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Pathophysiological Features of Asthma Develop in Parallel in House Dust Mite–Exposed Neonatal Mice

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Abstract

Asthma frequently commences in early life during airway and immune development and exposure to new environmental challenges. Endobronchial biopsies from children with asthma are abnormal, and lung function is maximally reduced by 6 years of age. As longitudinal biopsy studies are unethical in children, the relationship between development of pathology and reduced lung function is unknown. We aimed to establish a novel neonatal mouse model of allergic airways disease to investigate the developmental sequence of the pathophysiologic features of asthma. Neonatal Balb/c mice were challenged three times weekly from Day 3 of life using intranasal house dust mite (HDM) or saline for up to 12 weeks. Weekly assessments of airway inflammation and remodeling were made. Airway hyperresponsiveness (AHR) to methacholine was assessed from Week 2 onward. Total and eosinophilic inflammation was significantly increased in the lungs of HDM-exposed neonates from Week 2 onwards, and a peak was seen at 3 weeks. Goblet cells and peribronchiolar reticulin deposition were significantly increased in HDM-exposed neonates from Week 3, and peribronchiolar collagen was significantly greater from Week 4. HDM-exposed neonates had increased AHR from Week 2 onward. Although inflammation and AHR had subsided after 4 weeks without allergen challenge, the increased reticulin and collagen deposition persisted in HDM-exposed mice. Neonatal mice exposed to intranasal HDM developed eosinophilic inflammation, airway remodeling, and AHR as reported in pediatric asthma. Importantly, all abnormalities developed in parallel, not sequentially, between 2 and 3 weeks of age.

Keywords

pediatric; remodeling; asthma pathophysiology; mouse model; allergic airways disease

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Many individuals with asthma develop symptoms and reduced lung function during infancy and preschool age (1). These children may be born with normal lung function, which is significantly reduced by 3 years (2), is maximally reduced by 6 years (1), and tracks through adolescence (3) and adulthood (4–6). Pathologic abnormalities, including eosinophilic airway inflammation and remodeling, are present in school-aged children with asthma (7, 8). Endobronchial biopsy studies in infants and preschool children have shown no evidence of either eosinophilic inflammation or remodeling in infants (median age 1 yr) with wheeze and reversible airflow obstruction (9), but these are present in preschool wheezers at a median age of 3 years (10). The timing of development of abnormal lung function and pathology suggests that the pathophysiologic abnormalities of asthma develop early and in parallel, some time between infancy and 3 yr, and that this may be when an early intervention might alter the natural history of the disease. However, this needs confirmation in longitudinal studies assessing pathophysiologic abnormalities in the same cohort. Since serial biopsies cannot be performed in children, the relationship between onset of abnormal lung function, inflammation and remodeling, and their mechanisms cannot be explored in children. However, a neonatal mouse model of allergic airways disease (AAD) would allow these issues to be addressed.

This early period is also a peak time of airway growth and immune maturation to a predominant T-helper (Th1) phenotype from the Th2 environment *in utero* (11). It is also when new allergen, infective, and pollutant challenges are encountered, and early aeroallergen sensitization is a known risk factor for later asthma (12). A neonatal mouse model would thus allow the pathophysiologic abnormalities of asthma to be assessed in the critical context of lung growth and development and a maturing immune system. Furthermore, the use of an inhaled environmentally relevant allergen, such as house dust mite (HDM), would incorporate the influence of early allergic sensitization.

Pathophysiologic abnormalities are only seen in ovalbumin (OVA)-exposed neonatal mice when either an airpollutant aerosol is also co-administered (13, 14), or if intraperitoneal sensitization is performed before inhaled challenge (15). These models do not use an allergen that is representative of human exposures, and need systemic sensitization, thus biasing the immune system to a Th2 phenotype before challenge. Furthermore, previously reported infantile models of AAD have only demonstrated increased airway hyperresponsiveness (AHR) for the first 2 weeks after allergen challenge, and have not shown evidence of tissue eosinophilic inflammation beyond the first week after allergen challenge or any evidence of airway remodeling (16). Importantly, there have been no reports of eosinophilic inflammation, airway remodeling, or increased AHR in neonatal mice exposed only to inhaled allergen.

