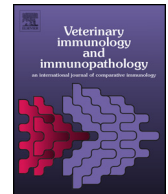




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# Veterinary Immunology and Immunopathology

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## Research paper

# Neonatal aerosol exposure to Bermuda grass allergen prevents subsequent induction of experimental allergic feline asthma: Evidence for establishing early immunologic tolerance

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## ARTICLE INFO

### Article history:

Received 6 December 2013

Received in revised form 27 February 2014

Accepted 10 March 2014

### Keywords:

Allergic asthma

Tolerance

Feline asthma

Neonatal immunity

Bermuda grass allergen

## ABSTRACT

Allergic asthma is increasing in industrialized countries, especially in children. Rodent and human studies suggest an opportunity to “prevent” asthma in the perinatal period. The aims of this study were to create a more “natural” model of feline asthma by exposing offspring of asthmatic queens to Bermuda grass allergen (BGA) by inhalation only, and to investigate maternal–fetal–infant interactions in the development of asthma. Kittens from asthmatic queens were divided into four groups: maternal exposure to aerosolized BGA during the third trimester, neonatal exposure to aerosolized BGA in the first three months of life, both maternal and neonatal exposure, or saline control. Kittens failing to achieve an asthmatic phenotype based on bronchoalveolar lavage fluid (BALF) analysis by 6 months underwent traditional sensitization: adjuvanted allergen injection, intranasal allergen, and aerosol challenges. BALF was collected at 3, 4 and 6 months, and after sensitization at 8 months, and analyzed for eosinophil counts and BGA-specific IgG and IgA. Intradermal testing (IDT) was performed at 6 and 7 months. At six months none of the kittens had airway eosinophilia, BGA-specific IgG or IgA, and were non-responsive to IDT. After sensitization, kittens receiving neonatal aerosolization failed to develop airway eosinophilia as seen in the controls. Kittens exposed to BGA aerosols, either in-utero or neonatally, continued to lack IDT response. Chronic exposure to BGA aerosols failed to induce asthma in kittens, and instead tolerized the kittens to BGA. This is the first evidence that neonatal intervention could potentially “prevent” allergic asthma in cats.

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## 1. Introduction

Asthma is one of the leading chronic childhood illnesses in the United States (Adams et al., 1996) and is associated with morbidity, mortality and a heavy economic burden. In the last two decades, the incidence of asthma in industrialized countries has been increasing to epidemic levels, especially in children (Redd, 2002; Tattersfield et al., 2002). Allergic asthma, the most common form of asthma in

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humans, is characterized by intermittent reversible airway obstruction, chronic airway inflammation, airway hyper-responsiveness, and airway remodeling (Yssel and Groux, 2000). Current therapeutic strategies for allergic asthma focus on modulating the immune response to allergen once allergy has been well established. An alternative and possibly more effective approach could be to prevent development of allergic hypersensitivity. There are compelling studies in humans and in rodent models of asthma that suggest the window of opportunity to “prevent” asthma is in the in utero/neonatal period (Matson et al., 2009; Almqvist et al., 2010; Verhasselt, 2010; von Mutius and Vercelli, 2010).

The driving force in the induction and maintenance of the allergic inflammatory cascade is ascribed to allergen-specific CD4<sup>+</sup> T helper 2 (Th2) cells (Romagnani, 2000). In non-allergic individuals, Th1 immunity predominates and is characterized by production of interferon  $\gamma$  which inhibits Th2 cell polarity. While the respiratory tract is continually exposed to a variety of nonpathogenic antigens such as allergens, most individuals are tolerant and do not develop active immunity against these allergens. What dictates whether tolerance or active immunity develops is multifactorial and involves both genetic and environmental contributions. There is compelling evidence that the immune system is “programmed” to be (or not to be) sensitized to allergens during the perinatal period (Holt, 1998; Devereux et al., 2002). That is, expression of allergic reactivity in adults is dependent on perinatal immune responses leading to long term Th2 polarized allergen-specific immunologic memory. Pregnancy is skewed toward a Th2 immune polarity in order to prevent fetal rejection, as Th1 cytokines could promote rejection of the fetus (Wegmann et al., 1993). In non-allergic individuals, there is a transition toward a Th1 memory cell profile after birth; in allergic individuals, failure to transition to Th1 memory cells favors allergen-specific Th2 memory cells (Holt, 1998; Prescott et al., 1998, 1999; Devereux et al., 2002). It is postulated that influences from the allergic mother may delay or prevent transition to a non-allergic (tolerant) immune response to aeroallergens. A recurring theme in animal and human studies is that the in utero/neonatal period is the window of opportunity in which immune responses might be able to be manipulated to prevent development of an asthmatic phenotype.

