

# A guinea pig model of acute and chronic asthma using permanently instrumented and unrestrained animals

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**To investigate mechanisms underlying allergen-induced asthmatic reactions, airway hyperresponsiveness and remodeling, we have developed a guinea pig model of acute and chronic asthma using unanesthetized, unrestrained animals. To measure airway function, ovalbumin (IgE)-sensitized animals are permanently instrumented with a balloon-catheter, which is implanted inside the pleural cavity and exposed at the neck of the animal. Via an external cannula, the balloon-catheter is connected to a pressure transducer, an amplifier, an A/D converter and a computer system, enabling on-line measurement of pleural pressure ( $P_{pl}$ )—closely correlating with airway resistance—for prolonged periods of time. Using aerosol inhalations, the method has been successfully applied to measure ovalbumin-induced early and late asthmatic reactions and airway hyperresponsiveness. Because airway function can be monitored repeatedly, intra-individual comparisons of airway responses (e.g., to study drug effects) are feasible. Moreover, this model is suitable to investigate chronic asthma and airway remodeling, which occurs after repeated allergen challenges. The protocol for establishing this model takes about 4 weeks.**

## INTRODUCTION

### Allergic asthma

Allergic asthma is a chronic inflammatory disease of the airways. Characteristic features of this disease are allergen-induced early and late bronchial obstructive reactions, associated with infiltration and activation of inflammatory cells in the airways—particularly Th2 lymphocytes and eosinophils—and the development of airway hyperresponsiveness to a variety of stimuli, including allergens, chemical irritants, cold air and pharmacological agents like histamine and methacholine<sup>1</sup>. Alterations in the neurogenic and non-neurogenic control of airway smooth muscle function, as well as physical changes in the airways, including epithelial damage, thickening of the mucosa due to edema and local vasodilation, and mucous secretions in the airway lumen, may be involved in the development of airway hyperresponsiveness after the early and late asthmatic reactions in acute asthma. In chronic asthma, airway remodeling due to irreversible structural changes of the airway wall, including thickening of the basement membrane, mucous gland hypertrophy, subepithelial fibrosis and increased airway smooth muscle mass, may be importantly involved in persistent airway hyperresponsiveness and decline of lung function. All of these changes are induced by a complex cascade of inflammatory reactions involving various allergic mediators, neurotransmitters, cytokines, growth factors and reactive oxygen and nitrogen species<sup>1,2</sup>.

### Animal models of asthma

Although clinically well characterized, the exact pathological mechanisms underlying the allergen-induced early and late asthmatic reactions, airway hyperresponsiveness and remodeling are still incompletely understood. These mechanisms cannot be investigated exhaustively in human subjects because of trivial experimental limitations; hence, there is considerable interest in animal models of human asthma, each with its strengths and weaknesses<sup>3</sup>.

Of these, the sensitized guinea pig has received considerable attention as a small laboratory animal that develops allergen-

induced asthmatic reactions; guinea pigs have been used to study pathophysiological mechanisms and pharmacological interventions in asthma since the 1930s<sup>4</sup>. From a physiological point of view, the sensitized guinea pig, being a non-rodent<sup>3,5</sup>, may be preferred as an animal model to investigate mechanisms of airway hyperresponsiveness in asthma as compared to commonly used rodents such as mice and rats. Although rodents have been invaluable for elucidating putative mechanisms of allergic airway inflammation, the mediators that govern airway smooth muscle tone and ultimately determine airway responsiveness in rodents differ appreciably from those found in humans and guinea pigs<sup>3</sup>. From a technical point of view, present disadvantages of the guinea pig as an experimental animal may be the unavailability of genetically modified animals and the relative scarcity of immunological tools for this species, although the latter circumstance is rapidly improving.

Various studies, including our own, indicate that sensitized guinea pigs develop early and late asthmatic reactions, airway hyperresponsiveness and airway inflammation upon a single exposure to an aerosolized allergen solution; it has also been shown that airway remodeling occurs after repeated inhalation challenges with aerosolized allergen solutions<sup>6–12</sup>.

### Techniques of airway function measurement

In order to study the complex relationship between these aspects within the same animal, it is necessary to perform airway function measurements continuously and repeatedly over prolonged periods of time. Most of the available methods for the assessment of airway function in guinea pigs are not very well suited for this purpose. Airway function can be measured in anesthetized, tracheotomized, mechanically ventilated animals by changes in ventilation circuit pressure or air overflow as the lungs are inflated<sup>13</sup>. Because these measurements require surgical intervention and anesthesia, these methods are not suited to long-term or repeat

studies. In addition, anesthesia may greatly affect airway resistance<sup>14,15</sup> as well as airway responsiveness to histamine<sup>16</sup>. Airway function can also be measured consciously using bodyplethysmography, either under physically restrained conditions using single- or double-chamber plethysmographs to measure respiratory mechanics (i.e., airway resistance and lung compliance)<sup>13</sup> or under unrestrained conditions using barometric whole-body plethysmography to measure enhanced pause (Penh), an empiric variable that is used to assess airway responsiveness<sup>17</sup>. Especially in guinea pigs, physical restraint creates a very stressful situation, which is likely to affect airway responsiveness<sup>18</sup>. On the other hand, the validity of Penh as a measure of airway function under unrestrained conditions has been seriously questioned recently<sup>19,20</sup>.