We aimed to establish a neonatal mouse model of AAD to investigate the temporal relationships between pathology and physiology in early life. We hypothesized that intermittent intranasal exposure of neonatal mice to the environmentally relevant allergen HDM would result in the key pathophysiologic abnormalities of asthma observed in severe preschool wheezers and children with asthma, and that these abnormalities would develop in parallel.

MATERIALS AND METHODS

Animals

Newborn Balb/c mice were purchased commercially from Harlan Laboratories (Bicester, UK) as litters with their mother mouse at 2 days of age. Each mother mouse was housed with its litter separately until weaned at 4 weeks. Mice were housed under specific pathogen-free conditions and a 12:12-hour light:dark cycle. All experiments described in

this study were approved by the UK Home Office, and guidelines for animal welfare based on Animals (Scientific Procedures) Act 1986 were strictly observed. Food and water were supplied *ad libitum*.

Allergen Challenge

From 3 days of age, pups were administered either 10 µg (10 µl of 1 mg/ml protein weight solution in PBS) HDM extract (Greer, Lenior, NC) or 10 µl saline intranasally, three times per week. The dose of HDM was increased to 15 µg (15 µl) from Week 3 onwards, the volume of saline was concurrently increased to 15 µl. Dosing was continued for up to 12 consecutive weeks, followed by a 4-week period without allergen challenge. Allergen or saline was administered without anesthesia for the first 2 weeks of life, and subsequently using recovery anesthesia with inhaled isoflurane.

Inflammation

Assessments of inflammation were made weekly at 3 and 24 hours after allergen challenge in two separate experiments; the first included allergen challenge for 8 consecutive weeks and the second for 12 consecutive weeks. Both periods were followed by an assessment after 4 weeks without challenge (Figure 1).

Airway lumen—Bronchoalveolar lavage (BAL) was performed by lavaging airways of mice three times with PBS via a tracheal cannula. The volume of lavage fluid was increased with age as follows. Week 1, 3 × 150 µl aliquots PBS; Week 2, 3 × 200 µl aliquots; Week 3, 3 × 300 µl aliquots; Week 4 onward, 3 × 400 µl aliquots. BAL fluid was centrifuged (200 µg, 5 min, 4°C). BAL fluid supernatants were removed and analyzed by enzyme-linked immunosorbent assay (ELISA) for cytokines. Cells were resuspended in 0.5 ml complete media (RPMI + 10% fetal calf serum [FCS], 2 mM L-glutamine, 100 U/ml penicillin/streptomycin). Cells were counted and pelleted onto glass slides by cytocentrifugation (5 × 10⁴ cells/slide). Differential cell counts were performed on Wright-Giemsa-stained cytopins. Percentages of eosinophils, lymphocyte/mononuclear cells, neutrophils, and macrophages were determined from a total of 400 cells. To obtain absolute numbers of each leukocyte subtype, these percentages were multiplied by the total number of cells obtained in the lavage fluid.

Lung parenchyma—To disaggregate the cells from the lung tissue, 2 lobes from the right lung were incubated at 37°C for 1 hour in digest reagent (0.15 mg/ml collagenase [type D; Roche Diagnostics, Burgess Hill, UK], 25 µg/ml DNase [type 1; Roche Diagnostics]) in complete media. The recovered cells were filtered through a 70-µm nylon sieve (Falcon, BD Biosciences, MA), washed twice, resuspended in complete media, and counted in a hemocytometer (Immune Systems, Paignton, UK). Cytocentrifuge preparations were made, Wright-Giemsa stained, and differential counts were performed as for BAL.

Lung Cell Isolation and Flow Cytometric Analysis of Lung Cells Subsets

Lung cells were isolated as described in LUNG PARENCHYMA above, and were stained for flow cytometric analysis. Suspensions of lung tissue cells were stained in staining buffer (PBS containing 1% FCS and 0.01% sodium azide). To reduce nonspecific binding, cells were incubated with rabbit serum (Sigma, Gillingham, UK) for 15 minutes before staining. Cells were stained with the following antibodies: anti-mouse CD4, anti-mouse CD8 (BD PharMingen, Oxford, UK), anti-mouse T1/ST2 (Morwell Diagnostics, Zurich, Switzerland) or relevant isotype controls for 20 minutes at 4°C. Cells were then washed twice and fixed in Cellfix (Becton Dickinson, Oxford, UK). Data were collected using a FACSCalibur (Becton Dickinson) and analyzed using CellQuest software.