Rodent models are most commonly employed to study asthma pathogenesis because of relative low cost, abundance of reagents to characterize immunopathology, and ease of manipulating their genetic background. However, extrapolation of results from rodents to asthmatic humans has major limitations (Hein and Griebel, 2003). Rodents do not naturally develop asthma and artificial induction of asthma requires very large allergen doses in combination with adjuvant administration. Many of the common antigens used to induce asthma (e.g., ovalbumin) are not clinically relevant allergens in humans. Indeed, there have been concerns about biologic relevance of these rodent models, especially with respect to novel therapeutics that have shown promise in rodents and failed clinical trials in humans (Holmes et al., 2011). In looking for a more suitable comparative model, the only animal species that naturally

**Table 1**

Experimental groups.

| Group | Maternal challenge | Neonatal sensitization/challenge | Number of kittens |
|-------|--------------------|----------------------------------|-------------------|
| 1     | Saline             | Saline                           | 5                 |
| 2     | Saline             | BGA                              | 4                 |
| 3     | BGA                | Saline                           | 4                 |
| 4     | BGA                | BGA                              | 4                 |

develops allergic asthma with the major features of the human disease (recurrent clinical signs referable to spontaneous bronchoconstriction, airway hyperresponsiveness, airway eosinophilia and airway remodeling) is the domestic cat (Moise et al., 1989; Dye et al., 1996; Norris Reiner et al., 2004). We have previously developed an experimental feline model of chronic allergic asthma using a clinically relevant allergen identified in pet cats (Bermuda grass allergen, BGA) helpful for studying immunopathogenic mechanisms of this disease and novel therapeutics (Norris Reiner et al., 2004; Reiner et al., 2005; Reiner et al., 2006; Dhand et al., 2008; Reiner et al., 2008). In this model, allergen sensitization and challenge results in allergen-specific IgE, IgG and IgA; airway hyperresponsiveness; airway eosinophilia; and acute T helper 2 cytokine profile and histologic evidence of airway remodeling (Norris Reiner et al., 2004). However, exposure to allergen in kittens in our model starts no earlier than 12 weeks of age, missing the window of opportunity to investigate mechanisms by which the maternal immune system can influence the development of the fetal/infant immune response to allergen. Furthermore, because cats are a species that spontaneously develops asthma, administration of allergens by aerosol to kittens without priming the immune system by allergen injection with adjuvants may be a superior model of the human disease. Thus, the aim of the current study was twofold: First, to create a more “natural” model of feline asthma by exposing offspring of experimentally asthmatic queens to BGA by inhalation only and second, to simultaneously investigate maternal–fetal–infant interactions as they relate to the development of asthma. We hypothesized that kittens born to experimentally asthmatic queens would themselves be susceptible to asthma development after chronic exposure to low concentrations of BGA by aerosol (both in utero and neonatally) without the use of injected antigens or adjuvants.

## 2. Materials and methods

### 2.1. Animals and sensitization protocols

Four female adult queens from a high responder asthmatic cat colony that were induced to have experimental asthma according to a previously published protocol served as the mothers for the kittens enrolled in the study (Norris Reiner et al., 2004). These queens gave birth to seventeen kittens; the litters were randomized into four groups challenged during the pregnancy or the neonatal period with either saline or Bermuda grass allergen (BGA) (Table 1). Kittens born in the same litter were kept in the same treatment group and were allowed to nurse from their mother until weaning (week 6). All cats were cared for in accordance

with the NIH Guide for the Care and Use of Laboratory Animals, with approval by the University of Missouri Animal Care and Use Committee (ACUC protocol #3390).