### Guinea pig model of allergic asthma using permanently instrumented, conscious and unrestrained animals

To avoid the complications of anesthesia and physical restraint, we have developed a method to assess airway function parameters in chronically instrumented, conscious and unrestrained guinea pigs<sup>8</sup>. This method relies on (i) the measurement of airflow with a specially designed pneumotachograph that is surgically implanted inside the trachea, and (ii) measurement of pleural pressure ( $P_{pl}$ ) using a saline-filled balloon-catheter placed inside the pleural cavity. Both devices are connected to external pressure transducers via silicon conducting tubes subcutaneously implanted and permanently attached to the neck of the animal<sup>8</sup>. Using these signals, control values for airway resistance ( $R_{AW}$ ), dynamic compliance,  $P_{pl}$ , tidal volume and respiratory frequency have been derived that are all in the range of results reported previously for this species. In IgE-sensitized animals, a highly significant correlation is found between  $R_{AW}$  and  $P_{pl}$  values measured at baseline and during allergen (ovalbumin, OA)- or histamine-induced airway obstruction, indicating that  $P_{pl}$  measurements alone can be used as a relatively simple and sensitive method to assess bronchial obstructive reactions in unrestrained guinea pigs<sup>8</sup>. Allergen, histamine and methacholine challenges, as well as drug treatments, are performed by inhalation of aerosolized solutions in a specially designed 9-l perspex provocation cage in which the animals can move freely. Prior to the experimental provocations, the animals are habituated to the procedure in three training sessions, in order to minimize stress. Using an on-line computer system connected to the pressure transducer through an amplifier and an A/D

converter,  $P_{pl}$  can be registered continuously for prolonged periods of time, allowing the measurement of bronchoconstrictions induced by contractile agonists (histamine, methacholine) as well as allergen-induced early and late asthmatic reactions<sup>8,9</sup>. Because chronically instrumented animals can be monitored repeatedly, intra-individual comparisons of airway responses to different pharmacological and immunological stimuli, and to drug treatment, are feasible. Moreover, this model is suitable for the study of chronic asthma, which can be established after repeated allergen challenges<sup>12</sup>.

### Mechanisms of allergen-induced airway hyperresponsiveness and remodeling in conscious, unrestrained guinea pigs

In the acute model, we have demonstrated allergen-induced early and late phase asthmatic reactions, airway hyperresponsiveness after both reactions and airway inflammation with a striking similarity to human allergic asthma, both in a qualitative and in a quantitative sense<sup>9,10</sup>. Recently, we established the development of chronic airway hyperresponsiveness (F.J.W., A.B.Z., J.L.L. Kimpen, J.Z. & H.M., unpublished observations) and airway remodeling<sup>12</sup> in this model by applying 12 weekly allergen aerosol challenges to the animals.

The guinea pig model has been successfully applied to investigate mechanisms of acute and chronic airway hyperresponsiveness and the effectiveness of pulmonary drug treatment. This has been performed both by inhalation studies *in vivo* and by measurement of airway responses after allergen challenge *ex vivo*, using isolated intact perfused tracheae as well as tracheal open-ring preparations. These studies have uncovered various mechanisms of airway hyperresponsiveness, including the involvement of increased cholinergic reflex activity<sup>21</sup>, reduced function of prejunctional M<sub>2</sub>-receptors<sup>22,23</sup>, neurogenic inflammation<sup>24,25</sup> and disturbance of NO metabolism<sup>26–28</sup>, the latter being due to altered L-arginine homeostasis induced by inflammatory cell-derived polycations<sup>29,30</sup> and induction of arginase activity<sup>2,31,32</sup>. Moreover, using chronic allergen exposure, it has recently been established that the long-acting muscarinic receptor antagonist tiotropium bromide attenuates airway smooth muscle remodeling in chronic asthma<sup>12</sup>. This suggests a significant role for endogenous acetylcholine in the development and/or progression of chronic asthma and reveals hitherto unknown potentially beneficial effects of anticholinergic drugs in the treatment of asthma and possibly of chronic obstructive pulmonary disease (COPD).