Histopathology

Inflammation and morphology—The largest lobe of the lungs was inflated with PBS and fixed in 10% normal buffered formalin. Four-micrometer paraffin-embedded sections were stained with hematoxylin and eosin. A semiquantitative scoring system was used to grade the size of lung infiltrates as previously described (17). Briefly, a score of 5 signified a large (> 3 cells deep) widespread inflammatory infiltrate around the majority of vessels and bronchioles, and a score of 1 represented a small (< 2 cells deep) number of inflammatory foci.

Goblet cell hyperplasia—Goblet cells were scored on sections stained with periodic acid–Schiff at $\times 200$ magnification, using a semi-quantitative scoring system where positively stained cells in the airway epithelium were measured (18) (0 = < 5% goblet cells; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = > 75%). The sum of the airway scores from each lung was divided by the number of airways examined (10–20 per mouse), and expressed as mucus score in arbitrary units.

Peribronchiolar collagen—Peribronchiolar collagen deposition was assessed in sections stained with picrosirius red as previously described (19). Image analysis was performed using Scion Image Analysis software (Scion Corporation, Frederick, MD). Digital photographs of four bronchioles per tissue section were taken at $\times 400$ magnification, and these images were converted into monochrome. Twenty-micrometer lines were drawn at a right angle from the basal surface of the epithelium into the submucosa, and the mean density of staining intensity along that length was recorded as pixels per μm^2 . Ten such measurements were made along each of the four bronchioles. The geometric mean of 40 measurements was used to represent collagen density for that section.

Peribronchiolar reticulin—The key feature of airway remodeling reported in pediatric biopsies is increased thickening of the RBM; however, rodents do not have a true reticular basement membrane (RBM). Therefore, to optimally mimic changes seen in human biopsies, the main structural component of the RBM, reticulin, was quantified in the subepithelial region of bronchioles. Paraffin sections were stained for reticulin using Gordon and Sweet's reticulin stain (20), and the thickness of the reticulin-stained zone (μm) beneath the bronchiolar epithelium was measured using Scion image analysis software, using a technique similar to that previously described (20). Briefly, at $\times 400$ magnification, photographs of four bronchioles per section were taken and converted to monochrome. The thickness of the reticulin zone beneath the epithelium was measured at right angles to the basal surface of the epithelium at 10 points along each photograph, and the mean of 40 measurements was used to represent reticulin thickness for that section.

Quantification of Cytokines and Immunoglobulins

Cytokines were analyzed in BAL and lung homogenate supernatants. Lung tissue was homogenized at 50 mg/ml in Tissue Protein Extraction Reagent (T-PER; Perbio Science UK, Cramlington, UK) containing protease inhibitor tablets (Roche Diagnostics) and centrifuged at $800 \times g$ for 10 minutes, and the supernatant was collected. Total IgE and IgG1 were measured in diluted serum. Paired antibodies for murine interleukin (IL)-4, IL-5, IFN- γ , TGF- β 1, IgE, IgG1 (PharMingen) and Activin A (R&D Systems, Abingdon, UK) were used in standardized sandwich ELISAs according to the manufacturer's protocol. Kits to measure IL-13 were purchased from R&D Systems. HDM-specific IgE and IgG1 ELISA plates were coated with 50 mg/ml HDM preparation in carbonate buffer overnight at 4°C. Diluted serum (1:10 for IgE and 1:10,000 for IgG1) was added. Plates were incubated overnight at 4°C and washed and developed with biotin-labeled IgE and IgG1, followed by

streptavidin HRP and K-Blue substrate according to the manufacturer's instructions. As there were no standards used, the units correspond to absorbance at 450 nm.

Airway Hyperresponsiveness

AHR was measured weekly from 2 weeks after allergen challenge.