Queens were not exposed to allergens or saline aerosols during mating or gestation except as reflected in Table 1. Exposure of the pregnant queen to allergen or saline (“maternal challenge”) took place during the last 20 days of gestation (after confirmation of fetal presence by serial palpation and transabdominal ultrasonography). Three aerosol challenges of 100 µg BGA were performed 6 days apart in awake and spontaneously breathing cats placed in a sealed plastic chamber. Compressed air at a pressure of 2.93 kg/cm<sup>2</sup> was supplied via an air compressor (Easy Air 15, Precision Medical, Inc., Northampton, PA) to a nebulizer (Acorn nebulizer, model 646, Devilbis Health Care, Somerset, PA) to deliver an aerosol of the allergen solution in an air flow rate of 9.3 l/min.

Unlike the traditional protocol, induction of asthma in neonatal cats (“neonatal sensitization/challenge”) took place only by delivery of BGA by the aerosol route. The intent was to mimic spontaneous feline asthma in which allergen exposure is via inhalation (and perhaps ingestion via grooming of fur). The neonatal sensitization/challenge protocol started at 3 days of age whereby an entire litter was removed from the mother for the 10 min aerosol treatment (100 µg BGA total; same delivery system as described for the queens). Aerosol treatment was performed twice weekly on the whole litter for 6 weeks. Once kittens reached a larger size (>1 kg) they were separated for individual treatments. Twice weekly aerosol treatments were continued until kittens were 3 months of age; aerosols were also administered 24 h prior to collection of bronchoalveolar lavage fluid. Cats were serially evaluated until 6 months of age (see “airway eosinophilia” below) without further exposure to allergen. At 6 months, cats failing to achieve an asthmatic phenotype received the traditional sensitization/challenge protocol involving injections of adjuvanted allergen and intranasal allergen administration, followed by seven aerosol challenges (also 100 µg/treatment) (Norris Reinero et al., 2004).

## 2.2. Airway eosinophilia

Bronchoalveolar lavage fluid (BALF) was collected at 3, 4, and 6 months of age, and again after traditional sensitization (at 8 months of age). Kittens were administered ketamine IV (5–10 mg/kg) and intubated for collection of BALF in a blind fashion. Briefly, a 3, 5 or 8 French red rubber catheter (depending on kitten size) was advanced down the endotracheal tube until gentle resistance was felt. A single 10–20 ml aliquot of sterile saline was instilled and subsequently retrieved into the syringe by gentle hand suction. Albuterol by metered dose inhalant and/or oxygen was administered as clinically indicated. A cytospin was used to make a cytological preparation, and cells were stained with a modified Wright’s stain. To determine the % eosinophils, a total of 200 nucleated cells were counted. Bronchoalveolar lavage fluid % eosinophils >17% was defined as compatible with an asthmatic phenotype. Remaining BALF was centrifuged at 350 × g for 5 min and supernatant harvested and banked in aliquots at –80 C until analysis.

## 2.3. BGA-specific immunoglobulin (Ig) G and A

Thawed BALF was analyzed for BGA-specific IgG and IgA according to a previously described enzyme linked immunosorbent assay (ELISA) protocol with minor modifications from samples collected at months 3, 4 and 8 (Norris et al., 2003). The positive control consisted of banked pooled BALF from cats in an unrelated study with a strong asthmatic phenotype and high optical densities, using the previously published ELISA conditions. A seven point doubling dilution of the pooled BALF was performed to ensure a sigmoidal curve; results from the current study fell within the linear range. For IgG, the positive control was diluted from 1:4 to 1:512 and for IgA, from 1:30 to 1:3840. The negative controls consisted of PBS only and banked pooled BALF from cats prior to induction of asthma. The dilutions of secondary antibody (goat anti-cat IgG Fc at 1:150,000 or anti-cat IgA at 1:100,000) conjugated with horseradish peroxidase (Bethyl Laboratories, Inc Montgomery, TX) were selected based on the highest positive control to negative control ratio. The optical density (OD) value of the most concentrated positive control sample was arbitrarily set to 1 ELISA unit and each serial dilution was half of the prior value. The OD values of samples were multiplied by 1000 and reported in these arbitrary ELISA units. Samples were all run neat.

