because the guinea pig is more difficult to anesthetize. Moreover, due to the big nasal cavity, the amount that reaches the lung can be more variable. Thus, the procedure for airway administration needs to be further developed into practical solutions that both are reliable and reproducible.

In the guinea pig allergic model of asthma, it is always possible to measure EAR. This is almost impossible to perform in mice. The EAR confirms a successful IgE activation and response, and can therefore be an important parameter for intervention studies. Non-invasive methods for measurement can be used to follow the IgE responses during the protocol. The LAR is a more difficult parameter to study due to the time needed to detect this and the inherent individual-to-individual temporal variabilty. However, there are several studies showing this in guinea pig models [26,102,106,115], whereas it is practically unfeasible to study in mice.

One objective for an asthma model is to allow studies of AHR. The AHR can be evaluated using direct acting agonists, such as histamine, muscarinic agonists and CysLTs, or indirect acting agonists, such as adenosine and mannitol [117,133,137,169]. This is clearly exemplified by the hyperresponsivity to inhaled adenosine in allergic guinea pigs [170]. In contrast, administration of muscarinic receptor agonists is almost the only agent that can be used in mice. An increasing awareness of the importance of the small airways in asthma has caused an increased interest for lung function measurement that separate the proximal and distal responses of the airways. The technique for doing these measurements has successfully been developed in mice using FOT, whereas it has almost never been used in guinea pig. It is possible that a different pattern of responses will be observed in guinea pigs compared with mice as the guinea pig has smooth muscle cells surrounding all branches down to the distal airways, whereas mice have no smooth muscle beyond large airways [73].

Further suggestions for the future

When the additional tools for investigation of the guinea pig asthma models have been established, there are several exciting possibilities for discoveries. If the use of the animal increases, the interest to develop more tools will increase. Having better tools, more researchers will be using the animal and a virtuous circle will be established. With the current clinical insight into asthma phenotypes, there is already a great need for asthma models of different phenotypes, e.g. non-T_H2 types of asthma, as well as the role of co-morbidities such as obesity. As guinea pigs share so many pulmonary features with humans, it is possible that this species has an explicit potential to be used for developing other phenotypes. We believe that guinea pig models will be important for further asthma studies into exacerbations, the role of the microbiome, epigenetic regulation, and impact of gender and age. Of particular relevance to the use of guinea pigs is the study of early onset asthma, as it is easier to study pups from guinea pigs than from mice. With an increased use, and an increased demand for breeding it is possible that inbreed strains will be created. With this, genetically modified guinea pigs also can eventually be generated. It is clear we are a long way from reaching the limits of what is possible using guinea pig models and realising their full potential.

As described in this review, there are many defined advantages to the use of guinea pigs in asthma research. Yet, there is also a requirement for more development to make models in this animal more effective, especially in regards to studies on inflammation, immunology and those that require genetically modified guinea pigs. In particular, the

guinea pig has a distribution of mast cells that is similar to humans, which should attract the researcher as mouse models fail to mimic the interactions between other inflammatory cells and mast cells that are fundamental in asthma.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BAL, bronchoalveolar lavage; ChAMP, Centre for Allergy Research Highlights Asthma Markers of Phenotype; CNS, central nervous system; COPD, chronic obstructive pulmonary disease; cysLT, cysteinyl-leukotriene; FOT, forced oscillation technique; LPS, lipopolysaccharide; PDE, phosphodiesterase; Penh, enhanced pause.

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Suppl. Table 1. Tools for ELISA

Target	Clone	Manufacturer	Product number
CCL3	polyclonal	R&D systems	AF-270/AF-450
CCL5	21418	R&D systems	MAB678
CCL11	43911	R&D systems	MAB320
CXCL8	6217	R&D systems	MAB208
IFN-γ	K3.53	R&D systems	MAB2852
IgG1	kit	My Biosource	MBS765111
IL-4	3010	R&D systems	MAB604
IL-5	TRFK5	BD Pharmingen/BD Biosciences	554392
IL-8	kit	Casubio	CSB-E13096Gp
IL-8	set	R&D systems	DY208
IL-10	127107	R&D systems	MAB2172
IL-13	kit	Casubio	CSB-E12916Gp
IL-13	kit	R&D systems	DY213
IL-17A	41809	R&D systems	MAB317
Neurokinin A	kit	Ray Biotech	EIA-NEA
PGE2	kit	R&D systems	KGE004B
Substance P	kit	ABCAM	AB133029
TGF-β1	BAF240	R&D systems	Polyclonal
TNF-α	set	R&D systems	DY5035