Assessment of lung function—Measurements of dynamic resistance and compliance were performed using a Flexivent system (Scireq, Montreal, Canada). After induction of anesthesia with an intraperitoneal injection of sodium pentobarbital (Sigma) at a dose of 50 mg/kg in saline and intramuscular injection of ketamine (90 mg/kg), mice were tracheostomized and connected to the flexivent ventilator via a blunt-ended 21-gauge needle (2–3 wk of age) or 19-gauge needle (4–12 wk of age). Mice were ventilated using the following settings; tidal volume of 10 ml/kg body weight, 150 breaths/minute; positive end-expiratory pressure approximately 2 cm H₂O. Mice were initially ventilated for 5 minutes. Standardization of the lung volume history was done by performing two deep inflations. Subsequently, measurements of airway function were made. Measurements of resistance and compliance were determined from a user defined protocol using the snapshot-150 perturbation, which is a single frequency sinusoidal wave at a frequency equivalent to ventilation rate (i.e., 2.5 Hz). Resultant data was fitted using multiple linear regression to the single compartment model in the form: pressure = resistance × flow + elastance × volume + fitting constant. Fitting the data to this model provides readings of resistance and compliance. Changes in resistance and compliance were measured in response to increasing concentrations of nebulized methacholine (MCh) from 3–100 mg/ml, delivered into the inspiratory line of the flexivent ventilator.

Statistical Analysis

Results were expressed as mean ± SEM, with a group size of 6 to 12 from two experiments. Data were analyzed using the nonparametric Mann Whitney U test and statistical significance accepted when $P < 0.05$. All data was analyzed using Graph Pad Prism version 4.

RESULTS

Inflammation

A significant increase in total and eosinophilic inflammation was seen in the LD of HDM-treated neonates from Week 2 onward, and in BAL from Week 3 onward (Figure 2A). A peak in total inflammatory cells and eosinophils was seen at Week 3 in LD (lung digest) and between Weeks 5 and 7 in the BAL. Inflammation was greater in HDM neonates at all time points in LD and BAL when assessed at 3 hours after allergen challenge compared with 24 hours after allergen challenge (Figure 2A).

Semiquantitative scoring of H&E-stained lung sections revealed significantly increased peribronchiolar inflammation in HDM-exposed neonates at Week 1, which peaked at Week 3. The tissue infiltrate was limited to the peribronchial and perivascular regions, with little or no infiltrate observed in the lung parenchyma (Figure 2Bii).

CD4⁺ cells in the lung were significantly increased from Week 1 in HDM-exposed neonates and reached a peak by Week 6. Approximately 10% of the total CD4⁺ population was also positive for the Th2 surface marker T1/ST2. An increase in CD8⁺ cells was also observed, and this followed a similar time course; however, at all time points there were at least 50% more CD4⁺ than CD8⁺ cells in the lungs of the HDM-exposed mice compared with saline

controls. In the absence of continued HDM challenge, the number of T cells in the lung returned to control levels (Figure 2C).

Impact of HDM Exposure on Serum Immunoglobulin Production

Total serum IgE was significantly higher in HDM exposed neonates compared with controls from Week 3 onward, and HDM-specific IgE was elevated from Week 4. Total serum IgG1 was higher in HDM-exposed neonates from Week 2 onward, and HDM-specific IgG1 was elevated from Week 3 onward. Immunoglobulin levels remained elevated after 4 weeks without allergen challenge (Figures 3A–3D).

Pulmonary Cytokine Production in HDM-Exposed Neonates

Cytokines were only detectable in BAL and lung homogenate supernatants when measured 3 hours after allergen challenge. The Th2 cytokines IL-4, IL-13, and IL-5 were all significantly increased in the lungs of HDM-exposed neonates from Week 2 onward (Figures 4A, 4C, and 4E). Levels of IL-4 and IL-13 peaked at Week 3 in the lungs and Week 5 in BAL (Figures 4B and 4D). IL-5 production was maximally increased in the lung at Week 2, but levels in BAL were below the limit of detection of the assay. After a peak in cytokine levels, pulmonary production of all three cytokines was reduced over the 12-week time course of the experiment; however, values in HDM-exposed neonates remained significantly higher than in age-matched controls at all time points during allergen exposure. Levels of IL-4, IL-5 and IL-13 decreased to baseline after 4 weeks without allergen challenge. There were no significant differences in the levels of the Th1 cytokine, IFN- γ , in the lungs of HDM neonates and PBS controls (Figure 4F). IFN- γ levels were below the limit of detection in BAL.