## MATERIALS

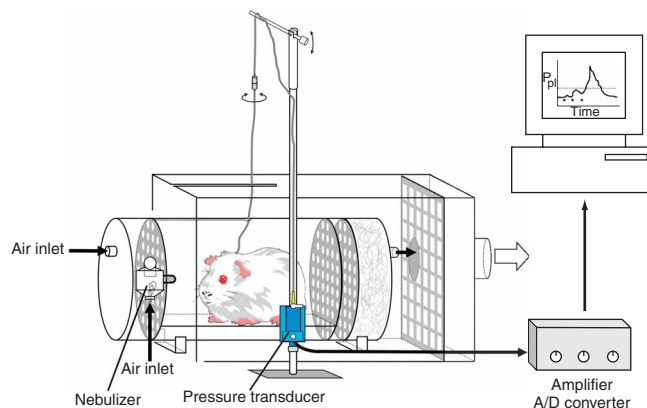
### REAGENTS

- Outbred specified pathogen-free Dunkin-Hartley guinea pigs weighing 250–300 g (Harlan) **! CAUTION** Experimenters must comply with national regulations concerning animals and their use.
- Ovalbumin (grade III; Sigma) **▲ CRITICAL** Ovalbumin solutions should be made up fresh before use.
- Al(OH)<sub>3</sub> (J.T. Baker Chemicals)
- Histamine hydrochloride (Sigma)
- Isoflurane (Forene, Abbott Laboratories)
- Buprenorphine, 0.3 mg ml<sup>-1</sup> (Temgesic, Schering-Plough)
- O<sub>2</sub> (Hoek Loos)
- N<sub>2</sub>O (Hoek Loos)
- Chlorohexidine (Blockland)
- Ethanol 70%

### EQUIPMENT

- Roller bank (Rock-n-roller; Breda Scientific)
- Polyethylene tubing, inner diameter 0.75 mm, outer diameter 1.45 mm (Rubber BV)
- Latex intraventricular balloon, size 3 (volume 0.03 ml; Hugo Sachs Electronics)
- Trelex Natural Mesh, 6 mm monofilament knitted polypropylene mesh (Boston Scientific, Medi-tech)
- Anesthesia and surgical equipment in operation room
- Electric clipper (elektra GH 204, Aesculap)
- Surgical instruments: dissection scissors, microdissection scissors, small and large straight anatomical forceps, straight jewellers forceps, curved anatomical forceps, needle holder (Barraquer-type) without lock, straight slender needle holder with lock (Lawton)

**Figure 1** | Experimental setup. The guinea pig is placed in a perspex 9-l provocation cage for aerosol challenge. During the experiment, an airflow of 8 l/min enters the cage via the air inlet at the front of the cage. Aerosol challenge is performed using the same airflow, through a DeVilbiss nebulizer, which is also positioned at the front of the cage. Using this airflow, the aerosol mass mean diameter is approximately 4.5  $\mu\text{m}$ , with a roughly 60% mean percentage of aerosol mass contained in droplets less than 5  $\mu\text{m}$  in diameter<sup>35</sup>. The compartment at the rear of the cage is packed with cotton wool, ensuring an even distribution of aerosol in the cage. The outer perspex cage placed over the provocation cage serves as a hood. Via a saline-filled external cannula and swivel, the implanted saline-filled balloon-catheter is connected to a fluid pressure transducer, which feeds the signal into a signal conditioner (amplifier plus A/D converter) interfaced to a personal computer.  $P_{\text{pl}}$  is measured in  $\text{cm H}_2\text{O}$ , with a sampling period of 10 s for each data point.



- Suture materials: silk (Perma Hand Seide, Silkam black), polyglactin (Vicryl) and polypropylene (Prolene) (Johnson and Johnson)
- Infant ventilator (Loosco M.K. 2, Hoek Loos)
- Perspex provocation cage with 9-l volume (custom made; Research Instrument Department, University Centre for Pharmacy, Groningen; see EQUIPMENT SETUP)
- Nebulizer (type 646, DeVilbiss)
- Pressure transducer (TC-XX, Viggo-Spectramed BV)
- Signal conditioner (custom-made; Electronics Department, Faculty of Science, University of Groningen, The Netherlands, deliverable on request). See EQUIPMENT SETUP for more details.
- Data handling software (Electronics Department, Faculty of Science, University of Groningen; programmed in LabVIEW 4.0 (National Instruments), available on request)

## EQUIPMENT SETUP

**Perspex provocation cage** A schematic representation of the cage is shown in **Figure 1**. The inner diameter of the cage is 19 cm,

the outer diameter is 20 cm. Lengths of individual inner compartments are: front compartment 4.6 cm, animal compartment 24 cm, intermediate compartment 3.4 cm, back compartment 6.0 cm. The total outer length of the cage is 39 cm. The front compartment also functions as a door, allowing easy transfer of the animal into the cage.

