Published in final edited form as:

Toxicol Appl Pharmacol. 2009 April 1; 236(1): 39–48. doi:10.1016/j.taap.2008.12.031.

Ozone and Allergen Exposure During Postnatal Development Alters the Frequency and Airway Distribution of CD25+ cells in Infant Rhesus Monkeys

Lisa A. Miller^{a,c,*}, Joan E. Gerriets^c, Nancy K. Tyler^c, Kristina Abel^{b,c}, Edward S. Schelegle^{a,c}, Charles G. Plopper^{a,c}, and Dallas M. Hyde^{a,c}

a Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA 95616

b Department of Internal Medicine, School of Medicine, University of California, Davis, CA 95616

c California National Primate Research Center, University of California, Davis, CA 95616

Abstract

The epidemiologic link between air pollutant exposure and asthma has been supported by experimental findings, but the mechanisms are not understood. In this study, we evaluated the impact of combined ozone and house dust mite (HDM) exposure on the immunophenotype of peripheral blood and airway lymphocytes from rhesus macaque monkeys during the postnatal period of development. Starting at 30 days of age, monkeys were exposed to 11 cycles of filtered air, ozone, HDM aerosol, or ozone + HDM aerosol. Each cycle consisted of ozone delivered at 0.5 ppm for 5 days (8h/day), followed by 9 days of filtered air; animals received HDM aerosol during the last 3 days of each ozone exposure period. Between 2–3 months of age, animals co-exposed to ozone + HDM exhibited a decline in total circulating leukocyte numbers and increased total circulating lymphocyte frequency. At 3 months of age, blood CD4+/CD25+ lymphocytes were increased with ozone + HDM. At 6 months of age, CD4+/CD25+ and CD8+/CD25+ lymphocyte populations increased in both blood and lavage of ozone + HDM animals. Overall volume of CD25+ cells within airway mucosa increased with HDM exposure. Ozone did not have an additive effect on volume of mucosal CD25+ cells in HDM-exposed animals, but did alter the anatomical distribution of this cell type throughout the proximal and distal airways. We conclude that a window of postnatal development is sensitive to air pollutant and allergen exposure, resulting in immunomodulation of peripheral blood and airway lymphocyte frequency and trafficking.

Keywords

lung; development; house dust mite; ozone; CD25; lymphocyte

*Address correspondence and reprint requests to: Lisa A. Miller, Ph.D. California National Primate Research Center, University of California, Davis, Davis, California 95616, Telephone: (530) 754-7546/FAX: (530) 752-2880, Email: E-mail: lmiller@ucdavis.edu.

CONFLICT OF INTEREST STATEMENT

The authors of this paper declare that they have no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

The natural progression of childhood asthma from persistent wheeze in infancy to the clinical diagnosis of asthma in school age children and adults is not well understood. Cross-sectional surveys have provided evidence suggesting that the genesis of asthma occurs within the first year of life, and that early exposure to certain environmental factors (such as air pollution or aeroallergens) may promote the asthma phenotype (Yunginger *et al.*, 1992; Rosenstreich *et al.*, 1997; Mortimer *et al.*, 2002). Factors that predict persistence or relapse of asthma in adulthood included sensitization to house dust mites, airway hyperresponsiveness, and early age at onset (Sears *et al.*, 2003). In general, longitudinal studies have supported the notion that events taking place early in life can be predictive of disease later on in life. However, the complex interaction of genetic constitution and environmental influences, such as air pollutant exposures, limit the ability to provide a definitive mechanism to explain the progression of clinical symptoms during infancy to the diagnosis of asthma in school age children.