Airway Remodeling

Goblet cell hyperplasia—Morphologic changes in the airway epithelium were evident, and there was a change to a mucus-secreting phenotype in HDM-exposed neonates. Mucus-secreting goblet cells were significantly increased in HDM-exposed neonates from Week 3 of challenge, and numbers were maintained over the 12-week time course. Goblet cell hyperplasia was no longer present after 4 weeks without allergen challenge (Figures 5A–5C).

Increased peribronchiolar collagen and reticulin deposition—Significantly increased collagen deposition was present in the subepithelial region of the airways in HDM-exposed mice from Week 4 onward. Importantly, this structural change persisted after 4 weeks without allergen challenge (Figures 5D–5F).

Unlike humans, rodents do not have a true RBM; however, subepithelial reticulin, the main component of the RBM, is still measurable. The thickness of the reticulin layer beneath the epithelium was significantly greater in HDM-exposed neonates from Week 3 onward, and this structural change also persisted without allergen challenge (Figures 5G–5I).

Increased pulmonary activin A levels—We determined levels of key fibrotic mediators in lung tissue. There was no difference in levels of total or active TGF- β 1 (Figure 5J) at any time point between HDM-exposed neonates and controls. However, levels of activin A (a member of the TGF- β superfamily) were significantly increased from Week 2 onward, with an early peak in production at Week 3 in HDM-exposed neonates (Figure 5K). This peak also coincided with the peak in lung inflammation and inflammatory cytokine production, and with the onset of increased reticulin deposition.

Increased AHR

To determine if HDM exposure resulted in physiologic changes to the lungs, we measured airways resistance and compliance in anesthetized, tracheostomized, and ventilated mice, and assessed AHR to increasing doses of MCh.

Significant increases in AHR were observed in HDM-exposed mice from 2 weeks onward during the period of allergen exposure. Airways resistance was significantly higher at all time points and compliance was significantly lower in HDM-exposed mice from Week 3 onward at a MCh dose of 30 mg/ml (Figures 6A and 6B). However, the HDM-induced increase in AHR was no longer seen in the absence of allergen exposure for 4 weeks, at Week 12 (Figures 6A and 6B).

DISCUSSION

The timing of onset of, and relationship between, inflammation and remodeling in pediatric asthma is an important unanswered question (21). To explore this further, and to explore the molecular mechanisms underlying the pathophysiologic abnormalities of asthma, we have developed a neonatal mouse model of AAD that demonstrates increased eosinophilic inflammation, goblet cell hyperplasia, peribronchiolar collagen and reticulin deposition, and increased AHR after intermittent exposure to intranasal HDM. Importantly, all of the changes appear at a similar time, in parallel, between 2 and 4 weeks after initiation of allergen challenge at Day 3 of life. This is the first demonstration of the temporal relationship between the onset of the pathologic and physiologic changes seen in allergic asthma in a neonatal mouse model. It is also the first report describing a neonatal mouse model of AAD using an environmentally relevant allergen delivered to naïve mice without prior systemic sensitization.

