Published in final edited form as:

Clin Exp Allergy. 2012 July ; 42(7): 1104–1118. doi:10.1111/j.1365-2222.2012.04005.x.

Lung effects of inhaled corticosteroids in a rhesus monkey model of childhood asthma

C. G. Plopper^{1,2}, J. P. Joad^{1,3}, L. A. Miller^{1,2}, E. S. Schelegle^{1,2}, M. V. Fanucchi^{1,2,*}, L. S. Van Winkle^{1,2,4}, N. K. Tyler¹, M. V. Avdalovic^{1,3}, M. J. Evans^{1,2}, W. L. Lasley^{1,2,4}, A. R. Buckpitt^{1,2}, K. E. Pinkerton^{1,2,3,4}, B. K. Tarkington¹, S. Davis¹, S. J. Nishio¹, L. J. Gershwin^{1,2}, R. Wu^{1,2,3}, and D. M. Hyde^{1,2}

¹California National Primate Research Center, University of California, Davis, CA, USA

²School of Veterinary Medicine, University of California, Davis, CA, USA

³School of Medicine, University of California, Davis, CA, USA

⁴Center for Health and the Environment, University of California, Davis, CA, USA

Summary

Background—The risks for infants and young children receiving inhaled corticosteroid (ICS) therapy are largely unknown. Recent clinical studies indicate that ICS therapy in pre-school children with symptoms of asthma result in decreased symptoms without influencing the clinical disease course, but potentially affect postnatal growth and development. The current study employs a primate experimental model to identify the risks posed by ICS therapy.

Objective—To (1) establish whether ICS therapy in developing primate lungs reverses pulmonary pathobiology associated with allergic airway disease (AAD) and (2) define the impact of ICS on postnatal lung growth and development in primates.

Methods—Infant rhesus monkeys were exposed, from 1 through 6 months, to filtered air (FA) with house dust mite allergen and ozone using a protocol that produces AAD (AAD monkeys), or to FA alone (Control monkeys). From three through 6 months, the monkeys were treated daily with ICS (budesonide) or saline.

Results—Several AAD manifestations (airflow restrictions, lavage eosinophilia, basement membrane zone thickening, epithelial mucin composition) were reduced with ICS treatment, without adverse effects on body growth or adrenal function; however, airway branching abnormalities and intraepithelial innervation were not reduced. In addition, several indicators of postnatal lung growth and differentiation: vital capacity, inspiratory capacity, compliance, non-parenchymal lung volume and alveolarization, were increased in both AAD and Control monkeys that received ICS treatment.

Conclusions and Clinical Relevance—Incomplete prevention of pathobiological changes in the airways and disruption of postnatal growth and differentiation of airways and lung parenchyma in response to ICS pose risks for developing primate lungs. These responses also represent two mechanisms that could compromise ICS therapy's ability to alter clinical disease course in young children.

© 2012 Blackwell Publishing Ltd

Correspondence: Charles G. Plopper, Respiratory Diseases Unit, California National Primate Research Center, University of California, One Shields Ave, Davis, CA 95616, USA., cgplopper@ucdavis.edu.

*Current address: School of Public Health, University of Alabama, Birmingham, AL, USA

Conflict of interest: The authors have no conflict of interest to declare.

Keywords

allergic asthma; animal models; gender; immune response

Introduction

Strong evidence supports the concept that asthma in children has its origin within the first year of postnatal life [1]. Exposure to environmental contaminants, such as air pollutants and aeroallergens, during this period of rapid postnatal lung development, is thought to be a causative factor in the progression of asthma symptoms (from wheeze in infants to asthma in young children) [2–7]. Recent studies have confirmed that onset of airway hyper-reactivity as early as 1 month of age was a predictive factor for asthma emergence at 1 year of age [8] and persistence into older children [9] and adults [10]. The therapeutic approach for childhood asthma is based on acute symptomology. Prospective studies suggest that much of the airway dysfunction in children occurs within the first 3–5 years of life and that greater early dysfunction is associated with increased duration of disease [11–15]. Intervention with inhaled corticosteroids (ICS) for up to 2 years (beginning as early as infancy) reduced symptom burden and frequency of exacerbations in children with high risk of developing persistent asthma compared with a similar cohort given a placebo [11–13, 16, 17]. Accordingly, NHLBI's Guidelines for the Diagnosis and Management of Asthma [18] recommends the use of ICS in infants and young children to control asthma symptoms and improve the quality of life. The extent and frequency of therapy are based on the careful monitoring of symptoms (episodes of wheezing) and history, such as personal and family history of asthma, atopic dermatitis, sensitivity to airborne or food allergens, and blood eosinophilia. However, the clinical study by Guilbert and colleagues [11] found that preventive ICS treatment of at-risk children was not successful for preventing the asthma phenotype once ICS treatment was discontinued. Clearly, a better understanding of disease changes in postnatal lungs is needed. Studies in experimental models of childhood asthma can be helpful in this regard and for assessing the impact of treatments.

The time period in which asthma appears to develop in children, 0–5 years of age, is also the time of greatest change in postnatal lung development. While the human lung is sufficiently developed at birth to perform its primary function of gas exchange, lung development continues for an extended period (8–12 years) after birth [19, 20]. In addition, lung function continues to change during adolescence [21]. We have determined previously that the infant rhesus monkey provides a useful experimental model of childhood asthma [22–41]. Like humans, rhesus monkeys have an extensive period of early postnatal airway growth and development [42], and adult rhesus monkey lungs exhibit the cellular and anatomical characteristics of adult human lungs [42, 43]. The general developmental stages in the rhesus monkey are the following: embryo, 21–45 days gestation; fetus, 45–165 days gestation; newborn, 24 h postnatal; neonate, 0–1 month; infant, 1–12 months; juvenile, 12–24 months; adolescent, 2–4 years; and young adult, 4–8 years [44]. The approximate conversion from rhesus monkey years to human years is 1–3. The characteristics used by the NHLBI guidelines as criteria for defining asthma in children [22, 23] can be reproduced in infant rhesus monkeys by exposing them, soon after birth and continuing through the period of active lung development (6 months), to a known human allergen, house dust mite, together with ozone, an oxidant air pollutant. Exposure to this allergen and ozone also has a negative effect on both airway growth and the organization of the cellular and acellular components of the airway wall that participate in normal airway function as part of the epithelial mesenchymal trophic unit (EMTU) [45]. The NHLBI Guidelines for ICS therapy in very young children emphasizes the need for regimens that hold risk to a minimum. While the potential adverse systemic and prenatal lung effects of ICS have been recognized

[46–55], the full extent of the potential risks of ICS in infants and young children has not been defined, especially with regard to normal postnatal lung development. The study presented here mimics the usual clinical scenario, providing an important parallel to the clinical study by Guilbert and colleagues [11], and addresses the issue of ICS therapy's potential adverse postnatal lung effects by studying the effects of ICS in infant rhesus monkeys with and without allergic airway disease (AAD). Our rhesus monkey experimental model of AAD induced by exposure to house dust mite allergen (HDMA) with ozone is used to address two issues: (1) to establish whether ICS therapy in developing primate lungs reverses pulmonary pathobiology associated with AAD; and (2) to define the impact of ICS on postnatal lung growth and development in primates.

Methods

Animals and production of allergic airway disease by allergen exposure

Figure 1 summarizes the experimental protocol we have used to produce AAD in rhesus monkeys. For the present study, a total of 24 colony-born male rhesus monkeys (*Macaca mulatta*), housed from birth in chambers ventilated with chemical, biological and radiological filtered air (FA), received priming injections [see (22, 24) for details] of HDMA (*Dermatophagoides farinae*) at 2 and 4 weeks of age. At 4 weeks of age (day 28), 12 of these monkeys, the AAD group, were exposed to 11 cycles (one cycle = 14 days) of allergen with ozone: HDMA aerosolized at 1 mg/mL, 2 h/day for days 3–5 [see (22, 24) for details] with ozone at 0.5 ppm, 8 h/day on days 1–5, followed by 9 days of FA (days 6–14). The other 12 monkeys, the Control group, remained in FA.