**Signal conditioner** Signals are amplified by an instrumentation amplifier, type INA110 (Burr-Brown), amplification steps: 10, 100, 200 and 500 times, digitally operated by a Lab-PC-1200AI i/o device (National Instruments) and adjusted in the data handling program. Signals are filtered by a first-order analog filter with cutoff frequencies of 0.01 Hz, 0.05 Hz, 0.1 Hz, 0.5 Hz, 1 Hz or 5 Hz, digitally operated by the Lab-PC-1200AI i/o device and adjusted in the data handling program.

**Data handling** Absolute  $P_{\text{pl}}$  data are sampled every 50 ms. Amplitude values of  $P_{\text{pl}}$  are calculated, averaged over 10-s periods for each data point and presented in a  $P_{\text{pl}}$  time curve, using the data handling program.

## PROCEDURE

### Sensitization

- 1| To sensitize animals to antigen with IgE-class antibodies, add 100 mg  $\text{Al}(\text{OH})_3$  per ml of OA solution in sterile saline ( $100 \mu\text{g ml}^{-1}$ ). Rotate the mixture gently on a roller bank for 60 min at room temperature ( $20\text{--}22^\circ\text{C}$ ) to obtain an alu-gel.
  - 2| Inject each animal with 1 ml antigen solution: 0.5 ml intraperitoneally and 0.5 ml intradermally divided over seven injection sites in the proximity of lymph nodes in the legs, lumbar regions and neck, using  $0.50 \times 16 \text{ mm}$  needles. Note that full development of the immune response takes 4 weeks from injection. During this time, OA-specific IgE as well as IgG<sub>1</sub> antibodies are generated<sup>33</sup>.
- ▲ **CRITICAL STEP** Gently rotate the 1-ml syringe filled with alu-gel before injections to prevent precipitation of excess of  $\text{Al}(\text{OH})_3$  particulates.

### Surgery

- 3| The animals should be provided with an intrapleural balloon-catheter two weeks after sensitization. First, prepare a polyethylene tubing with three small bulges (diameter 3–4 mm) by locally heating (using a small gas flame) and gently compressing two ends of the tube while the lumen is filled with a small metal rod with the approximate diameter of the tube. One bulge should be positioned at the very end of the tube, and the other bulges at 0.8 and 12 cm from this bulge, respectively. The total length of the tubing should amount to approximately 16 cm. Place the latex balloon with its opening over the bulge at the end of the catheter and tighten with a surgical knot, using 5-0 silk (Silkam black).
- ▲ **CRITICAL STEP** Cut the end of the cannula at the outer bulge perpendicularly and carefully to prevent possible damage to the latex balloon, which has to be slit over the bulge at this end for fixation. It is important to test the connection between balloon and cannula for leakage, as this will prohibit  $P_{\text{pl}}$  measurement. This is performed by filling the balloon and tubing with saline, using a syringe with 21G needle, and putting the system under slight pressure.
- 4| Sterilize the balloon-catheter and a macron mesh ( $20 \times 20 \text{ mm}$ ) by incubation in 70% ethanol for 15 min. Sterilize the surgical instruments by autoclaving for 20 min at 3 Bar. Maintain an aseptic environment by cleaning the operation area on the operating table, including the heating mattress on which the animal lies during surgery, using 1% aqueous chlorohexidine solution or 70% ethanol.