Epidemiologic studies correlate high levels of ozone with exacerbation and development of asthma in school-aged children (Gauderman *et al.*, 2002; McConnell *et al.*, 2002; Gent *et al.*, 2003). In the ovalbumin mouse model of asthma, chronic exposure to ozone levels greater than 0.13 ppm result in greater anaphylactic sensitivity to intravenous challenge with allergen (Osebold *et al.*, 1988). In the same study, ozone exposure had an additive effect on numbers of IgE expressing cells in allergen challenged animals. Enhanced allergic sensitization via ozone is further supported by U. Neuhaus-Steinmetz and colleagues (Neuhaus-Steinmetz *et al.*, 2000), demonstrating a shift towards a Th2 cytokine profile in both IgE-high responder (Balb/c) and IgE-low responder (C57BL/6) mice following a combination of ozone and allergen exposures. These findings suggest a potential immune mediator for the adjuvant properties of ozone, but it should be noted that the mouse ovalbumin model used in the aforementioned studies is representative of an adult immune phenotype. A longitudinal study of lymphocyte populations in healthy human infants from birth through 1 year of age suggests that the first year of life is a dynamic phase of immune system maturation, with fluctuations of lymphocyte numbers and phenotypes (de Vries *et al.*, 2000). As such, it is imperative to investigate the immunomodulatory properties of ozone exposure during the postnatal period of development, when both the innate and adaptive arms of the immune system undergo substantial maturation.

The functional contribution of airway T lymphocytes that produce IL-4 and IL-5 (Th2 cytokines) in the pathogenesis of allergic asthma is well documented in both rodent models and adult human subjects (Holgate, 2008). In children, the role of specialized T lymphocyte subpopulations in the lung is somewhat controversial, with studies correlating the presence of IFN γ -producing (but not IL-4) CD3+ cells, as well as T regulatory cells and invariant NK-T cells with the development of the asthma phenotype (Brown *et al.*, 2003; Pham-Thi *et al.*, 2006; Hartl *et al.*, 2007; Jartti *et al.*, 2007). Because of the invasive nature of bronchoalveolar lavage, much of what is known about the asthma immune mechanisms during the neonatal period is restricted to prospective analysis of peripheral blood lymphocytes. Although a consistent cytokine repertoire in circulating T lymphocytes has not been observed during longitudinal evaluation of different childhood asthma cohorts, increased expression of activation markers (such as CD25) on CD4+ T helper cells is a frequent finding, particularly in association with acute asthma (Gemou-Engesaeth *et al.*, 1994; Gemou-Engesaeth *et al.*, 2002; Macaubas *et al.*, 2003; Neaville *et al.*, 2003; Heaton *et al.*, 2005; Antunez *et al.*, 2006; Bottcher *et al.*, 2006). The aim of our study was to determine if chronic ozone and allergen exposure during the postnatal period of development could alter peripheral blood and airway T lymphocyte phenotypes. Because activation of T lymphocytes is an important step in the establishment of a cytokine effector response, we proposed that ozone could promote the

development of allergic airways disease during early life by increasing the activation of circulating and pulmonary lymphocytes.

To address this hypothesis, we used a non-human primate model of allergic airways disease that has been previously characterized (Schelegle *et al.*, 2001; Schelegle *et al.*, 2003). We have reported that episodic exposure to combined ozone and house dust mite (HDM) during the first six months of life in rhesus monkeys resulted in a marked increase in plasma histamine and airways eosinophilia (Schelegle *et al.*, 2003). Further, ozone and HDM co-exposure in infant monkeys resulted in airways remodeling in association with increased airways resistance and reactivity to histamine challenge (Schelegle *et al.*, 2003). In this current study, animals were evaluated at 1–6 months of age to measure the impact of ozone and HDM co-exposure on circulating and pulmonary lymphocyte frequency, as well as lymphocyte expression of the activation marker, CD25. In addition, we also assessed histologic specimens to determine whether ozone exposure during postnatal development alters the overall abundance and anatomic distribution of CD4+ and CD25+ cells recruited into the tracheobronchial airway tree of aeroallergen-challenged monkeys.

METHODS

Animal and experimental protocol

Briefly, 30 day-old male rhesus macaque (*Macaca mulatta*) infant monkeys were exposed to 11 cycles of filtered air (n=6), HDM (n=6), ozone (n=6), or ozone + HDM (n=6) (Figure 1). Each cycle consisted of ozone exposure for 5 days, followed by 9 days of filtered air (0.5 ppm at 8h/day). Animal groups not exposed to ozone remained in filtered air throughout each cycle. HDM aerosol exposures were on day 3–5 (2 h/day) of either filtered air exposure or ozone exposure. All monkeys that received HDM aerosol were sensitized to HDM via subcutaneous injection with adjuvant at age 14 days and 28 days; 11/12 monkeys developed positive intradermal reactivity to HDM (≥ 3 mm) by skin prick testing prior to the start of cycle 1 (Schelegle *et al.*, 2001; Schelegle *et al.*, 2003). Non-sensitized monkeys were exposed to either filtered air or ozone. An additional control group of four infant monkeys received systemic HDM sensitization but no aerosol exposures. Complete blood counts (CBC) were measured using a Beckman Coulter analyzer (Beckman Coulter Inc., Miami, FL) and differential counts were obtained from blood smears. All animals were necropsied at approximately 175 days of age, at 3–5 days following the last ozone or allergen exposure (cycle 11). Care and housing of animals before, during and after treatment complied with the provisions of the Institute of Laboratory Animal Resources and conforms to practices established by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Ozone and HDM exposures