Inhaled corticosteroid treatment

From the beginning of exposure cycle 6 (average 90 days of age) until the end of cycle 11 (180 days of age), Pari LC Plus[®] nebulizers (PARI Respiratory Equipment, Inc., Midlothian, VA, USA) were used to deliver the following ICS treatment, via face mask, to 6 Control and 6 AAD monkeys: a daily 5-min aerosol of budesonide (0.25 mg/mL concentration, Pulmicort Respules[®]; AstraZeneca US, Wilmington, DE, USA, 0.33 mg per 5 min exposure). The other 6 Control and 6 AAD monkeys received an aerosol of sterile normal saline.

Determination of inhaled corticosteroids treatment levels

Inhaled corticosteroids treatment levels were determined by measuring circulating budesonide and its metabolites, hydroxyprednisolone and hydroxybudesonide. These levels were measured by liquid chromatography negative electrospray ionization tandem mass spectrometry in blood sampled prior to and 20 min after aerosol delivery on treatment days 1, 28 and 70 (90, 118 and 167 days of age). Plasma concentrations of budesonide and hydroxyprednisolone (mean + 1 SD) after ICS treatment for days 1 were as follows: Control ICS, 6.5 + 2.0 pmol/mL; AAD ICS, 8.1 + 3.0 pmol/mL. As the monkeys grew, the circulating levels of budesonide decreased by 25% on treatment day 28 (Control ICS, 4.7 + 2.6 pmol/mL; AAD ICS, 6.3 + 3.5 pmol/mL) and 57% on treatment day 70 (Control ICS, 3.3 + 1.5 pmol/mL; AAD ICS, 3.0 + 1.9 pmol/mL). Levels of hydroxyprednisolone did not change substantially during the experiment. Concentrations of hydroxybudesonide were below the limit of quantification 20 min after treatment. Neither budesonide nor its metabolites were detected in any sample taken before ICS treatment started or in any sample from infants receiving saline.

The measurements were performed in the state-certified Analytical Chemistry Laboratory at UCD, using the method of Wang et al. [56], who validated the assay over a linear range from 0.1 to 10 ng/mL for all three analytes.

Assessment plan for determining inhaled corticosteroids' impacts on infant rhesus monkeys (see below for method details)

Two groups of infant rhesus monkeys were established: monkeys exposed to FA with HDMA and ozone (AAD monkeys), and monkeys exposed to FA only (Control monkeys). Half of each group was given the ICS treatment (AAD ICS monkeys, Control ICS monkeys), the remaining monkeys received saline (AAD saline monkeys, Control saline monkeys). ICS impact on postnatal lung growth was assessed by evaluating the following parameters: lung volume, alveolarization and airway branching number. ICS impact on pulmonary function decrements associated with AAD was assessed by measuring airflow and quasistatic compliance. ICS impact on airway eosinophilia associated with AAD was assessed by differential count in bronchoalveolar lavage and in the airway wall tissue by morphometry of major basic protein expressing cells. ICS impact on the pathobiology of the EMTU in the airway wall was assessed in the epithelial and basement membrane zone compartments. ICS impacts on body growth and adrenal function were also assessed.

Pulmonary function

Forced expiratory flow were measured in sedated and anaesthetized monkeys that were intubated and placed in a whole body plethysmograph with the cuffed endotracheal tube attached to a pneumotachograph assembly. Measurements included quasistatic respiratory system compliance, total lung capacity, vital capacity, inspiratory capacity, functional residual capacity, residual volume, forced expiratory flow at 25% vital capacity and forced expiratory flow at 50% vital capacity.

Necropsy, tissue preparation and bronchoalveolar lavage

After euthanasia by sodium pentobarbital overdose, the thoracic contents of each monkey were removed *enbloc* by midline incision and the bronchus of each lung lobe was cannulated separately. The left cranial and right middle lobes were fixed by airway infusion with paraformaldehyde (1%) or glutaraldehyde/paraformaldehyde (1%/1%), the axial path of the airway trees were exposed by microdissection, the airway branches were recorded and then blocks were cut perpendicular to the airway long axis and embedded in paraffin or Araldite. The left caudal lobe was infused with a 50/50 mixture of OCT medium and PBS, embedded in OCT medium and rapidly frozen in liquid nitrogen for sectioning and immunohistochemistry. Bronchoalveolar lavage, performed immediately following removal and cannulation of the right caudal lobe, was evaluated for total cell number and leucocyte differentials.

Morphometry of lung parenchyma and epithelial mesenchymal trophic unit

Paraffin or frozen sections and differential staining were used to identify basement membrane zone (collagen I), mucous cells (Periodic acid Schiff/Alcian blue), proliferating cells based on Proliferating Cell Nuclear Antigen expression (PCNA) or eosinophils in airway wall tissue (mouse anti-human major basic protein) (see above). Volume density of cells within the epithelium and interstitium of airway walls and the relative volume of parenchyma and non-parenchyma were estimated by point and/or intercept counting [57]. Alveolar number was estimated in the right middle lobe using the method of Hyde, et al. [41]. Neural components were mapped three-dimensionally in the caudal portion of the right cranial lobe [58].

Body growth

Body growth was assessed by direct measurement of body weight and crown-rump length at 60, 90, 160 and 180 days of age.

Adrenal cortical function

Adrenal cortical function was assessed by injecting the monkeys with Cortrosyn (0.025 mg/kg) prior to aerosol delivery on treatment days 1 (90 days of age) and 77 (167 days of age) then comparing cortisol measurements in blood sampled prior to and 30 min after aerosol delivery.

Statistics

We analysed data for overall effects of AAD or ICS by two-way ANOVA and for differences between individual groups by one-way ANOVA combined with Fisher's least significant difference test (SYSTAT 10, SPSS, Chicago, IL, USA). Significance was accepted at $P = 0.05$.

Results

Inhaled corticosteroids' impact on postnatal lung growth

Physiological and fixed lung volumes—In Control saline and AAD saline monkeys, total lung capacity did not differ. However, there were significant reductions in both vital capacity (18%) and inspiratory capacity (28%) of AAD saline monkeys compared with Control saline monkeys (Table 1). In Control ICS monkeys, total lung capacity was significantly higher (28%) than in Control saline monkeys. Total lung capacity in AAD ICS monkeys averaged only slightly higher than in AAD saline monkeys. ICS treatment produced a significant elevation in vital capacity and inspiratory capacity in both Control and AAD monkeys (for the overall effect of ICS, $P < 0.05$ by two-way ANOVA) (Table 1).

The fixed lung volume of the right middle lobe did not change regardless of exposure or ICS treatment (Table 1). Compared with Control saline monkeys, the volume occupied by non-parenchymal tissues (conducting airways, blood vessels) within the lobe did not change in AAD saline monkeys (Table 1). Compared with Control saline monkeys, the non-parenchymal volume was significantly larger in control ICS monkeys and in AAD ICS monkeys. ICS treatment produced a statistically significant increase in non-parenchymal volume in both control and AAD monkeys ($P < 0.05$ by two-way ANOVA) (Table 1).

Alveolarization of lung parenchyma—Based on size and configuration of alveoli and organization of the interalveolar septa, there was little discernible difference in the histological appearance of alveolar tissue in the lungs of either Control saline monkeys or AAD saline monkeys (Figs 2a and c). The number of alveoli estimated by morphometry was the same in both (Fig. 2e). In contrast, the alveoli of both Control ICS monkeys and AAD ICS monkeys appeared smaller, with more focal areas of slightly thickened interalveolar septa, compared with either Control saline monkeys or AAD saline monkeys (Figs 2b and d). Furthermore, the number of alveoli in both the Control ICS and the AAD ICS monkeys more than doubled compared with Control saline and AAD saline monkeys (Fig. 2e). ICS treatment produced a statistically significant increase in alveolar number ($P < 0.05$ by two-way ANOVA) (Fig. 2e).