- 5| Administer buprenorphine (0.015 mg/kg) by intramuscular injection in the right hind leg for epi- and postoperative analgesia.
- 6| Induce anesthesia by placing the guinea pig in an induction chamber filled with a N<sub>2</sub>O (500 ml/min)/O<sub>2</sub> (500 ml/min)/5% isoflurane mixture. After the animal has lost consciousness, maintain anesthesia via a face mask at the same flow rate, but with a 1.5–2% concentration of isoflurane.  
**▲ CRITICAL STEP** Fine-tuning of this concentration should be done on every single animal. Depth of anesthesia should be checked by the absence of the pedal-withdrawal reflex and cornea reflex. Waste and excess anesthetic should be scavenged.
- 7| Shave the dorsal and ventral surface of the neck and the right side of the chest. Gently scrub the shaved area with 1% chlorohexidine solution and place the animal on an electrically heated mattress (37 °C).
- 8| Make an incision in the ventral surface of the neck and expose the trachea, preparing it free from muscles and esophagus, and moisten the tissue with warm (37 °C) sterile saline solution.
- 9| Make an incision in the ventral surface of the chest halfway between the right foreleg and the xyphoid cartilage of the sternum in the fourth intercostal space. Expose the external intercostal muscle and place a four-stitch purse-string suture using 6-0 Prolene. Moisten the exposed tissue with warm (37 °C) sterile saline solution.
- 10| Make an incision in the trachea, intubate the trachea and attach the animal to the mechanical ventilator. Ventilate the animal at 40 respirations per minute with an inspiration:expiration ratio of 1:2.
- 11| Penetrate the thoracic wall in the middle of the purse-string with a dissecting forceps and insert the balloon-catheter up to the second bulge. Tighten the purse-string while compressing the chest wall to prevent pneumothorax and fix the second bulge of the cannula with the free ends of the purse-string suture. Close the outer muscle layer with a surgical knot using 6-0 Prolene.  
**▲ CRITICAL STEP** Airtight closure of the thoracic cavity can be tested by applying saline over the surgical spot and gently pressing the chest. In this way, possible air leakage can be observed.
- 12| Stop the ventilation and close the trachea with a surgical knot using 7-0 silk (Perma Hand Seide). Also close the outer muscle layer covering the trachea with a surgical knot using 6-0 Prolene.  
**▲ CRITICAL STEP** Some blood may accumulate in the trachea and should be carefully removed with small forceps wrapped up with cottonwool.
- 13| Tunnel the catheter subcutaneously to the ventral surface of the neck using artery forceps.
- 14| Close the skin of the chest with four horizontal mattress sutures using 3-0 Vicryl.
- 15| Make an incision at the dorsal surface of the neck and tunnel the catheter subcutaneously to this place using artery forceps.
- 16| Slide the end of the catheter over the end of a hollow needle (diameter 0.90 mm) and drive the needle through the macron mesh (20 x 20 mm) until the third bulge for subcutaneous fixation.
- 17| Close the skin at the dorsal surface of the neck by four interrupted sutures with 3-0 Vicryl, concomitantly fixing the subcutaneous mesh.
- 18| Close the skin at the ventral surface of the neck with two horizontal mattress sutures using 3-0 Vicryl.
- 19| Stop the administration of isoflurane and N<sub>2</sub>O and continue the administration of O<sub>2</sub> (1 l/min) until the return of righting reflex.
- 20| Place the animal in an individual recovery cage in a climate-controlled room (20–22 °C), maintaining a 12-h light/12-h dark cycle. Allow recovery for at least one week, until preoperative weight has been restored. Give water and food *ad libitum*.  
**▲ CRITICAL STEP** Since the neck area may be sensitive after surgery, the animals should be able to reach their drinking water without stretching their neck.

#### **Measurement of allergen-induced early and late asthmatic reactions and airway hyperresponsiveness to histamine**

- 21| Place the animal in the provocation cage (**Fig. 1**) to start the habituation session with the catheter unconnected to the measurement system. Allow an airflow of 8 l/min through the cage via the air inlet at the front for 30 min. Fill the nebulizer with 5 ml saline and start nebulization with the same airflow using a three-way valve, to obtain an aerosol output of 0.33 ml/min. Nebulize for 3 min, and subsequently change the airflow to the front inlet. Repeat the nebulization twice with 7-min intervals, after which the animal is placed in the individual home cage (0.16 m<sup>2</sup>).

**22|** Next day: fill the implanted balloon-catheter with saline, using a thin polyethylene cannula slid into the balloon and connected to a 5-ml syringe. Connect the filled balloon-catheter to an external fluid-filled cannula, which is slid over the hollow needle at the end of it. Connect the external cannula via a swivel to a fluid pressure transducer, which signals the  $P_{pl}$  changes via an amplifier and A/D converter to a personal computer for on-line registration and data analysis (**Fig. 1**).

#### ? TROUBLESHOOTING

**▲ CRITICAL STEP** Take care that no air is trapped in the system, as it will dampen the pressure signal.

**23|** Repeat the habituation protocol as in step 21, but now with the balloon-catheter connected to the measurement system.

**24|** Next day: perform a histamine challenge for habituation. Connect the guinea pig to the measuring system. Start the airflow through the inlet at the front of the cage for adaptation and register  $P_{pl}$  changes during 30 min. In the meantime, fill the nebulizer with 5 ml saline to perform two saline control challenges as described in step 21. After these inhalations, fill the nebulizer with 5 ml of  $25 \mu\text{g ml}^{-1}$  histamine solution in saline. Nebulize for up to 3 min. If no dyspnea develops, refill the nebulizer with an increased concentration of histamine by one dosage step of  $25 \mu\text{g ml}^{-1}$  and start a new aerosol challenge after 7 min. Repeat this procedure with increasing dosage steps until respiratory distress is observed and  $P_{pl}$  increases by more than 100% for at least three consecutive minutes, as monitored by the computer. Disconnect the animal after 30 min, when baseline  $P_{pl}$  has been reached again.

**▲ CRITICAL STEP** The sensitivity to histamine may be dependent on the guinea pig strain used as well as on their immune status. We have found that both Dunkin-Hartley and BFA tri-color guinea pigs have a basal  $PC_{100}$  value well above  $25 \mu\text{g ml}^{-1}$  (approximately  $100 \mu\text{g ml}^{-1}$ ).