It was important to establish a neonatal mouse model and not just explore relationships in described adult models of AAD, as the influences of allergen exposure on normal postnatal immune maturation and lung growth and development had to be considered. In this respect, the neonatal model differed from reported adult HDM models of AAD in that lung inflammation, airway remodeling, and AHR occur in parallel in neonates, but in adult mice peak inflammation precedes airway remodeling (22, 23) and AHR (19, 23). In the adult HDM model of AAD reported by Johnson and coworkers, peak inflammation was seen after 3 weeks of allergen exposure, whereas remodeling was not seen until 5 weeks after allergen exposure (23). Subsequently, in the chronic HDM model of AAD reported by Southam and colleagues, increased BAL and tissue inflammation was seen in HDM-exposed mice from Week 1 after allergen exposure, but increased peribronchiolar collagen was not seen until 6 weeks after exposure (22). In our previous studies with a chronic ovalbumin-driven model, peak inflammation was reported at 24 days after challenge, whereas remodeling was not seen until 55 days after allergen challenge (19). We observed an increase in tissue inflammation in HDM-exposed neonates at 2 weeks that peaked at 3 weeks, and we did not see an increase in BAL inflammation until 3 weeks; this peaked at 5 weeks after exposure. However, these time-points coincided with the increase in reticulin deposition in HDM-exposed neonates seen at Week 3, and with increased peribronchiolar collagen that was seen at Week 4. In contrast to the reported adult HDM model of AAD from Johnson and coworkers, in which increased AHR was not reported before 5 weeks of continuous HDM exposure (23), in neonates AHR was increased as early as 2 weeks (the earliest point at which lung function could technically and reliably be measured). Importantly, AHR did not persist in the neonatal model after 4 weeks without allergen challenge, even though airway remodeling remained. In contrast, persistent AHR was seen without allergen challenge in the adult HDM models of AAD, and this was thought to be a result of persistent airway remodeling (22, 23). However, other studies have also found that remodeling and AHR may

not necessarily be predictive of each other. A recent study has shown that while anti-TGF- β reduces airway remodeling in a mouse model, it actually increases AHR (24). The relationships between AHR, inflammation, and remodeling remain poorly understood, and we aim to use our neonatal model to investigate these in future studies.

This is the first report of abnormal lung function in diseased neonatal mice demonstrated using invasive measurement techniques. A previously reported infantile mouse model of AAD that used OVA only used noninvasive measures of AHR and did not show persistence of AHR beyond 2 weeks of challenge (16). Importantly, lung function abnormalities in the current neonatal model are similar to reports from pediatric studies, whereby persistent wheezers develop abnormal lung function in the first year of life (3). This abnormality increases at 3 years and reaches a maximal reduction by approximately 6 years of age, and subsequently persists through to early adulthood (3, 6). Also, the development of pathologic changes in parallel in the current model is reflective of findings from biopsy studies in infants and preschool wheezers that have shown the absence of both eosinophilic inflammation and RBM thickening at about a year of age (9), but the presence of both changes by 3 years (10). Although similarities between the current mouse model and findings from studies in children are apparent, we acknowledge that findings in a mouse model should be extrapolated with caution to humans. However, the limitation of not being able to perform serial biopsies in children leaves little alternative but to explore the development of pathophysiology in an animal model.

Previous infantile mouse models of AAD have only used OVA (15) and have required systemic sensitization before inhaled allergen challenge to elicit inflammation and AHR (13, 15). This is not an optimal approach in the context of a neonatal model, as the immune system is still maturing postnatally and systemic sensitization results in active manipulation and bias of this immune maturation toward a Th2 phenotype. Furthermore, use of adjuvants such as alum stimulate the immune system without having any specific antigenic effect (25), and would result in further skewing of the maturing immune system. We therefore administered intranasal HDM to naïve neonatal mice to mimic the route of pulmonary sensitization as occurs in humans, since activation of the immune system via the pulmonary antigen-presenting cell network rather than peritoneal dendritic cells may subsequently trigger different immune responses. Furthermore, HDM is a common environmental allergen to which all infants are exposed from birth, and early sensitization to HDM has been shown to impact on later asthma development in children (12). A crude HDM extract, which contains endotoxin in addition to *Dermatophagoides pteronyssinus* (Der p) antigen, was used in the present study as opposed to purified Der p protein because we wanted to use an allergen that reflects human exposure. We demonstrated a humoral response to HDM in allergen-exposed mice indicated by elevated levels of total and HDM-specific IgE and IgG1 which subsequently persisted even in the absence of allergen exposure. These data are in contrast to findings in an adult mouse model of Der p1 exposure (26), but reflect human atopic manifestation to indoor environmental allergens that may not be complete until approximately 3 years of age (27) and that subsequently persist.