## 2.4. Intradermal testing (IDT)

Evaluation of biologically relevant BGA-specific IgE bound to mast cells was assessed using intradermal testing at months 6 and 7, pre and post traditional sensitization. IDT has been shown to be more sensitive for detection of IgE from aeroallergens than serum testing (Lee-Fowler et al., 2009). Cats were sedated with ketamine (5 mg/kg IV) for intradermal injections of histamine (0.275 mg/ml, 1:10 dilution; positive control), PBS (negative control) and BGA (2.78 mg/ml, 1:20 dilution) all at a volume of 0.1 ml. After 20 min, the width and length of wheals were measured in millimeters. Cats were considered to have a positive test result if the wheal formed in response to BGA injection was greater than or equal to halfway between the positive and negative controls.

## 3. Results

### 3.1. Airway eosinophilia

None of the kittens exposed to aerosols of BGA or saline in the early neonatal period (regardless of maternal challenge with BGA or saline while in utero) developed airway eosinophilia (Fig. 2). To determine if these cats with early-life exposure to allergen were tolerized, all cats subsequently underwent “traditional” allergen sensitization and challenge at 6 months of age. Kittens who received BGA aerosols in the first 4 months of life (groups 2 and 4; Fig. 1) failed to later develop airway eosinophilia, even with adjuvanted allergen administration. However, those cats who had received saline, in place of BGA, as kittens mounted robust eosinophilic responses in their airways after traditional sensitization. Development of tolerance in response

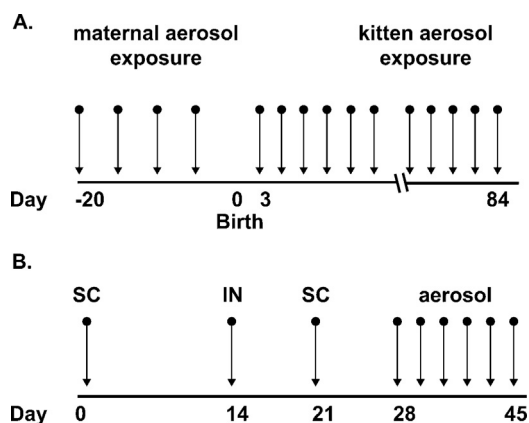


**Fig. 1.** Timeline of asthma induction protocol for kittens in this study. Maternal aerosol BGA exposure was performed every 6 days starting at 20 days pre-partum (A). After birth kittens were exposed by aerosol twice weekly for 12 weeks (84 days) and evaluated through 6 months. Kittens who failed to develop asthma by 6 months received traditional sensitization protocol (B), which involved subcutaneous injection of 10  $\mu$ g BGA in 10 mg alum adjuvant on day 0, 75  $\mu$ g intranasal BGA on day 14, and a second subcutaneous injection of BGA in alum on day 21, followed by 7 rounds of aerosol exposures administered every 2 days.

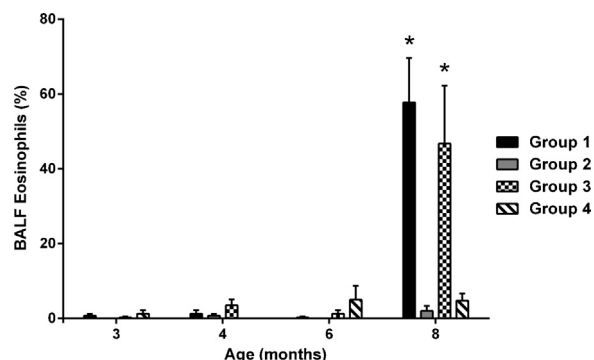


**Fig. 2.** Eosinophils in BALF before and after traditional sensitization. Bronchoalveolar lavage fluid (BALF) was collected at 3, 4, and 6 months of age, and again at 8 months of age after traditional sensitization. After traditional sensitization treatment, cats from groups 1 and 3 had over 17% BALF eosinophils which is consistent with successful induction of asthma. Cats from groups 2 and 4 who underwent neonatal sensitization with BGA aerosols lacked biologically relevant increases in BALF % eosinophils (i.e., mean and range % BALF eosinophils for group 2 was 2 (0–6)% and for group 4 was 5 (0–9%). Eosinophils PAS were significantly higher in group 1 compared to groups 2 and 4 ( $p < 0.01$ ), and in group 3 compared to groups 2 and 4 ( $p < 0.05$ ).

to traditional sensitization was not dependent on maternal exposure to n (i.e., kittens in both groups 2 and 4 failed to develop an asthmatic phenotype at month 6). Additionally, maternal BGA exposure by itself did not prevent successful asthma induction in kittens undergoing traditional sensitization as group 3 cats all developed airway eosinophilia.