**25|** Start the experimental protocol one week after the first habituation session, by repeating step 24. From the collected data, the histamine  $PC_{100}$  (i.e., the provocation concentration causing 100% increase in  $P_{pl}$ ) can be derived by linear intrapolation of the concentration- $P_{pl}$  response curve, as a measure of basal airway responsiveness to histamine (**Fig. 2**).

**26|** Next day: perform an OA challenge to measure allergen-induced early and late asthmatic reactions and airway hyperresponsiveness after these reactions. Connect the guinea pig to the measuring system. Start the airflow through the inlet at the front of the cage for an adaptation period of 30 min. Perform two saline control challenges subsequently, as described in step 21. After these inhalations, perform an allergen challenge by inhalation of stepwise increasing concentrations of 0.05%, 0.1%, 0.3%, 0.5% and 1.0% (w/v) OA solution in saline for 3 min, separated by 7-min intervals. Immediately stop nebulization as soon as the first signs of respiratory distress are observed and  $P_{pl}$  increases by more than 100%. Continue  $P_{pl}$  measurement while the guinea pig remains in the provocation cage.

**▲ CRITICAL STEP** Carefully observe the animals during the allergen challenge to avoid anaphylactic shock. When performed properly, no pretreatment with an antihistamine is needed. This is important, as these agents affect allergen-induced asthmatic reactions, inflammation and airway hyperresponsiveness<sup>34</sup>.

**27|** At 6 h after the allergen provocation, when the early asthmatic reaction has resolved (**Fig. 2**), determine histamine responsiveness ( $PC_{100}$ ) according to steps 24 and 25. Place the animal in its home cage when the histamine-induced bronchoconstriction has resolved, while keeping the animal connected to the measuring system for assessment of the late asthmatic reaction. Give water and food *ad libitum*. The magnitudes of the early and late asthmatic reactions can be assessed as areas under the  $P_{pl}$  time-response curve between 0 and 6 h after allergen provocation for the early asthmatic reaction, and between 8 and 24 h after provocation for the late asthmatic reaction (**Fig. 2**).

**Figure 2 |** Allergen-induced early and late asthmatic reactions and airway hyperresponsiveness. **(a)** Representative examples of pleural pressure ( $P_{pl}$ ) airway responses after single saline or ovalbumin (OA) challenge. Results are expressed as percentage of baseline, which is calculated as the mean of all data points obtained during the last 20 min of the adaptation period. Challenges were performed at 0 h. For improved visualization, the responses are presented as moving average over three consecutive sampling periods. **(b)** Examples of histamine  $PC_{100}$  measurements as a measure of airway responsiveness, performed before OA challenge and after the early asthmatic reaction (6 h after OA) and after the late asthmatic reaction (24 h after OA).  $PC_{100}$  is defined as the provocation concentration causing 100% increase in  $P_{pl}$ , and is derived by linear intrapolation of the concentration- $P_{pl}$  response curve. In this representative example a 3.6-fold increase in airway responsiveness is found after the early asthmatic reaction, whereas a 1.4-fold increase is found after the late asthmatic response.

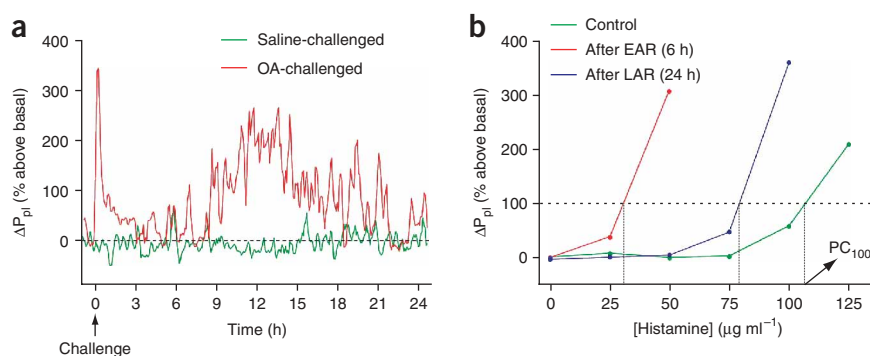
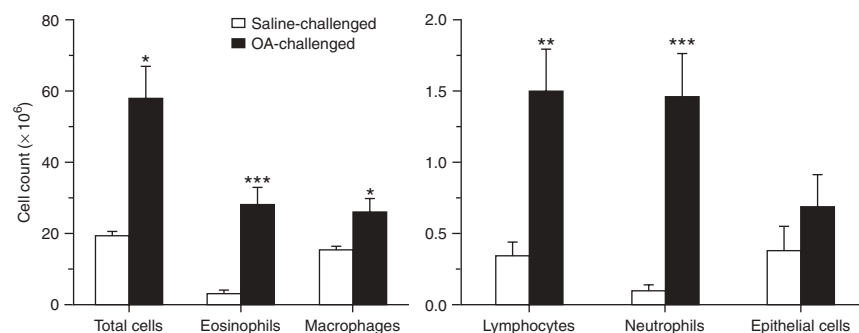