Details of ozone and HDM exposure methods for this study were previously reported (Schelegle *et al.*, 2003). In brief, ozone was generated a previously described (Wilson *et al.*, 1984) and concentration was monitored using a Dasibi 1003-AH ozone analyzer (Dasibi Environmental Corporation, Glendale, CA). HDM aerosols were generated with a lyophilized extract of *Dermatophagoides farinae* purchased from Greer Laboratories (Lenoir, NC) diluted in phosphate buffered saline (PBS), and nebulized with a high-flow-rate nebulizer as previously described (Schelegle *et al.*, 2001). Animals were exposed to ozone and HDM aerosols while housed in a 4.2 m³ exposure chamber; data for generation of HDM mass concentration and aerodynamic size distribution have been reported in (Schelegle *et al.*, 2001). We have demonstrated that protein concentration of HDM aerosols in chamber exposures consist of 506 $\pm$ 38 μ g/m³ per day (n=6), a concentration comparable to that previously used to induce symptoms of allergic asthma in adult rhesus monkeys (Miller *et al.*, 2003). Filtered air

conditions were established with a CBR (chemical, biological and radiological) filtration system, which consists of a prefilter, HEPA filter and a carbon filter.

Immunophenotyping of leukocytes

Lavage specimens and peripheral blood mononuclear cells (PBMC) were prepared for immunostaining as previously described (Schelegle *et al.*, 2001). Mouse anti-human monoclonal antibodies used for flow cytometry were as follows: (1) CD2 fluorescein isothiocyanate (FITC), CD4 phycoerythrin (PE), CD8 PE, CD25, CD45 (DAKO, Carpinteria, CA); (2) CD20 PE (Caltag, Burlingame, CA); (3) CD19 PE (Becton Dickinson, San Jose, CA) (5) CD3 FITC (Pharmingen, San Diego, CA). PE-Cy5-conjugated goat F(ab')² anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was used as a secondary reagent. Two and three color analysis was performed on a FACScan, acquiring 30,000–50,000 events per samples and analyzed with CELLQuest software (Becton Dickinson). Lymphocyte gates were defined by forward and side-light scatter properties.

Histopathology and immunohistochemistry

Following necropsy, cross-sections of the trachea and left caudal lobe of each animal were embedded in Optimal Cutting Temperature compound embedding media (OCT, Sakura Finetek, Torrance, CA). The left caudal lobe was inflated with a 1:1 mixture of OCT and PBS and sliced perpendicular to the long axis of the intrapulmonary airway. Each left caudal lobe slice was numbered in sequence from proximal to distal direction prior to freezing in OCT molds. Left caudal lobe slices were approximately 7–8 mm in thickness, the entire lung lobe consisted of 10–11 OCT blocks. Cryosections from alternately numbered OCT blocks were used for immunofluorescence and immunohistochemical staining. For immunofluorescence staining, 5 micron cryosections of the left caudal lobe and trachea were fixed in ice-cold acetone for 10 minutes. Non-specific binding of antibodies was blocked by a 10 minute incubation of cryosections with purified goat IgG (10 mg/ml; Sigma, St. Louis, MO) prior to addition of primary antibodies. Cryosections were stained with mouse anti-human CD4 (clone OKT4; ATCC, Manassas VA) and FITC-conjugated mouse anti-human CD25 (1 µg/ml; Becton Dickinson). ALEXA 568-conjugated goat anti-mouse IgG was used as a secondary antibody (1:1000 dilution). Purified mouse IgG₁ (MOPC 21, ATCC) and FITC-conjugated mouse IgG₁ (Pharmingen) isotype control antibodies were used to test for non-specific staining. All monoclonal antibodies used for this study have been confirmed to produce immunoprofiles by FACS analysis identical to that of human peripheral blood leukocytes (data not shown).