### 3.2. BGA-specific immunoglobulins (ELISA and IDT)

Local induction of BGA-specific IgG and IgA, as detected by ELISA in BALF, did not occur in any kitten during the early neonatal exposure period through 6 months. Similarly, BGA-specific IgE was not detected using intradermal

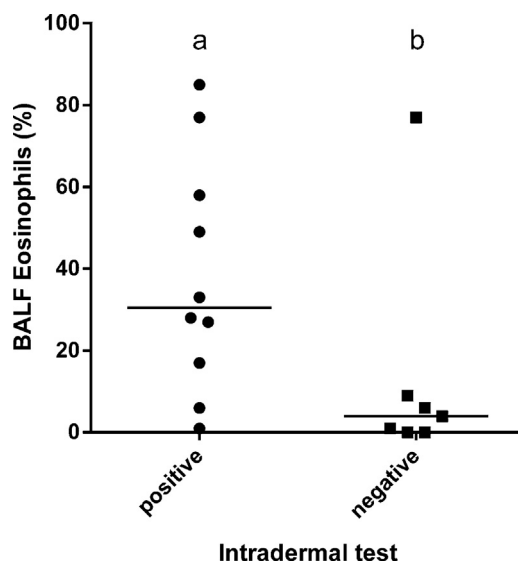


**Fig. 3.** Eosinophils in BALF and IDT results after traditional sensitization. Individual results with median are shown. Cats with no response to BGA specific intradermal test had a significantly lower percentage of eosinophils in BALF ( $p = 0.039$ ).

testing in any kitten during this time. There were positive BGA-specific IDT responses after traditional sensitization depending on group. All kittens in group 4 that received both in utero and neonatal BGA aerosols continued to lack BGA-specific IgE responses after traditional sensitization. Half of the cats receiving saline in utero and BGA neonatally also failed to develop positive IDT results. However, 80 and 100% (group 1 and 3, respectively) of kittens receiving saline in the neonatal period (regardless of type of maternal challenge) had detectable BGA-specific IgE responses. Airway eosinophilia results agreed with the IDT results; Cats with positive IDT responses to BGA post-sensitization were more likely to have increased airway eosinophilia ( $p = 0.039$ ; Fig. 3).

## 4. Discussion

Contrary to the study hypothesis, chronic administration of low concentrations of BGA aerosols to kittens (regardless of whether queens received BGA aerosol challenges during the 3rd trimester of pregnancy) failed to produce an asthmatic phenotype in the offspring. Interestingly, exposure of kittens to these aerosols of BGA during the neonatal period induced tolerance to allergen as evidenced by the later inability to create an asthmatic phenotype with the traditional, adjuvanted protocol. Moreover, induction of tolerance was dependent on neonatal and not maternal (in utero) exposure to allergen, as kittens born to mothers exposed to BGA during the 3rd trimester but having no BGA exposure themselves in the neonatal period were still “susceptible” to asthma development after experimental sensitization/challenge. This is the first study in cats to document that intervention in the neonatal period has potential to “prevent” development of asthma.

The window of influence for programming susceptibility to offspring can occur in utero or during the neonatal

period. In utero, maternal immune cells are responsible for instructing fetal cells on how to balance self-defense and self-tolerance. Fetal response to allergens during pregnancy is influenced by the timing of exposure (i.e. during which trimester). Unfortunately, there are no clear cut conclusions regarding the timing and nature of gestational allergen exposure as published studies report conflicting results. In one human study, maternal exposure to allergen later in gestation (>22 weeks) was associated with higher cord blood mononuclear cell (CBMC) proliferation to allergen than in mothers exposed to allergen early in gestation (Jones et al., 1996). These findings suggest in-utero sensitization and infant T cell priming results from allergen crossing the placenta during a critical window of time later in gestation. Contradictory results were reported in another study where most positive CBMC responses were associated with maternal allergen contact in the first six months of pregnancy (Van Duren-Schmidt et al., 1997). These authors hypothesized the placental barrier was less permeable for the studied allergens later in gestation or that fetal lymphocytes were more sensitive to the priming effects of allergens during the earlier phase of pregnancy. However, when infants were followed in a longitudinal study, lymphoproliferative responses assessed in cord blood did not predict immune responses at one year of age (Smillie et al., 2001). Additionally, maternal allergen exposure during pregnancy did not correlate with immunoproliferative responses in one year old infants or development of an asthmatic phenotype at one or two years of age (Chan-Yeung et al., 1999; Smillie et al., 2001). In the current study, maternal allergen exposure during the third trimester did not exert an influence on ability to induce asthma with neonatal exposure of allergen, nor did it prevent tolerance induction in cats with neonatal exposure to allergen later undergoing traditional sensitization. It is unknown if exposure to allergen to queens earlier in gestation would have altered the study results.