**Figure 3** | Bronchoalveolar lavage (BAL) cell counts at 25 h after single saline or ovalbumin (OA) challenge. The animals were anesthetized with 20 mg/kg Brieatal, 35 mg/kg Ketalar and 6 mg/kg Rompun, administered intraperitoneally. The trachea was exposed and cannulated, and the lungs were lavaged gently using 5 ml of sterile saline at 37 °C, followed by three subsequent aliquots of 8 ml saline. The recovered BAL samples were cooled on ice, and centrifuged at 200g for 10 min at 4 °C. The pellets were combined and resuspended to a final volume of 1.0 ml in PBS and total cell numbers were counted using a Coulter Counter. For cytological examination, cytospin preparations were stained with May-Grünwald and Giemsa stain. A cell differentiation was performed by counting at least 400 cells in duplicate. Results represent mean values  $\pm$  s.e.m. of 5–7 animals. \* $P$  < 0.05; \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 compared to saline-challenged controls. Adapted from Schuiling *et al.*<sup>24</sup>.



**▲ CRITICAL STEP** Owing to increased sensitivity to histamine after allergen challenge, particularly after the early asthmatic reaction, it may be necessary to start at a lower concentration than 25  $\mu\text{g ml}^{-1}$  to measure  $\text{PC}_{100}$  (e.g., a starting dose of 6.25  $\mu\text{g ml}^{-1}$  followed by 12.5  $\mu\text{g ml}^{-1}$ ).

**28** | Place the animal back in the provocation cage at 24 h after allergen challenge to measure histamine  $\text{PC}_{100}$  after the late asthmatic reaction.

**▲ CRITICAL STEP** See step 27.

**29** | For measurement of airway hyperresponsiveness and airway remodeling in chronic asthma, perform 12 weekly allergen challenges as described in step 26.

### ● TIMING

Day 1: Sensitization of animals to antigen (1–2 min per animal)

Day 15: Insertion of balloon-catheter (30–45 min per animal)

Day 22: Habituation procedure with no connection to the measurement system (60 min per animal)

Day 23: Habituation procedure while connected to the measurement system (60 min per animal)

Day 24: Histamine challenge for habituation (80–100 min per animal)

Day 29: Measurement of airway responsiveness to histamine or methacholine (80–100 min per animal)

Day 30: Measurement of early and late asthmatic reactions (24 h per animal)

### ? TROUBLESHOOTING

In step 22, if the  $P_{\text{pl}}$  signal is small or nonexistent, the intrapleural balloon-catheter could be leaky because (i) the balloon is damaged or (ii) there is a poor connection between the balloon and catheter (see step 3). In addition, there could be air in the catheter and/or the external cannula. If the problem is air in the cannula, this can be resolved by refilling the system with saline.

### ANTICIPATED RESULTS

An example of an OA-induced dual asthmatic reaction, in comparison with a saline-induced control, is shown in **Figure 2a**. The early reaction starts with a sharp increase in  $P_{\text{pl}}$ , which is maximal within 20 min and lasts for approximately 2–3 h. The late response starts at approximately 8 h after allergen challenge and is usually reversible within 24 h after challenge. Marked oscillations in  $P_{\text{pl}}$  are observed during the late asthmatic reaction, stressing the importance of continuous registration of this response for quantitative analysis. By definition, all animals respond with an early asthmatic reaction using this protocol.

Approximately 70% of these animals develop a late reaction as well, which is remarkably similar to human provocation studies<sup>9</sup>. By challenging with subeffective OA doses that do not induce an immediate response, isolated late asthmatic reactions may be observed<sup>9</sup>. Changes in airway reactivity to

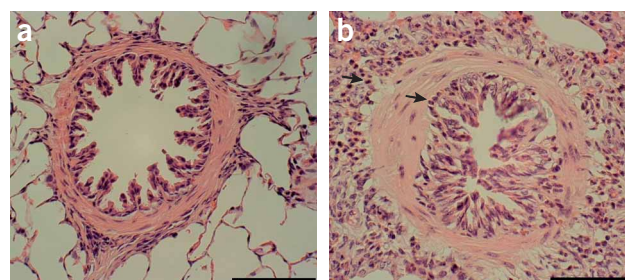
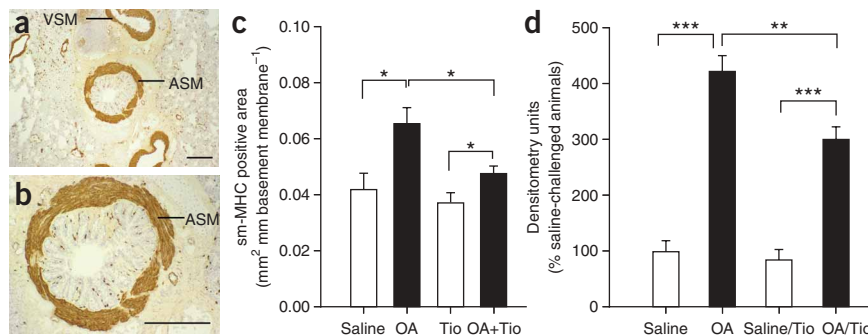