Morphometry

Five micron cryosections of immunostained CD4⁺ cells and CD25⁺ cells were imaged using the appropriate excitation and emission filters for the cellular labeled fluorochromes (listed above) on an Olympus Provis Microscope at 600X. Images were captured using a Zeiss camera at a resolution of 150 pixels/inch in a 1300 by 1130 pixel image for each of 10 fields for a selected airway, using stratified sampling with a random start within each block. The images were imported into the Stereology Toolbox® (version 1.1, Morphometrix, Davis, CA) for estimation of volume density of each of the immunostained cells in airway epithelium or interstitium using a 125 point grid to achieve a count of about 200 points on each cell type. Points which fell on fluorescence positive cells were counted as (P_{cells}) and those points which fell on either epithelium or interstitium were counted as the reference volume of epithelium (P_{epi}) or interstitium (P_{int}). The volume density of fluorescence positive cells per volume of epithelium or interstitium was calculated as

$$V_{V_{\text{cells, (epi or int)}}} = \frac{\sum P_{\text{cells}}}{\sum P_{\text{epi or } \sum P_{\text{int}}}}$$

The surface of epithelial basal lamina per unit volume of epithelium ($S_{V_{\text{bl, epi}}}$) or interstitium ($S_{V_{\text{bl, int}}}$) was calculated on cross sections of airways at 100X using an Olympus BH-2 microscope with the CAST version 2.00.04 software (Olympus, Denmark) as

$$S_{V_{\text{bl, (epi or int)}}} = \frac{\pi \sum I_{\text{bl}}}{2(I/p)(P_{\text{epi or } P_{\text{int}}})}$$

Where I/p = length per test point on 4 lines oriented either horizontally or vertically in a counting frame, I_{bl} is the number of line intersections of the epithelial basal lamina and $P_{\text{epi or } P_{\text{int}}}$, the number of points that hit epithelium or interstitium, respectively. The volume of fluorescence positive cells within the epithelial or interstitial compartment per surface area of basement membrane (mm^3/mm^2) was then calculated as

$$V_{S_{\text{eos, bl}}} = \frac{V_{V_{\text{eos, (epi or int)}}}}{S_{V_{\text{bl, (epi or int)}}}}$$

Relative quantitation of CD4 and FoxP3 mRNA expression levels

Total RNA was isolated from midlevel airway samples using TRIzol reagent as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Real-time PCR was performed as previously described (Abel *et al.*, 2003; Abel *et al.*, 2004). The primer-probe pairs for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) have been previously reported (Abel *et al.*, 2001). The following primer-probe pair (5'-3') was used to amplify CD4 transcripts: forward primer, CAA GGA TGC TTT TCC ATG ATC A; reverse primer, AGC AGG TGG GTG TCA GAG TTG; probe, CAG TCA ATC CGA ACA CCA GCA ATT CCA-TAMRA. The following primer-probe pair (5'-3') was used to amplify FoxP3 transcripts: forward primer, GGG CAG GGC ACA ATG TCT; reverse primer, ATG GCA CTC AGC TTC TTC TTC T; probe, TGG TAC AGT CTC TGG AGC AGC AGC -TAMRA. Samples were tested in duplicate, and reactions for housekeeping GAPDH gene and the target gene from each sample were run in parallel on the same plate. The reaction was carried out on a 96-well optical plate (Applied Biosystems, Foster City, Calif.) in a 25- μl reaction volume containing 5 μl of cDNA plus 20 μl of Mastermix (Applied Biosystems). All sequences were amplified using the 7700 default amplification program: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The results were analyzed with the SDS 7700 system software, version 1.6.3 (Applied Biosystems). Relative gene expression for CD4 and FoxP3 mRNA was calculated as recommended by User Bulletin no. 2, ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Statistics

Unless indicated, all data are reported as mean $\pm$ SE. Treatment groups and differences by airway level were compared using either two-way or one-way ANOVA (GraphPad Prism, La Jolla, CA).