Airway branching—In AAD saline monkeys, the number of airway branches in the axial path of both the right middle and left cranial lobes was significantly reduced, by an average of 4 compared with Control saline monkeys (Table 1). There was no change in airway number in Control ICS monkeys compared with Control saline monkeys. In contrast, in AAD ICS monkeys, the number of branches was reduced, by 2 in the right middle lobe and 4 in the left cranial lobe compared with Control saline monkeys (Table 1). In agreement with our previous study [36], exposure to HDMA with ozone (AAD monkeys) produced a

statistically significant decrease in airway branch number in the right middle and left cranial lung lobes compared with Control monkeys ($P < 0.05$ by two-way ANOVA) (Table 1).

Inhaled corticosteroids' impact on pulmonary function

Airflow—Two measures of airflow were evaluated: forced expiratory flow at 25% vital capacity (FEF₂₅) and forced expiratory flow at 50% vital capacity (FEF₅₀). The AAD saline monkeys had significantly reduced FEF₂₅ and FEF₅₀ compared with Control saline monkeys (Table 2). ICS treatment reversed this reduction in FEF₂₅ and FEF₅₀ in AAD ICS monkeys, but did not affect Control ICS monkeys (Table 2). The allergen exposure effect (AAD) showed an overall statistically significant reduction in FEF₅₀ ($P < 0.05$ by two-way ANOVA) (Table 2).

Quasistatic compliance—There was a moderate decrease in compliance in AAD saline monkeys compared with Control saline monkeys (Table 2). Compared with Control saline monkeys, compliance was elevated in Control ICS monkeys. There was no difference in compliance in AAD ICS monkeys compared with Control saline monkeys. ICS treatment produced a statistically significant overall increase in pulmonary compliance in both Control ICS and AAD ICS monkeys ($P < 0.05$ by two-way ANOVA) (Table 2).

Impact of Inhaled corticosteroids on airway eosinophilia

Bronchoalveolar lavage—The frequency of eosinophils in lavage significantly increased (13-fold) in AAD saline monkeys compared with Control saline monkeys (Table 3). Allergen exposure (AAD) had an overall statistically significant effect of increasing eosinophils in lavage ($P < 0.05$ by two-way ANOVA) (Table 3). The frequency of eosinophils in Control ICS monkeys was significantly higher (over 3-fold) than in Control saline monkeys (Table 3). ICS treatment limited the increase to 8-fold in AAD ICS monkeys compared with Control saline monkeys (Table 3).

Airway epithelium—The density of eosinophils in the epithelium in AAD saline monkeys was significantly increased (16-fold) compared with Control saline monkeys (Table 3). Allergen exposure (AAD) had an overall statistically significant effect of increasing the density of eosinophils in airway epithelium ($P < 0.05$ by two-way ANOVA) (Table 3). Treatment with ICS had no effect on eosinophil density in Control ICS monkeys compared with Control saline monkeys. In contrast, eosinophil density was partially reduced (42%) in AAD ICS monkeys, compared with AAD saline monkeys, but was much higher (9-fold) than in Control saline monkeys. ICS therapy had no overall effect.

Airway interstitium—Eosinophil populations in the airway interstitium were increased significantly, but not to the same extent as the increases in the epithelial compartment (6-fold vs. 16-fold respectively) in AAD saline monkeys compared with Control saline monkeys (Table 3). Allergen exposure (AAD) had an overall statistically significant effect of increasing the density of eosinophils in airway interstitium ($P < 0.05$ by two-way ANOVA) (Table 3). ICS did not significantly alter eosinophil density in Control ICS animals compared with Control saline monkeys. In contrast, ICS treatment reduced interstitial eosinophils in AAD ICS monkeys to a level that was not significantly higher than that of Control saline monkeys. ICS treatment also had an overall statistically significant effect of decreasing the density of eosinophils in airway interstitium ($P < 0.05$ by two-way ANOVA) (Table 3).

Inhaled corticosteroids' impact on epithelial mesenchymal trophic unit pathobiology in the airway wall

Epithelial mucous cell abundance and mucin composition—In mid-level bronchi of AAD saline monkeys, total epithelial mass was reduced by approximately one-third and the mass of histochemically detectable mucins was reduced by about 50%, compared with Control saline monkeys ($P < 0.05$) (Figs 3a and b). In Control saline monkeys, the majority of the mucins were neutral (Periodic acid Schiff positive) and, with the exception of a very small proportion of acidic mucins (Alcian Blue positive), the remainder were mixed (both Alcian Blue and Periodic acid Schiff positive) (Figs 3b and d). In contrast, in AAD saline monkeys, mixed mucins predominated and the remainder of the mucins were neutral (Figs 3b and f). In Control ICS and AAD ICS monkeys, total epithelial mass and the mass of histochemically detectable mucins were not statistically different from those of Control saline monkeys (Figs 3a and b). Neutral mucins predominated in both Control ICS (Figs 3b and e) and AAD ICS monkeys (Figs 3b and g).

Epithelial proliferation—In Control saline monkeys, approximately 20% of the epithelial cells were proliferating (Fig. 3c). In comparison, in AAD saline monkeys, the percentage of proliferating cells was reduced by approximately 40%. Compared with Control saline monkeys, the percentage of proliferating epithelial cells nearly doubled in Control ICS monkeys ($P < 0.05$). In contrast, in AAD ICS monkeys, the percentage of proliferating cells was reduced by approximately 40% compared with Control saline monkeys. In all four groups, the majority of the proliferating cells were columnar and the rest were basal cells.

Basement membrane zone—The basement membrane zone in the trachea of AAD saline monkeys (Fig. 4c) had extensive areas of altered thickness with irregular profiles compared with Control saline monkeys (Fig. 4a). The percentage of the basement membrane zone less than 2 μm in thickness (Fig. 4e) was significantly greater in the AAD saline monkeys ($P < 0.05$) than in the AAD ICS monkeys. The intensity of immunoreactive perlecan in the basement membrane zone was also markedly reduced in AAD saline monkeys compared with Control saline monkeys (Fig. 4f). In both groups of monkeys treated with ICS, the appearance of the basement membrane zone (Figs 4b and d), and the presence of immunoreactive perlecan were not different compared with Control saline monkeys (Fig. 4f).

Intraepithelial innervation—There was a marked decrease in intraepithelial nerve fibres immunoreactive for Protein Gene Product 9.5 in the midlevel airways of AAD saline monkeys (Fig. 5c) when compared with Control saline monkeys (Fig. 5a). In addition, clusters of immunoreactive ciliated cells were present in AAD saline monkeys but not in Control saline monkeys. The distribution of nerve fibres in Control ICS monkeys resembled that of AAD saline monkeys, but with a greater abundance of immunoreactive ciliated cells (Fig. 5b). ICS treatment increased the clusters of immunoreactive ciliated cells and reduced the density of nerve fibres in AAD ICS monkeys (Fig. 5d) compared with either Control saline or AAD saline monkeys (Figs 5a and c).

Inhaled corticosteroids' impact on body growth and adrenal function

Body weight and crown-rump length—Neither the pattern nor the extent of growth during the period of this experiment, whether measured by body weight or crown-rump length, was significantly altered by allergen exposure or treatment with ICS. Body weight increases: Control saline, 0.90 ± 0.07 to 1.63 ± 0.13 kg; AAD saline 0.92 ± 0.05 to 1.58 ± 0.08 kg; Control ICS 0.93 ± 0.07 to 1.60 ± 0.21 kg; AAD ICS 0.85 ± 0.16 to 1.54 ± 0.20 kg. Crown-rump length increases: Control saline 249.5 ± 9.0 to 310.3 ± 13.6 mm; AAD saline

252.7 $\pm$ 7.5 to 311.2 $\pm$ 7.9 mm; Control ICS 249.3 $\pm$ 11.4 to 316.5 $\pm$ 15.8 mm; AAD ICS 236.5 $\pm$ 14.8 to 303.7 $\pm$ 14.2 mm.