Post-natal factors contributing to allergen sensitization in neonates, such as breast feeding, have also been studied. In a murine model of asthma, both offspring from OVA-sensitized mothers that nurse on non-sensitized mothers, as well as offspring from non-sensitized mothers that nurse on OVA-sensitized mothers develop allergic reactions (Leme et al., 2006). This suggests that breast milk is not absolutely required for sensitization, but that there are factors in breast milk sufficient to induce an asthmatic phenotype. In contrast, other studies have shown a protective effect of breast milk from allergic mothers, suggesting it may be possible to intervene in the neonatal period to prevent allergic responses (Matson et al., 2009; Verhasselt, 2010). Lactating mice exposed to aerosols of OVA, or oral OVA, throughout lactation, had offspring who were partially protected from development of allergic airway disease as adults (Verhasselt, 2010). This protection was antigen specific and was dependent on the presence of antigen and TGF $\beta$  in breast milk. A similar mechanism could be taking place in the kittens in this study, who were nursing during the first six weeks of aerosol exposures. Although the kittens in this study were exposed to BGA allergen via aerosols, studies in rabbits show

antigen distribution following aerosolization as 2–4% in the lung parenchyma and 65–80% in the digestive tract 1–2 h post exposure (Willoughby and Willoughby, 1977). Additionally, allergen deposited on the fur of the kittens may have been ingested during grooming (either maternal or neonatal) allowing BGA allergen to be presented to the gastrointestinal tract in concert with breast milk. It is unknown if feline milk contains TGF $\beta$  as in cows, humans and mice; this was not measured in the present study (Oddy and McMahon, 2011).

Post-natal exposures that modulate the infant's immune system, aside from environmental allergens, include pollutants (e.g., cigarette smoke, ozone), and infectious agents (Lodrup Carlsen and Carlsen, 2001; Devereux et al., 2002; Barrett, 2008). A study in humans showed for a cohort at high risk of atopy and asthma, cat or dog ownership within the first 5 years of life reduced the risk of subsequent allergic disease (Almqvist et al., 2010). Children who grow up on farms and are exposed to multiple animal species, farm microbes and plant allergens have a decreased risk of asthma or atopy when they reach school age; T regulatory cells may play a role in this tolerance (von Mutius and Vercelli, 2010). The cats in the current study were all housed in an identical indoor laboratory animal-facility environment in rooms containing only cats. None of the cats in the facility demonstrated evidence of any infection during the entire study period.

A maternal influence of asthma susceptibility or allergen tolerance was not identified in this study. The development of tolerance to allergen, preventing induction of allergic asthma after traditional sensitization in these cats was dependent solely on neonatal exposure to allergen. The differences in maternal influence in this study compared to the human literature could be due to differences in placentation and transfer of immunoglobulin to the fetus or to timing of allergen exposure. In humans, immunoglobulins are actively transported across the placenta to the fetus, and neonates have IgG concentrations 20–30% higher than their mothers at birth (Palmeira et al., 2012). Newborn kittens, on the other hand, have very little transfer of immunoglobulins across the placenta and derive 90% of their immunoglobulins from colostrum ingestion (Claus et al., 2006). In our study, allergen exposure was limited to the third trimester of gestation; further study would be required to determine whether exposure earlier in gestation may have produced different results. While the factors that influence development of allergic asthma in the perinatal period are many and likely have complex interactions, this study demonstrates that neonatal exposure to aeroallergen in kittens shows promise to prevent subsequent development of allergic asthma to the same allergen. More study is needed to determine the mechanism involved and its applicability to other species.

## Acknowledgements

We would like to thank Drs. Dietrich Volkmann and Dawna Voelkl for assistance with the breeding program and accurate determination of the timeline 3rd trimester.

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