Importantly, the current neonatal model using HDM exposure mimics human disease more accurately than a previously reported infantile model of AAD that used OVA, for the following reasons. First, it demonstrates persistent AHR throughout the period of allergen challenge, whereas the OVA model only demonstrated AHR for the first 2 weeks of allergen challenge (16). Second, the neonatal HDM model shows persistently elevated tissue eosinophils throughout the period of allergen challenge, whereas the OVA model only showed tissue eosinophilia in the first week after allergen challenge (16). Biopsy studies in preschool and school-aged children with asthma have shown a predominance of tissue eosinophilic inflammation (7, 10), and tissue eosinophilia persists through to adulthood (28).

Inflammation in the current model was predominantly of the Th2 cell phenotype, as evidenced by an increase in T1/ST2+ CD4+ cells in the lung and increased pulmonary levels of the Th2 cytokines IL-4, IL-5, and IL-13 in HDM-exposed pups, in the absence of changes to the Th1 cytokine IFN- γ .

It is important to note that a significant proportion of neutrophils were present in the inflammatory infiltrate (data not shown). This likely represents the crude HDM extract used. However, it is not dissimilar to the pattern of inflammation reported in BAL of asthmatic children and infant wheezers, in whom some neutrophils (> 10%) have been found, in up to one third of individuals with asthma and half of infants with wheeze (29).

This is the first neonatal mouse model of AAD that has demonstrated the presence of increased airway reticulin and collagen deposition. In terms of airway structural changes, goblet cell hyperplasia was apparent from 3 weeks onward in HDM-exposed mice, and persisted throughout the period of allergen challenge. The structural change most consistently and repeatedly reported in biopsies from children with asthma is increased thickening of the RBM. A limitation of any murine model is the absence of a true RBM in mice. However, the RBM is composed of reticulin (30), which is a member of the collagen family (31). We therefore measured subepithelial, peribronchiolar reticulin deposition as a marker of RBM thickening. This was increased in HDM-exposed neonates from Week 3 onward, and persisted even after 4 weeks without allergen challenge. Peribronchiolar collagen deposition was also increased in HDM-exposed neonates from Week 4 onward and persisted in the absence of continued allergen challenge. TGF- β (32) and activin (a member of the TGF- β superfamily) (33) are known to be critical mediators of normal airway branching and lung morphogenesis, and are thought to play a role in the development of the pathophysiologic abnormalities of asthma (34, 35). We therefore explored these as possible mediators of AAD in our neonatal model. Levels of total and active TGF- β 1 measured by ELISA in lung were not different in HDM-exposed and control mice (Figure 5J). However, TGF- β may be differentially expressed in the epithelium and underlying inflammatory infiltrate in allergen-challenged and naïve mice, while total levels may remain unchanged (19). In contrast, levels of activin A were significantly higher in HDM-exposed pups from Week 2 (Figure 5K), with a peak in levels at Week 3, which also corresponded with the time of maximal lung inflammation, and the onset of peribronchiolar reticulin deposition and goblet cell hyperplasia. A previous report of activin A expression in T cells from individuals with asthma has shown increased activin A mRNA expression in individuals with moderate asthma and increased serum activin A levels in steroid-naïve patients compared with control subjects (36), whereas TGF- β expression in individuals with asthma and control subjects was not different, suggesting that activin A is an acute allergen responsive cytokine that provides a link to TGF- β -mediated remodeling. Activin A may therefore be involved in initiating the events leading to the pathogenesis of asthma. This needs to be explored further in the neonatal model described here, and subsequently to be confirmed in airway biopsies from children with asthma.

Evidence for the concurrent development of inflammation and remodeling in the current neonatal model also supports the hypothesis for the reactivation of the epithelial mesenchymal trophic unit in asthmatic airways (37). It has been proposed that an interaction between the host's genetic background and environmental and allergen exposures determines the initiation of tissue damage and aberrant repair mechanisms that result in airway inflammation and remodeling in asthma (38). One advantage of a mouse model is that each factor can be manipulated and explored separately. The use of inbred mice allowed us to keep the genetic background constant, but we have explored the role of a clinically relevant allergen, HDM, in a neonatal environment, and revealed the timing and pattern of development of inflammation and remodeling in AAD.