Adrenal cortex function—At 3 months of age, immediately prior to the beginning of ICS therapy, stimulation with ACTH produced an average increase of approximately 45 mcg/dL in plasma cortisol in both Control and AAD monkeys (Control saline 49.9 $\pm$ 9.3 mcg/dL; AAD saline 43.7 $\pm$ 15.7 mcg/dL; Control ICS 45.3 $\pm$ 11.8 mcg/dL; AAD ICS 44.7 $\pm$ 7.7 mcg/dL.). At 6 months of age, ACTH stimulation increased plasma cortisol by an average of approximately 30 mcg/dL in both Control and AAD monkeys, whether they were treated with saline or ICS (Control saline 32.5 $\pm$ 9.2 mcg/dL; AAD saline 28.5 $\pm$ 9.0 mcg/dL; Control ICS 32.7 $\pm$ 13.2 mcg/dL; AAD ICS 26.8 $\pm$ 5.3 mcg/dL.).

Discussion

The effects of ICSs on the developing human lung are poorly understood. By employing a primate experimental model of childhood asthma with a clinically modelled regimen of ICS treatment and examining both AAD manifestations and postnatal lung development parameters, this study reveals a more complex picture of the impact of ICS therapy.

The goals of our study were to address two issues regarding the use of ICS in very young subjects: to (1) establish whether ICS therapy in developing primate lungs reverses pulmonary pathobiology associated with AAD and (2) define the impact of ICS on postnatal lung growth and development in primates. Our observations regarding these two goals are summarized in Table 4. ICS therapy in AAD monkeys substantially reversed two of the principal symptoms of asthma observed in children, namely, restrictions in airway flow and eosinophilia in airway fluids. However, ICS therapy did not prevent all of the pathobiological alterations associated with AAD produced by the allergen exposure regimen. Furthermore, with regard to early postnatal lung development, the impact of ICS on growth, organization and differentiation of cellular and acellular constituents of the tracheobronchial airway walls and lung parenchyma was mixed, with significant alterations, potentially both positive and negative, to many parameters of normal growth and development.

In our study, infant rhesus monkeys were exposed cyclically for 5 of the first 6 months of life to HDMA with ozone and half of the monkeys received ICS treatment consisting of daily inhalation of budesonide for the last 3 months of allergen exposure. This study design closely parallels the clinical design used by Guilbert and colleagues, who examined whether preventative ICS treatment of an at-risk population of young children would be successful in preventing asthma as the children grew older [11].

A major concern in the choice of dose and regimen for therapy in young children is minimizing the potential for negative systemic effects on growth and development [11, 46, 49, 52, 55]. Our rationale for the therapeutic approach used in this study was to establish a regimen where the dose would produce plasma budesonide levels in the range reported in young children, and which would markedly reduce eosinophilia and airway hyper-reactivity, while minimizing the additional stress on the infant monkeys created by repeated handling during the day. Since a single treatment per day addressed symptomology initially, we continued that regimen throughout the study. The budesonide plasma levels in the infant rhesus monkeys produced by the therapeutic regimen used here were comparable to those reported for human children treated with budesonide aerosols [59, 60] and are within the range provided in the prescribing information from AstraZeneca (2003) for the Pari LC Plus[®] nebulized Pulmicort Respules[®]. In addition, the regimen used in the current study did

not inhibit overall body growth or development of adrenal function in the infant rhesus monkey.

The parameters of airway biology and pathobiology we assessed in the current study were chosen based on our previous studies with rhesus monkeys exposed to HDMA and ozone, which have established that the model exhibits the criteria for asthma as defined by the NHLBI's Guidelines for Diagnosis and Management of Asthma [18, 24]. We found extensive changes in the organization of the cellular and acellular components within the airway wall, which appear to act in concert, functioning as a unit called the EMTU to modulate airway function [45, 61]. We have found this concept to be critical to understanding asthma in rhesus monkeys [22, 23, 40, 61] and it has proven useful in analysis of asthma in human lungs [62]. The interactive components, all of which are altered in AAD, include (1) luminal and glandular epithelium, (2) the interstitium, including basement membrane zone, fibroblasts and smooth muscle, (3) afferent and efferent nerve processes plus the central regulating neurons in the brainstem, (4) the vasculature, including capillaries, arterioles, venules, and lymphatic vessels and (5) migratory and resident inflammatory and immune cells. The structural and functional properties of the EMTU characteristic of airways in adults are established over an extensive period of postnatal development that appear to involve all the elements [62], including secretory epithelium [33, 42], the basement membrane zone [26], smooth muscle [31], innervation [29], chemokine networks and immune cell trafficking [28, 63] and capillary beds [37]. Postnatal exposure to allergen and/or ozone markedly disrupts these normal developmental events [23–25, 27, 28, 30, 32, 34–37, 39].

If a major goal of ICS therapy is prevention of the pathobiological changes thought to be the root cause of AAD, recent clinical studies suggest that this is not fully achieved [11, 15, 50, 52]. The current study and the companion *in vitro* physiological study of tissue from the same monkeys [40] demonstrate experimentally that a dose of ICS which ameliorates symptoms may not be successful in fully preventing the pathological reorganization of the EMTU that accompanies AAD and, in fact, may exacerbate some pathological changes. ICS treatment of AAD monkeys had a mixed effect on the epithelial compartment by reversing changes in mucous cell composition, but significantly altering epithelial proliferation rates. Intraepithelial innervation, which is disrupted by allergen and ozone exposure [29] and worsens with age [39], was actually worsened by ICS. ICS therapy prevents one of the major alterations in asthmatic airways, the thickened basement membrane zone [61, 62], without altering the normal progress of its postnatal development. While ICS markedly decreased eosinophils in airway luminal fluid and airway wall interstitium, it was only partially effective in preventing multi-fold increases in eosinophil accumulation in the epithelial compartment. In our companion study of tissue from the same monkeys [40], ICS treatment ameliorated smooth muscle thickening in AAD monkeys, but produced thickened smooth muscle in monkeys exposed only to FA [40]. This differential effect of steroids on airway smooth muscle has clinical implications. Conceivably, prolonged steroid treatment in an asthmatic child has the potential to perpetuate smooth muscle remodelling even after initial improvement. These subtle pathobiological changes are difficult to test for in the normal clinical situation.

In addition to the clinical and pathological conditions that comprise the criteria for asthma, we have identified a number of other aspects of airway pathobiology that contribute to AAD in the developing lung. Using a rigorous sampling scheme involving microdissection of the airway tree [38, 64], we have described previously two changes in airway architecture [23, 36], which were also found in the current study (Table 1). First, there is a marked reduction in the number of airway generations between the trachea and respiratory bronchioles. Second, the distal airways (bronchioles and respiratory bronchioles) have increased lengths

and reduced diameters. These would mean that the airflow in the remaining distal airways would be affected by the increased volume of air moving through them to and from the parenchyma if total volume of the gas exchange area stayed the same. These changes, which are refractory to ICS therapy, appear to result from disruption of normal airway growth [31, 33, 42].