RESULTS

Ozone and HDM exposure alters maturational shifts in peripheral blood leukocytes

To determine if ozone and HDM exposure during the postnatal period of growth has an effect on circulating leukocyte populations, peripheral blood samples were collected from rhesus monkeys on a bi-monthly basis throughout the 6-month study period. Blood samples were collected on the fifth day of each cycle, immediately following an ozone and/or HDM exposure. In filtered air control animals, there was a slight decline in total white blood cell counts (WBC) during the first 1.5 months of life, then a progressive increase to maximal values at 3.5 months of age, followed by a decline at 6 months of age (Fig 2, A). For ozone or HDM exposures alone, total WBC counts closely paralleled that of control animals throughout the 6-month study period; overall there were no significant differences with treatment as compared with control animals. Total WBC values for ozone and HDM co-exposed animals showed a significant interaction between age and experimental condition ($p < 0.008$ by two-way ANOVA as compared with filtered air control animals), with attenuation of peak WBC values beginning at 3 months of age. Coinciding with a decline in WBC counts during the first 1.5 months of life, peripheral blood lymphocyte frequency is observed to increase in control animals, followed by a rapid decline at 2 months, then a progressive increase that is maximal at 5 months of age (Fig 2, B). There were no differences in lymphocyte frequency between HDM alone and filtered air control animals. Ozone exposure, either alone or in combination with HDM, had a significant effect on lymphocyte frequency; there was a striking increase in lymphocyte frequency from 2 to 2.5 months of age as compared with control animals. There were no differences in lymphocyte frequency between ozone alone and ozone +HDM animals.

Ozone and HDM exposure promotes CD25 expression on peripheral blood and lavage lymphocytes

To determine if ozone and HDM exposure during postnatal maturation has an effect on specific lymphocyte populations, we evaluated the immunophenotype of peripheral blood lymphocytes at cycle 5 (3 months), cycle 8 (4.5 months) and at necropsy (6 months). Lavage samples were also evaluated at necropsy. For cycles 5 and 8, blood samples were collected immediately following the last ozone and/or HDM aerosol exposure of the cycle. For necropsy, blood and lavage samples were collected between 4–5 days following the last ozone and/or HDM aerosol exposure. At 3 months, 4.5 months, and 6 months of age, there was no significant effect of exposure on the overall frequency of CD3+ (pan-T lymphocyte marker) and CD19+/CD20+ (B lymphocyte marker) populations (data not shown). Frequency of CD2+, CD4+ and CD8+ lymphocyte populations within peripheral blood were also not affected by ozone and/or HDM exposure (data not shown). At 3 months of age, ozone or HDM alone had no effect on CD25 cell surface expression within CD4+ lymphocyte populations in peripheral blood, but combined ozone + HDM exposure significantly increased the frequency of this population (Fig 3, A). The percentage of CD4+CD25+ lymphocytes in peripheral blood was progressively attenuated with age, but combined ozone + HDM exposure retained a modest, but significant increase in this cell population at 6 months of age. Within CD8+ lymphocyte populations, there was a significant increase in CD25 expression with HDM alone at 3 months and 4.5 months of age (Fig 3, B). At 6 months of age, HDM alone no longer had an effect on CD8+CD25+ lymphocytes, but combined ozone + HDM resulted in a significant increase in frequency for this population. As in peripheral blood at 6 months of age, ozone + HDM exposure increased the frequency of CD25 expression within CD4+ and CD8+ lymphocyte populations in lavage as compared with filtered air or ozone control groups.

Ozone and HDM exposure affects the distribution of CD25+ cells in airway mucosa

In parallel with immunophenotypic analysis of peripheral blood and lavage lymphocytes, we assessed histological samples from five airway generations obtained from ozone and/or HDM

exposed 6-month old monkeys for volume of CD4+ and CD25+ cells by immunofluorescence staining. We focused on CD4+ cells as this was the predominant CD25+ lymphocyte population found in early peripheral blood samples collected from ozone and HDM co-exposed animals. Within monkey airways, CD4+ cell populations were overall more abundant within the interstitial compartment, as compared with the epithelium (Fig 4, A). Exposure to either ozone and/or HDM did not have an effect on the cumulative volume or distribution of CD4+ cells within either epithelial or interstitial compartments. This finding is consistent with the lack of effect for ozone and/or HDM exposure on the overall frequency of lavage CD4+ lymphocytes from the same animals (data not shown). Independent of exposure, CD4+ cells preferentially accumulated within the trachea and most proximal intrapulmonary airways within epithelial and interstitial compartments (epithelium $p < 0.005$, interstitium $p < 0.00001$ by one-way ANOVA with Bonferroni post test) (Fig 4, B).