Suppl. Table 2. Tools for flow cytometry

Target	Clone	Labelling	Manufacturer	Product number
CD25	740290	PE	BD Bioscience	M-A251
CD4	CT7	FITC and PE	AbDSerotec	MCA749F
FOXP3	PCH101	PE-Cy5	eBioscience	14-4776-80
IFN-γ	B27	APC	BD Bioscience	554702
IL-10	JES3-19F1	PE	eBioscience	554706
IL-13	JES10-5A2	APC	Biolegend	501908
IL-17A	SCPL1362	PE	BD Bioscience	560438
PAN T-cell	CT5	FITC and APC	AbDSerotec	MCA751
TGF-β1	9016	PE	R&D systems	IC240P

Suppl. Table 3. Tools for immunohistochemistry

Target	Clone	Manufacturer	Product number
β-Actin	AC-15	Sigma-Aldrich	A5441
β-catenin	8E7	Millipore	05-665
CBS	G-1	Santa-Cruz	sc-271886
c-kit/CD-117	polyclonal	Biorbyt	orb156377
laminin	polyclonal	Sigma-Aldrich	#L-9393
MUC5AC	45M1	Thermoscientific	#ms145p
Nrf2	polyclonal	Abcam	ab31163
sm-MHC	SM-M10	Neomarkers	MS-1348-P0
tryptase	AA1	Dako	M705229-2
Type I MyHC	A4.840	Developmental Studies Hybridoma Bank	A4.840

Suppl. Table 4. Primers for PCR

β-actin	TGGCTACAGTTTCACCACCA	GGAAGGAGGGCTGGAAGA
CRTH2	TACACAAGGGGAAGCTCGG	CCAGTTTGGTCTCAGCCTTC
FoxA2	CCTTCAACGACTGCTTCCTC	TAGCTGCTTCTCGCACTTGA
FoxA3	TTCGAGAATGGCTGCTACCT	AAATAGGGCGTGGAGGAAGT
GAPDH	GTCGGTTGTGGATCTGACCT	TGCTGTAGCCGAACTCATTG
GAPDH	CATCACCATCTTCCAGGAGCGA	GTCTTCTGGGTGGCAGTGATGG
IL-4	TGCTGCACCATACAGCCTAC	CGGAGTTCACTCACTGGACA
IL-5	TACACAAGGGGAAGCTCGG	CCAGTTTGGTCTCAGCCTTC
IL-13	TCACCCAGGATCAGAAGACC	CCACCTCGATCTTGGTGTCT
MUC5AC	CCCTTCAGTGAGACCTCTGC	CTGACCCAGCAGGATAGAGC
P2X2	GCTGCTCATCCTGCTCTACTTT	GGGCTTCACATACTCCTCCAC
P2X3	AGGGAGGCTGAGAACTTCAC	CAGCCAATCTTGATGCCCAG
PTGDS	CCCTTCAGTGAGACCTCTGC	CTGACCCAGCAGGATAGAGC
SPDEF	CCCTGTCTGAGGAGCAGTTC	GATGCCCTTCTCCTTGTTGA
TRPA1	AGAGATCAAGGCGCTGGAA	CCCATAACTGGCTGCAAAAT
TRPA1	CATTTTGCTGCAACCCAAGGAGCCACTG	ATAAGTGGAGAGCGTCCTTCAGAATC
TRPV1	CCAACAAGAAGGGGTTCACA	ACAGGTCATAGAGCGAGGAG
TRPV4	TGGGCAAGAACTCAGATGGC	TCCACAGTCCTAGAGGGGAG