In this study, we report several marked changes in ICS-treated lungs, including increases in compliance and the number of alveoli and moderate increases in lung volume. Previous studies of systemically administered corticosteroid effects on primate lungs indicate that the degree and nature of the impact on parenchymal growth and alveolarization depends, to a large extent, on the phase of pre- or postnatal development at the time of corticosteroid treatment [47, 65–67]. The early Childhood Asthma Management Program (CAMP) [14, 16] and Prevention of Early Asthma in Kids [11] studies concluded that ICS improves airway responsiveness in young asthmatic children without improving lung function when compared with placebo groups. In addition, ICS treatment was shown to slightly inhibit body growth. Both findings were reinforced by the recent 5-year follow-up study of the same cohort from the CAMP study [17]. In our study, using ICS at a dose that is effective in ameliorating AAD-associated symptoms without altering normal body growth or adrenal function, appears to alter the normal process of primate postnatal lung growth, especially alveolarization.

As documented previously, alveolarization is a postnatal event in both humans and non-human primates [19, 20]. In rhesus monkeys, the number of alveoli increases at a rapid rate, without changing in size, during the first year of life, and then continues to increase at a slower rate until full somatic growth is reached at about 7 years of age [41]. As lung volume is the most reliable predictor of alveolar number in young rhesus monkeys [41], the increased number of alveoli should have been associated with significantly greater lung volumes in ICS-treated monkeys compared with saline-treated monkeys. However, the alveoli appeared to be smaller in the ICS-treated monkeys. Consequently, part of the increase in TLC may be attributable to the increased number of smaller alveoli. Qualitative evaluation of interalveolar septa did not show differences in capillary density, extracellular fibres or epithelial cells between any of the treatment groups (data not shown). While we did not measure diffusion capacity in these monkeys, we would expect an increase, because membrane diffusion capacity and capillary blood volume in lambs showed an age-related increase consistent with alveolarization from birth to 8 weeks of age [68]. The nature and impact of alveolar remodelling in the ICS-treated monkeys observed in our study warrants further investigation, because studies in postnatal rodent [69, 70] and rabbit [67] lungs show decreased lung growth and alveolarization with glucocorticoid treatment. Differential regulation during developmental alveologenesis is evidenced from studies in retinoic acid receptor (RAR)- β knockout mice that show alveoli form twice as fast as wild-type mice during the first 2 postnatal weeks, but at the same rate as wild-type mice [71], whereas RAR- α knockout mice form alveoli at the same rate as wild-type mice during the first 2 postnatal weeks, but at a slower rate than wild-type mice thereafter [72]. These studies indicate that molecular signalling regulating alveolar formation differs during and after the period of rapid postnatal septation. Postnatal alveolar septation in rhesus monkeys is not well understood other than a rapid growth phase during the first 12–18 months and then a slower growth phase to full somatic growth at about 7 years of age [41]. Budesonide delivered via aerosol from 3 to 6 months of age in rhesus monkeys in the current study showed a significant increase in alveoli, while corticosteroid delivered Sub Q from 4 to 13 days of age inhibited interalveolar septation in rats [73]. These studies had different susceptible time windows and routes of administration that may account for the opposite results on alveolarization.

In summary, our study addressed two major issues raised by recent clinical studies of ICS therapy for very young children exhibiting early symptoms of asthma. The first is the apparent ineffectiveness of ICS therapy for inhibiting the progression of the disease. The second issue is the potential for adverse effects by ICS on organ systems undergoing postnatal growth and development. Overall, the results of this study reinforce that careful consideration should be given to the risks and benefits of early and prolonged ICS therapy for asthma during childhood, and further study of persistent developmental alterations resulting from ICS treatment appears warranted.

Acknowledgments

Development of this model was the product of the interactions of the entire faculty and staff members of the Respiratory Diseases Unit at the California National Primate Research Center, whose members, in addition to those listed above, include J. Bric, J. Garrets, K. Kott, D. Morin, M. Plancarte, L. Putney, W. Walby and A. J. Weir. The assistance of A. Chang and L. Orlando with data compilation and analysis and organization of the manuscript is gratefully acknowledged. The editorial assistance of Ms Heather Rizzino and Dr. J. St. George is gratefully acknowledged. The measurement of budesonide and its metabolites by the laboratory of Dr. S. D. Stanley at the California Animal Health and Safety Laboratory is gratefully acknowledged. The support of Primate Services at the California National Primate Research Center for animal handling, care and coordination was critical to this study and is gratefully acknowledged.

Sources of support: Supported in part by NIEHS P01 ES00628 and NCRR RR00169. At the time of this study, UC Davis was an NIEHS Center for Environmental Health Sciences (ES 05707) and the support for some of the core facilities used in this study is gratefully acknowledged.

References

1. Sly PD. The early origins of asthma: who is really at risk? *Curr Opin Allergy Clin Immunol.* 2011; 11:24–8. [PubMed: 21150438]
2. Mortimer KM, Neas LM, Dockery DW, Redline S, Tager IB. The effect of air pollution on inner-city children with asthma. *Eur Respir J.* 2002; 19:699–705. [PubMed: 11999000]
3. Yunginger JW, Reed CE, O'Connell EJ, Melton LJ 3rd, O'Fallon WM, Silverstein MD. A community-based study of the epidemiology of asthma. Incidence rates, 1964–1983. *Am Rev Respir Dis.* 1992; 146:888–94. [PubMed: 1416415]
4. Rosenstreich DL, Eggleston P, Kattan M, et al. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N Engl J Med.* 1997; 336:1356–63. [PubMed: 9134876]
5. McConnell R, Islam T, Shankardass K, et al. Childhood incident asthma and traffic-related air pollution at home and school. *Environ Health Perspect.* 2010; 118:1021–6. [PubMed: 20371422]
6. Clark NA, Demers PA, Karr CJ, et al. Effect of early life exposure to air pollution on development of childhood asthma. *Environ Health Perspect.* 2010; 118:284–90. [PubMed: 20123607]
7. Harley KG, Macher JM, Lipsett M, et al. Fungi and pollen exposure in the first months of life and risk of early childhood wheezing. *Thorax.* 2009; 64:353–8. [PubMed: 19240083]
8. Turner SW, Young S, Goldblatt J, Landau LI, Le Souef PN. Childhood asthma and increased airway responsiveness: a relationship that begins in infancy. *Am J Respir Crit Care Med.* 2009; 179:98–104. [PubMed: 18990677]
9. Malmstrom K, Pelkonen AS, Malmberg LP, et al. Lung function, airway remodelling and inflammation in symptomatic infants: outcome at 3 years. *Thorax.* 2011; 66:157–62. [PubMed: 21199817]
10. Sears MR, Greene JM, Willan AR, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med.* 2003; 349:1414–22. [PubMed: 14534334]
11. Guilbert TW, Morgan WJ, Zeiger RS, et al. Long-term inhaled corticosteroids in preschool children at high risk for asthma. *N Engl J Med.* 2006; 354:1985–97. [PubMed: 16687711]
12. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med.* 1995; 332:133–8. [PubMed: 7800004]