In summary, we have established a neonatal mouse model of AAD that demonstrates the key pathophysiologic features of early-onset asthma that have been reported in both preschool wheezers and school-aged children with asthma. After initiating allergen challenge with inhaled HDM at Day 3 of life, increased eosinophilic inflammation and AHR were present in HDM exposed pups at 2 weeks, and increased airway remodeling was observed from Week 3. Importantly, the airway remodeling persisted after 4 weeks without allergen challenge. This is the first description of the temporal relationship between the onset of abnormal lung function, airway inflammation, and remodeling in a neonatal model of AAD, showing their development at a similar time and in parallel.

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Figure 1.

Experimental plan. (A) Intranasal house dust mite (HDM) was administered from 3 days of age three times weekly (↑↑↑) for 8 consecutive weeks. (B) Intranasal HDM administered three times weekly (↑↑↑) for 12 consecutive weeks. Lung function and airway remodeling were assessed weekly (↓) and after 28 days without challenge. Bronchoalveolar lavage (BAL) and tissue inflammation were assessed weekly (↓) 24 hours after challenge in A and 3 hours after challenge in B, and after 28 days without challenge.

Figure 2.

(A) Total and eosinophilic inflammation in lung digest and BAL. Comparison of PBS controls (*open bars*) with HDM-exposed mice at 3 (*solid bars*) and 24 (*shaded bars*) hours after allergen challenge. Allergen challenge was not performed at Week 16. Data represent mean $\pm$ SEM ($n = 6-12$ per group). $*P < 0.05$ PBS versus HDM 3 hours. (B) Sections from 3-week-old neonates stained with hematoxylin and eosin showing increased airway inflammation in HDM-exposed neonates (ii) compared with saline controls (i). (C) Proportion of CD4+, CD8+ and T1/ST2+ cells in lung digest at 24 hours after allergen challenge. $*P < 0.05$ PBS (*open bars*) versus HDM (*solid bars*). ND, not determined.

Figure 3. (A and B) Increased total and HDM-specific IgE in HDM-exposed neonates (*solid bars*) compared with saline controls (*open bars*) from Week 3 onward. (C and D) Increased total and HDM-specific IgG1 in HDM-treated neonates from Week 3 onward. Data represent mean $\pm$ SEM ($n = 6-8$ per group). * $P < 0.05$ PBS versus HDM.

Figure 4.

Significantly elevated Th2 cytokines (IL-4, -13 and -5) in lung homogenate supernatant (LH) (A, C, E) and BAL supernatant (B, D) of HDM-exposed neonates (solid bars) compared with saline controls (open bars) from Week 2 onward. (F) Similar levels of IFN- γ in lung homogenate supernatant from HDM-exposed neonates and controls. Data represent mean $\pm$ SEM ($n = 6-8$ per group). * $P < 0.05$ PBS versus HDM.

Figure 5.

(A and B) Sections from 3-week-old neonates stained with periodic acid–Schiff showing increased goblet cells in the airways from (B) an HDM-exposed neonate compared with (A) an age-matched saline control. (C) Scoring confirmed goblet cell hyperplasia in HDM mice from Week 3 onward during the period of allergen challenge. (D and E) Sections from 4-week-old neonates stained with picrosirius red showing increased peribronchiolar collagen deposition in (E) an HDM-exposed neonate compared with (D) a saline control. (F) Image analysis quantification of mean collagen density confirmed increased peribronchiolar collagen deposition in HDM mice from Week 4 onward. (G and H) Reticulin stained sections from 3-week-old neonates showing increased subepithelial reticulin in (H) an HDM-exposed neonate compared with (G) a saline control. (I) Quantification of peribronchiolar reticulin thickness showed increased deposition in HDM mice from Week 3 onward. (J) Similar levels of active TGF- β 1 in HDM and control mice. (K) Significantly increased activin A levels in HDM-exposed neonates from Week 2 onward. * $P < 0.05$ PBS (open bars) versus HDM (solid bars).

Figure 6.

Airways resistance and compliance at a dose of 30 mg/ml methacholine (MCh) in HDM-exposed neonatal mice compared with saline controls (PBS) from 2 weeks of age. * $P < 0.05$, ** $P < 0.01$ PBS (open bars) versus HDM (solid bars).