In contrast with CD4+ cells, CD25+ cells were infrequently observed within the airway mucosa, but abundance of this phenotype was increased by HDM exposure (alone or with ozone) in both epithelial and interstitial compartments (Fig 5, A). Filtered air and ozone animals had few CD25+ cells in epithelial and interstitial compartments of sampled lung tissue section; this phenotype was often not detected within individually sampled airway generations (Fig 5, B). In addition, midlevel intrapulmonary airways (blocks 3 and 5) were also evaluated in filtered air sensitized control monkeys; we could not detect significant differences in CD25+ cell volume as compared with filtered air animals that were not sensitized (data not shown). The cumulative volume of CD25+ cells in airway epithelium was increased with ozone and HDM co-exposure as compared with filtered air control animals, but was reduced in comparison with HDM alone (Fig 5, A). The cumulative volume of CD25+ cells in airway interstitium was also increased with ozone and HDM co-exposure, but was not significantly different from HDM alone. By airway generation, there were no significant differences in the distribution of CD25+ cells within the epithelial compartment of either HDM or ozone + HDM animals. Further, the distribution of CD25+ cells by airway generation within epithelial vs. interstitial compartments was comparable in HDM alone animals. In ozone + HDM animals, we found that interstitial distribution of CD25+ cells by airway generation was different from that of the epithelial compartment, shifting from distal to more proximal airways ($p < 0.01$ as compared with epithelium by two-way ANOVA). Immunofluorescence staining images from a representative ozone + HDM animal demonstrate CD4+ and CD25+ cells within both the epithelial and interstitial compartments of the airways, double labeling indicates that most of CD25+ cells within airway mucosa are also CD4+ (Fig 6).

DISCUSSION

We have previously reported that episodic exposure to ozone has a synergistic effect on multiple parameters of allergic airways disease and asthma during postnatal development (Schelegle *et al.*, 2003). Exposure to ozone in conjunction with HDM aeroallergen exposure significantly increased plasma histamine, baseline airways resistance, airways responsiveness to histamine, and airway eosinophils in 6 month-old rhesus monkeys, relative to HDM alone. Here, we have expanded our investigation of the adjuvant effects of ozone exposure during early life by progressively evaluating the lymphocyte phenotype of infant monkeys during postnatal maturation. Using peripheral blood, lavage, and tissue specimens, we determined if ozone and HDM co-exposure can modulate both systemic and pulmonary lymphocytes in young animals, focusing on changes in cell surface expression of CD25, a marker of activation. Significant effects with ozone and HDM co-exposure included an early increase in percentage of circulating CD4+CD25+ lymphocytes, which was also observed at 6 months of age in blood and lavage. Although HDM alone during postnatal development does not promote airways reactivity in infant monkeys (Miller *et al.*, 2003; Schelegle *et al.*, 2003), our data suggests that the lymphocyte repertoire of airway mucosa is significantly affected with chronic allergen

exposure. Ozone and HDM co-exposure does promote airways reactivity in infant monkeys, but did not increase the number of CD4+ or CD25+ cells within the airway mucosa relative to HDM alone. Rather, the immunomodulatory effects of ozone may be due to the observed “shifting” of interstitial CD25+ cell populations from distal to more proximal airways.

To date, little is known about the development of pulmonary mucosal immune cell populations in the human infant; much has been extrapolated from cord blood and peripheral blood analysis. In human fetal airway tissues with no apparent lung abnormalities, T cells, mast cells, and macrophages may be observed as early as the pseudoglandular stage of development (Hubeau *et al.*, 2001). Surprisingly, despite the young age of the animals evaluated in this study, the overall volume of mucosal CD4+ cells in 6 month-old conducting airways (Fig 4) was very similar to what we have previously reported in normal adult rhesus monkeys (Miller *et al.*, 2005); these findings suggest that resident CD4+ lymphocytes of the airways are established at infancy. It should be noted that the infant monkeys within this study were continuously housed in a filtered air clean environment immediately following birth, such that animals were not exposed to the complete myriad of antigens that a healthy human infant inhales on a daily basis. Regardless, infant monkeys housed in filtered air do show evidence of a maturing adaptive immune response, as evidenced by progressive expansion of memory T helper populations in peripheral blood as early as 2 months of age (Miller *et al.*, 2003).