13. Morgan WJ, Stern DA, Sherrill DL, et al. Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. *Am J Respir Crit Care Med*. 2005; 172:1253–8. [PubMed: 16109980]
14. Zeiger RS, Dawson C, Weiss S. Relationships between duration of asthma and asthma severity among children in the Childhood Asthma Management Program (CAMP). *J Allergy Clin Immunol*. 1999; 103:376–87. [PubMed: 10069869]
15. Group TCAMPR. Long-term effects of budesonide or nedocromil in children with asthma. *N Engl J Med*. 2000; 343:1054–63. [PubMed: 11027739]
16. Castro-Rodriguez JA, Rodrigo GJ. Efficacy of inhaled corticosteroids in infants and preschoolers with recurrent wheezing and asthma: a systematic review with meta-analysis. *Pediatrics*. 2009; 123:e519–25. [PubMed: 19254986]
17. Strunk RC, Sternberg AL, Szeffler SJ, Zeiger RS, Bender B, Tonascia J. Long-term budesonide or nedocromil treatment, once discontinued, does not alter the course of mild to moderate asthma in children and adolescents. *J Pediatr*. 2009; 154:682–7. [PubMed: 19167726]
18. NAEP Program. Expert panel report 3: guidelines for the diagnosis and management of asthma, full report. Bethesda: National Heart, Lung and Blood Institute; 2007.
19. Burri, PH. Postnatal development and growth. In: Crystal, JBWRG.; Weibel, ER.; Barnes, PJ., editors. *The lung: scientific foundations*. Philadelphia: Lippincott-Raven Publishers; 1997. p. 1013-26.
20. Balinotti JE, Tiller CJ, Llapur CJ, et al. Growth of the lung parenchyma early in life. *Am J Respir Crit Care Med*. 2009; 179:134–7. [PubMed: 18996997]
21. Gauderman WJ, McConnell R, Gilliland F, et al. Association between air pollution and lung function growth in southern California children. *Am J Respir Crit Care Med*. 2000; 162:1383–90. [PubMed: 11029349]
22. Schelegle ES, Miller LA, Gershwin LJ, et al. Repeated episodes of ozone inhalation amplifies the effects of allergen sensitization and inhalation on airway immune and structural development in Rhesus monkeys. *Toxicol Appl Pharmacol*. 2003; 191:74–85. [PubMed: 12915105]
23. Plopper CG, Smiley-Jewell SM, Miller LA, et al. Asthma/allergic airways disease: does postnatal exposure to environmental toxicants promote airway pathobiology? *Toxicol Pathol*. 2007; 35:97–110. [PubMed: 17325978]
24. Schelegle ES, Gershwin LJ, Miller LA, et al. Allergic asthma induced in rhesus monkeys by house dust mite (*Dermatophagoides farinae*). *Am J Pathol*. 2001; 158:333–41. [PubMed: 11141508]
25. Evans MJ, Van Winkle LS, Fanucchi MV, et al. Fibroblast growth factor-2 in remodeling of the developing basement membrane zone in the trachea of infant rhesus monkeys sensitized and challenged with allergen. *Lab Invest*. 2002; 82:1747–54. [PubMed: 12480924]
26. Evans MJ, Fanucchi MV, Van Winkle LS, et al. Fibroblast growth factor-2 during postnatal development of the tracheal basement membrane zone. *Am J Physiol Lung Cell Mol Physiol*. 2002; 283:L1263–70. [PubMed: 12388330]
27. Evans MJ, Fanucchi MV, Baker GL, et al. Atypical development of the tracheal basement membrane zone of infant rhesus monkeys exposed to ozone and allergen. *Am J Physiol Lung Cell Mol Physiol*. 2003; 285:L931–9. [PubMed: 12832283]
28. Miller LA, Plopper CG, Hyde DM, et al. Immune and airway effects of house dust mite aeroallergen exposures during postnatal development of the infant rhesus monkey. *Clin Exp Allergy*. 2003; 33:1686–94. [PubMed: 14656356]
29. Larson SD, Schelegle ES, Walby WF, et al. Postnatal remodeling of the neural components of the epithelial-mesenchymal trophic unit in the proximal airways of infant rhesus monkeys exposed to ozone and allergen. *Toxicol Appl Pharmacol*. 2004; 194:211–20. [PubMed: 14761677]
30. Evans MJ, Fanucchi MV, Baker GL, et al. The remodelled tracheal basement membrane zone of infant rhesus monkeys after 6 months of recovery. *Clin Exp Allergy*. 2004; 34:1131–6. [PubMed: 15248862]
31. Tran MU, Weir AJ, Fanucchi MV, et al. Smooth muscle development during postnatal growth of distal bronchioles in infant rhesus monkeys. *J Appl Physiol*. 2004; 97:2364–71. discussion 54. [PubMed: 15347627]

32. Tran MU, Weir AJ, Fanucchi MV, et al. Smooth muscle hypertrophy in distal airways of sensitized infant rhesus monkeys exposed to house dust mite allergen. *Clin Exp Allergy*. 2004; 34:1627–33. [PubMed: 15479280]
33. Van Winkle LS, Fanucchi MV, Miller LA, et al. Epithelial cell distribution and abundance in rhesus monkey airways during postnatal lung growth and development. *J Appl Physiol*. 2004; 97:2355–63. discussion 54. [PubMed: 15298983]
34. Chou DL, Daugherty BL, McKenna EK, et al. Chronic aeroallergen during infancy enhances eotaxin-3 expression in airway epithelium and nerves. *Am J Respir Cell Mol Biol*. 2005; 33:1–8. [PubMed: 15834047]
35. Joad JP, Kott KS, Bric JM, et al. Structural and functional localization of airway effects from episodic exposure of infant monkeys to allergen and/or ozone. *Toxicol Appl Pharmacol*. 2006; 214:237–43. [PubMed: 16466656]
36. Fanucchi MV, Plopper CG, Evans MJ, et al. Cyclic exposure to ozone alters distal airway development in infant rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol*. 2006; 291:L644–50. [PubMed: 16648242]
37. Avdalovic MV, Putney LF, Schelegle ES, et al. Vascular remodeling is airway generation-specific in a primate model of chronic asthma. *Am J Respir Crit Care Med*. 2006; 174:1069–76. [PubMed: 16931639]
38. Hyde DM, Harkema JR, Tyler NK, Plopper CG. Design-based sampling and quantitation of the respiratory airways. *Toxicol Pathol*. 2006; 34:286–95. [PubMed: 16698728]
39. Kajekar R, Pieczarka EM, Smiley-Jewell SM, Schelegle ES, Fanucchi MV, Plopper CG. Early postnatal exposure to allergen and ozone leads to hyper-innervation of the pulmonary epithelium. *Respir Physiol Neurobiol*. 2007; 155:55–63. [PubMed: 16616710]
40. Joad JP, Kott KS, Bric JM, et al. The effects of inhaled corticosteroids on intrinsic responsiveness and histology of airways from infant monkeys exposed to house dust mite allergen and ozone. *Toxicol Appl Pharmacol*. 2008; 226:153–60. [PubMed: 17991502]
41. Hyde DM, Blozis SA, Avdalovic MV, et al. Alveoli increase in number but not size from birth to adulthood in rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol*. 2007; 293:L570–9. [PubMed: 17586691]
42. Plopper, CG.; Fanucchi, MV. Development of airway epithelium. In: Harding, R.; Pinkerton, K.; Plopper, C., editors. *The lung; development, aging and the environment*. London: Elsevier Academic Press; 2004. p. 13-32.
43. Plopper, CG.; Harkema, JR. The respiratory system and its use in research. In: Wolfe-Coote, S., editor. *The laboratory primate*. London: Elsevier Academic Press; 2005. p. 503-27.
44. Golub MS, Gershwin ME, Hurley LS, Saito WY, Hendrickx AG. Studies of marginal zinc deprivation in rhesus monkeys. IV. Growth of infants in the first year. *Am J Clin Nutr*. 1984; 40:1192–202. [PubMed: 6507341]
45. Evans MJ, Van Winkle LS, Fanucchi MV, Plopper CG. The attenuated - fibroblast sheath of the respiratory tract epithelial-mesenchymal trophic unit. *Am J Respir Cell Mol Biol*. 1999; 21:655–7. [PubMed: 10572061]
46. Lipworth BJ. Systemic adverse effects of inhaled corticosteroid therapy: a systematic review and meta-analysis. *Arch Intern Med*. 1999; 159:941–55. [PubMed: 10326936]
47. Bolt RJ, van Weissenbruch MM, Lafeber HN, Delemarre-van de Waal HA. Glucocorticoids and lung development in the fetus and preterm infant. *Pediatr Pulmonol*. 2001; 32:76–91. [PubMed: 11416880]
48. Cagney M, MacIntyre CR, McIntyre PB, Peat J. Childhood asthma diagnosis and use of asthma medication. *Aust Fam Physician*. 2005; 34:193–6. [PubMed: 15799674]
49. Gold DR, Fuhlbrigge AL. Inhaled corticosteroids do not prevent subsequent development of asthma. *J Pediatr*. 2006; 149:728–9. [PubMed: 17095358]
50. Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent inhaled corticosteroids in infants with episodic wheezing. *N Engl J Med*. 2006; 354:1998–2005. [PubMed: 16687712]
51. Weinberger M. Should corticosteroids be used for first-time young wheezers? *J Allergy Clin Immunol*. 2007; 119:567–9. [PubMed: 17258307]