**Figure 4** | Representative histological photomicrographs of formalin-fixed paraffin-embedded lung sections containing transverse cross-sections of peripheral airways, obtained (a) after single saline challenge or (b) after single OA challenge, at 17 h after the challenges. Sections were stained with haematoxylin-eosin. Eosinophils are indicated by arrows. Calibration bars represent 100  $\mu\text{m}$ .

**Figure 5** | Airway smooth muscle remodeling after chronic allergen exposure. (a,b) Representative photomicrographs of a cryostat lung section containing a transverse cross-section of a main bronchus, stained for smooth muscle myosin heavy chain (sm-MHC). Only the area in the smooth muscle layers of the airways and blood vessels stained positive for this protein. ASM, airway smooth muscle; VSM, vascular smooth muscle. Calibration bars represent 250  $\mu$ m.



(c) Morphometric analysis of sm-MHC expression in peripheral, non-cartilaginous airways obtained after 12 consecutive weekly challenges with saline or ovalbumin (OA), with or without treatment with the long-acting muscarinic receptor antagonist tiotropium bromide. For tiotropium treatment animals received a nebulized dose of tiotropium bromide in saline (0.1 mM, 3 min), 0.5 h prior to each challenge with saline or OA. Results shown represent mean  $\pm$  s.e.m. of 5–8 animals. Three to five airways were analyzed per animal. \* $P$  < 0.05. (d) Effects of repeated OA challenges and tiotropium treatment on sm-MHC expression in the guinea pig lung. Protein-matched lung homogenates were analyzed by western blotting for sm-MHC. Data shown represent densitometric means  $\pm$  s.e.m. of 5–8 animals. \*\* $P$  < 0.01; \*\*\* $P$  < 0.001. Taken from Gosens *et al.*<sup>12</sup>.

histamine at 6 h (after the early asthmatic reaction) and 24 h (after the late asthmatic reaction) in dual responders after OA challenge are shown in **Figure 2b**. Usually a 3–4-fold increase in PC<sub>100</sub> is observed after the early asthmatic reaction, while a smaller, but significant, 1.5–2-fold shift is usually observed after the late asthmatic reaction. Remarkably, no significant airway hyperresponsiveness is observed with single early asthmatic reactions. Saline challenge does not affect airway responsiveness at all. In both a qualitative and in a quantitative sense the allergen-induced asthmatic reactions and airway hyperresponsiveness closely resemble those observed in asthmatic patients<sup>9</sup>.

The OA-induced asthmatic reactions are associated with marked inflammatory responses in the airways. As shown in **Figure 3**, significant increases in total cell number, eosinophils, neutrophils, lymphocytes and macrophages are observed in the bronchoalveolar lavage (BAL) performed 25 h after allergen challenge. An increase in ciliated epithelial cells is usually also present. It should be mentioned that, depending on the guinea pig strain, significant alterations in numbers of lymphocytes and macrophages are not always detected. Infiltration of cells into the airway lumen is time dependent<sup>9</sup>, which is also reflected by the pattern of inflammatory cell trafficking in the airway tissue<sup>10</sup>. Histological examples of small airways obtained from a saline-challenged and an OA-challenged guinea pig are shown in **Figure 4**. It is demonstrated that the OA-challenged airway (**Fig. 4b**) is heavily infiltrated with inflammatory cells, particularly eosinophils. The airway smooth muscle is contracted and the airway wall is swollen. After chronic allergen exposure to the airway, airway remodeling occurs. **Figure 5a** and **b** show representative sm-MHC immunostainings of smooth muscle in transverse sections of the main bronchus. Morphometric analysis of the sm-MHC-positive area identified a significant increase in airway smooth muscle mass after 12 weekly repeated OA challenges, particularly in the small airways (**Fig. 5c**). Remarkably, the allergen-induced increase in airway smooth muscle mass is inhibited by pretreatment of the animals with the long-acting muscarinic receptor antagonist tiotropium bromide before each challenge, indicating a prominent role for endogenous acetylcholine in airway smooth muscle remodeling. These changes are reflected by changes in sm-MHC expression in total lung as established by western blotting (**Fig. 5d**).

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