52. Ducharme FM, Lemire C, Noya FJ, et al. Preemptive use of high-dose fluticasone for virus-induced wheezing in young children. *N Engl J Med*. 2009; 360:339–53. [PubMed: 19164187]
53. Agertoft L, Pedersen S. Short-term lower leg growth rate in children with rhinitis treated with intranasal mometasone furoate and budesonide. *J Allergy Clin Immunol*. 1999; 104:948–52. [PubMed: 10550737]
54. Panickar J, Lakhanpaul M, Lambert PC, et al. Oral prednisolone for preschool children with acute virus-induced wheezing. *N Engl J Med*. 2009; 360:329–38. [PubMed: 19164186]
55. Gold DR, Fuhlbrigge AL. Inhaled corticosteroids for young children with wheezing. *N Engl J Med*. 2006; 354:2058–60. [PubMed: 16687719]
56. Wang Y, Tang Y, Moellmann H, Hochhaus G. Simultaneous quantification of budesonide and its two metabolites, 6beta-hydroxybudesonide and 16alpha-hydroxyprednisolone, in human plasma by liquid chromatography negative electrospray ionization tandem mass spectrometry. *Biomed Chromatogr*. 2003; 17:158–64. [PubMed: 12717805]
57. Hyde DM, Tyler NK, Plopper CG. Morphometry of the respiratory tract: avoiding the sampling, size, orientation, and reference traps. *Toxicol Pathol*. 2007; 35:41–8. [PubMed: 17325971]
58. Larson SD, Schelegle ES, Hyde DM, Plopper CG. The three-dimensional distribution of nerves along the entire intrapulmonary airway tree of the adult rat and the anatomical relationship between nerves and neuroepithelial bodies. *Am J Respir Cell Mol Biol*. 2003; 28:592–9. [PubMed: 12707015]
59. Agertoft L, Pedersen S. Lung deposition and systemic availability of fluticasone Diskus and budesonide Turbuhaler in children. *Am J Respir Crit Care Med*. 2003; 168:779–82. [PubMed: 12893646]
60. Agertoft L, Andersen A, Weibull E, Pedersen S. Systemic availability and pharmacokinetics of nebulised budesonide in preschool children. *Arch Dis Child*. 1999; 80:241–7. [PubMed: 10325704]
61. Evans MJ, Fanucchi MV, Plopper CG. Basement membrane zone in asthma. *Curr Respir Med Rev*. 2006; 2:331–7.
62. Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol*. 2000; 105:193–204. [PubMed: 10669837]
63. Clay CC, Rodrigues DS, Brignolo LL, et al. Chemokine networks and in vivo T-lymphocyte trafficking in nonhuman primates. *J Immunol Methods*. 2004; 293:23–42. [PubMed: 15541274]
64. Plopper, CG. Structural methods for studying bronchiolar epithelial cells. In: Gil, J., editor. *Models of lung disease: microscopy and structural methods*. New York: Marcel Dekker, Inc; 1990. p. 537–59.
65. Bunton TE, Plopper CG. Triamcinolone- induced structural alterations in the development of the lung of the fetal rhesus macaque. *Am J Obstet Gynecol*. 1984; 148:203–15. [PubMed: 6691397]
66. Kovar J, Willet KE, Hislop A, Sly PD. Impact of postnatal glucocorticoids on early lung development. *J Appl Physiol*. 2005; 98:881–8. [PubMed: 15465892]
67. Mitzner W, Johnson JW, Beck J, London W, Sly D. Influence of beta-methasone on the development of mechanical properties in the fetal rhesus monkey lung. *Am Rev Respir Dis*. 1982; 125:233–8. [PubMed: 7065528]
68. Davey MG, Johns DP, Harding R. Postnatal development of respiratory function in lambs studied serially between birth and 8 weeks. *Respir Physiol*. 1998; 113:83–93. [PubMed: 9776554]
69. Massaro D, Massaro GD. Critical period for alveologenesis and early determinants of adult pulmonary disease. *Am J Physiol Lung Cell Mol Physiol*. 2004; 287:L715–7. [PubMed: 15355862]
70. Tschanz SA, Makanya AN, Haenni B, Burri PH. Effects of neonatal high-dose short-term glucocorticoid treatment on the lung: a morphologic and morphometric study in the rat. *Pediatr Res*. 2003; 53:72–80. [PubMed: 12508084]
71. Massaro GD, Massaro D, Chan WY, et al. Retinoic acid receptor-beta: an endogenous inhibitor of the perinatal formation of pulmonary alveoli. *Physiol Genomics*. 2000; 4:51–7. [PubMed: 11074013]

72. Massaro GD, Massaro D, Chambon P. Retinoic acid receptor-alpha regulates pulmonary alveolus formation in mice after, but not during, perinatal period. *Am J Physiol Lung Cell Mol Physiol.* 2003; 284:L431–3. [PubMed: 12533315]
73. Massaro D, Teich N, Maxwell S, Massaro GD, Whitney P. Postnatal development of alveoli. Regulation and evidence for a critical period in rats. *J Clin Invest.* 1985; 76:1297–305. [PubMed: 4056033]

Fig. 1.

Diagram of experimental protocol for production of allergic airway disease (AAD) in infant rhesus monkeys and their treatment with budesonide (ICS). Infant monkeys were divided into two groups and housed in filtered air (FA) from birth. One group (AAD monkeys) was sensitized to house dust mite allergen (HDMA) at 14 and 28 days of age and exposed to HDMA (days 3–5) with ozone (days 1–5) for eleven 14-day cycles beginning at 28 days of age. The other group (Control monkeys) received FA only. Half of the Control monkeys and half of the AAD monkeys received budesonide daily from the beginning of the sixth exposure cycle.

Fig. 2. Comparison of the histological appearance of alveoli in the lung parenchyma (right middle lobe) of infant rhesus monkeys exposed to filtered air (Ctrl) or house dust mite allergen with ozone to produce allergic airways disease (AAD) and treated with ICS or saline. (a) Control saline. (b) Control ICS. (c) AAD saline. (d) AAD ICS. (e) Alveolar number (Nalv) was estimated by morphometry. * $P < 0.05$ significantly different from saline groups; one-way ANOVA. There was a significant ($P < 0.05$) overall effect of ICS treatment on alveolar number by two-way ANOVA. Scale = 50 μ m. $n = 6$ per group.

Fig. 3.

Comparison of the bronchial epithelium of midlevel airways in infant rhesus monkeys exposed to filtered air (Ctrl) or house dust mite allergen with ozone to produce allergic airways disease (AAD) and treated with ICS or saline. (a) Morphometric comparison of the mass (volume per surface) of the epithelium (gray bars) and of the histochemically detectable mucin stored in the epithelium (dark bars). (b) Comparison of the relative abundance of stored mucins by composition, determined histochemically based on their reaction to Alcian blue (AB) and Periodic acid Schiff's (PAS) reagent. (d, e, f, g) Histochemical comparison of the epithelium in midlevel airways of Control saline monkeys (d) or Control ICS monkeys (e) with AAD saline monkeys (f) or AAD ICS monkeys (g). (c) Rate of proliferation of epithelial cells as a percentage of PCNA positive cells in the epithelium. * $P < 0.05$ significantly different from Control saline group; one-way ANOVA. Scale = 50 μ m. Mean $\pm$ one standard deviation. $n = 6$ per group.

Fig. 4.

Comparison of the tracheal basement membrane zone (BMZ) of infant rhesus monkeys exposed to filtered air (Ctrl monkeys) or house dust mite allergen with ozone to produce allergic airways disease (AAD monkeys) and treated with saline or ICS. Characterization of the basement membrane zone was based on the relative intensity and distribution of immunoreactive collagen I and perlecan (a, b, c, d). Distribution of immunoreactive collagen I (red signal) in Control saline (a), Control ICS (b), AAD saline (c) and AAD ICS (d) monkeys. The percentage of the basement membrane zone less than 2 μm in thickness (e) was significantly greater in the AAD saline monkeys ($P < 0.05$) than the AAD ICS monkeys. Expression of immunoreactive perlecan in the basement membrane zone (f) was significantly less in the AAD saline monkeys ($P < 0.05$) than the AAD ICS monkeys. These data demonstrate that ICS treatment did inhibit the deleterious effects of HDMA + O₃ on the developing BMZ but did not affect the normal development of the BMZ. Scale = 50 μm. Mean ± 1 SD. $n = 6$ per group.

Fig. 5. Comparison of the abundance of intraepithelial nerve fibres associated with postnatal innervation of the mucosal epithelium in midlevel bronchi of infant rhesus monkeys exposed to filtered air (Ctrl) or house dust mite allergen with ozone to produce allergic airways disease (AAD) and treated with ICS or saline. (a, b, c, d) Distribution of immunoreactive Protein Gene Product 9.5 (red signal) in intraepithelial nerve fibres and epithelial cells of Control saline (a), Control ICS (B), AAD saline (c) and AAD ICS (d) monkeys. 50 μ m. $n = 6$ per group.

Table 1
Measurements of lung growth: physiological lung volumes, fixed lung volumes, and airway branching number

Measurement	Allergen Exposure/Treatment Group			Two-way ANOVA	
	Control			P Values	
	Saline	ICS	Saline	Exposure Effect	Steroid Effect
Total Lung Capacity (mL)	79.75 ± 8.95 ^{‡*}	102.27 ± 6.26 [‡]	80.38 ± 8.48 [*]	89.79 ± 7.91	P = 0.471
Vital Capacity (mL)	57.70 ± 8.80 ^{‡*}	77.10 ± 4.20 [‡]	47.60 ± 9.30 [*]	59.10 ± 1.10	P = 0.059
Residual Volume (mL)	22.30 ± 2.20	24.60 ± 4.30	32.80 ± 2.00	30.70 ± 7.40	P = 0.075
Inspiratory Capacity (mL)	43.50 ± 6.20 ^{‡*}	55.00 ± 2.90 [‡]	31.10 ± 5.70 [*]	40.10 ± 2.50	P = 0.010
Functional Residual Capacity (mL)	36.50 ± 3.50	47.20 ± 4.50	49.00 ± 4.00	49.90 ± 8.00	P = 0.147
Volume of Fixed Rt Middle Lobe (mL)	8.80 ± 0.50	9.23 ± 0.80	9.27 ± 0.42	10.12 ± 1.14	P = 0.351
Non-parenchymal Volume of Rt Middle Lobe (mL)	0.27 ± 0.01 ^{‡†}	0.41 ± 0.09 [‡]	0.29 ± 0.03 [‡]	0.56 ± 0.01	P = 0.222
Number of Airway Branches to Respiratory Bronchiole Rt Middle Lobe (Number of branches)	16.60 ± 1.51 [*]	16.83 ± 1.17	13.42 ± 2.50 [*]	15.00 ± 2.00	P = 0.024
Number of Airway Branches to Respiratory Bronchiole Lf Cranial Lobe (Number of branches)	17.10 ± 1.73 ^{‡†}	18.17 ± 1.47	13.50 ± 2.32 [*]	13.83 ± 2.23 [‡]	P = 0.0001

Significant differences between groups (one-way ANOVA).

* (P = 0.01) Control Saline versus AAD Saline.

[‡] (P = 0.05) Control Saline versus AAD ICS.

^{‡†} (P = 0.01) Control Saline versus Control ICS.

Table 2

Measurements of pulmonary function: quasistatic lung compliance and air flow

Measurement	Allergen Exposure/Treatment Group				Two-way ANOVA	
	Control		AAD		P Values	
	Saline	ICS	Saline	ICS	Exposure Effect	Steroid Effect
Compliance (C _{chord}) (cm H ₂ O/mL)	2.3550 ± 0.4340	3.1780 ± 0.2390	1.6620 ± 0.4660	2.5560 ± 0.1000	P = 0.075	P = 0.015
Forced expiratory flow at 25% VC (FEF ₂₅) (mL/s)	114.2050 ± 12.5260*	110.6650 ± 8.0580	73.0120 ± 16.8810*	92.3400 ± 21.9710	P = 0.062	P = 0.812
Forced expiratory flow at 50% FEF ₅₀ (mL/s)	151.8200 ± 5.1450*	146.1920 ± 3.0960	122.4330 ± 12.6630*	140.5780 ± 8.7260	P = 0.043	P = 0.703

Significant differences between groups (one-way ANOVA).

* (P = 0.01) Control Saline versus AAD Saline.

† (P = 0.05) Control Saline versus AAD ICS.

‡ (P = 0.01) Control Saline versus Control ICS.

Table 3

Measurements of abundance of eosinophils in bronchoalveolar lavage and in airway walls

Measurement	Allergen Exposure/Treatment Group				Two-way ANOVA	
	Control		AAD		P values	
	Saline	ICS	Saline	ICS	Exposure Effect	Steroid Effect
Frequency in lavage (% of leucocytes)	0.68 ± 0.31 ^{*,†}	2.32 ± 2.29 [‡]	8.92 ± 3.89 [*]	5.47 ± 2.45 [†]	P = 0.0001	P = 0.507
Total number in lavage (×10 ³)	51.30 ± 39.88 ^{*,†}	220.16 ± 221.92 [‡]	575.60 ± 165.57 [*]	417.59 ± 106.53 [†]	P = 0.0001	P = 0.95
Density in airway epithelium (Vs, ×10 ⁴ ; mm ³ /mm ²)	2.01 ± 0.72 ^{*,†}	4.06 ± 2.33	32.42 ± 5.18 [*]	19.01 ± 3.81 [†]	P = 0.0001	P = 0.105
Density in airway interstitium (Vs, ×10 ⁴ ; mm ³ /mm ²)	4.23 ± 1.77 [*]	2.74 ± 1.07	24.31 ± 6.81 [*]	9.88 ± 1.77	P = 0.001	P = 0.038

Significant differences between groups(one-way ANOVA).

^{*} (P 0.01), Control Saline versus AAD Saline.

[†] (P 0.05), Control Saline versus AAD ICS.

[‡] (P 0.05), Control Saline versus Control ICS.

Table 4

A summary of airway disease parameters and developmental processes impacted and not impacted by corticosteroid treatment

ICS Beneficial Impacts on Allergic Airways Disease	No Impact of ICS on Disease	Developmental Process	ICS Impact on Postnatal Development
Prevented decreases in vital and inspiratory capacities	Loss of airway branching	Adrenal response to ACTH	No effect
Prevented basement membrane zone changes	Disrupted airway innervation	Body growth (length and weight)	No effect
Decreased eosinophils in bronchoalveolar lavage	Increased airway epithelial eosinophils	Total lung capacity	Increased
Prevented reduction in airflow		Vital capacity	Increased
Decreased airway interstitial eosinophils		Inspiratory capacity	Increased
Reversed abnormal mucous cell density and composition		Non-parenchymal volume	Increased
Reversed smooth muscle thickening in bronchi*		Lung compliance	Increased
*From reference [40].		Number of alveoli	Increased
		Postnatal airway innervation	Disrupted
		Airway epithelial proliferation	Increased
		Airway epithelial eosinophils	Increased
		Airway smooth muscle	Increased*
*From reference [40].			