The notion of a window of susceptibility for immunocompetence and lung function has been supported by several longitudinal birth-cohort studies, suggesting that early life events, including viral infections, play a critical role in the establishment of disease later in life (reviewed by (Holt *et al.*, 2005)). In rodent models of development, the postnatal lung has been shown to be highly susceptible to environmental insult (Johnston *et al.*, 2005; Johnston *et al.*, 2006), and postnatal stressors can significantly enhance airways inflammation in an ovalbumin model of asthma (Kruschinski *et al.*, 2008). In this study, we have found that combined ozone and HDM exposure can shift the frequency of circulating lymphocytes at 2–3 months of age (Fig 2). Antigenic stimulation via HDM may contribute to changes in circulating leukocytes, as evidenced by elevated CD25 expression on CD8 cells at 3 months (Fig 3, B), but HDM aerosol alone did not have a significant effect on the total WBC and lymphocyte population. The distinction between effects of HDM alone and ozone + HDM on circulating CD4+ and CD8+ cells at 3 months of age (Fig 3) suggests that the combination of an oxidant stress and antigenic stimulation does not result in a synergistic or additive immune response. Rather, the systemic and pulmonary mucosal lymphocyte profile of ozone and HDM co-exposure in early life suggests an immune mechanism that is similar but distinct from HDM alone.

Until recently, cell surface expression of the IL-2 receptor (CD25) on T lymphocyte populations has been associated with antigenic stimulation or activation. Now confounding the identification of this phenotype is the recognition of a CD4+CD25^{bright} T lymphocyte population with immunosuppressive properties (T regulatory cell). Regulatory T cells as defined by CD4+CD25^{bright} FoxP3+ markers are abundant in the human fetus and infant macaque (Cupedo *et al.*, 2005; Hartigan-O’ Connor *et al.*, 2007), but functional studies in blood CD4+CD25^{bright} from atopic young school-aged children suggest that this population may be a mixture of both activated and regulatory T cells (Jartti *et al.*, 2007). Recent studies in school-age children with asthma report a reduction in airway lavage CD4+CD25^{bright} cells and low FoxP3 mRNA expression as compared with control subjects, supporting the immunosuppressive function of this cell phenotype in asthma (Hartl *et al.*, 2007). In our study, we observed an increased frequency of circulating CD4+CD25+ lymphocytes starting at 3 months of age, immediately following completion of ozone + HDM exposure (Fig 3, A). Although we were not able to determine the contribution of T regulatory cells in our samples with the use of additional markers, the correlation of airways hyperresponsiveness with ozone

+ HDM exposure in the infant monkey would suggest that the CD4+CD25+ lymphocyte population associated with the co-exposure phenotype is not immunosuppressive in function. The increased frequency of CD4+CD25+ cells is most likely the result of T helper cell activation via recent HDM antigenic stimulation, which is important for development of a cytokine effector response to promote eosinophilic airways inflammation (which has been previously demonstrated in this animal model (Schelegle *et al.*, 2003)).

In conjunction with peripheral blood and lavage analysis, we determined if CD4+ cells and CD25+ cells within the airway mucosa were affected by ozone and/or HDM exposure. In adult monkeys sensitized and challenged with HDM aerosol, volume of CD4+ cells within the airway mucosa is significantly increased over control animals (Miller *et al.*, 2005). In infant monkeys, ozone and/or HDM had no effect on the abundance or distribution of CD4+ cells within infant monkey airways, suggesting that T cell responsiveness to environmental challenge is age-dependent (Fig 4). Functional changes in local resident populations in response to environmental challenge may in fact drive the differences in effector responses between treatment groups. This notion is supported by findings in rodent models that demonstrate the presence of long-lived airway dendritic cell populations with potent antigen presenting function, indicating that activation of effector T cells does not necessarily have to be initiated within secondary lymphoid organs (Julia *et al.*, 2002; Huh *et al.*, 2003).

Although the frequency of CD4+/CD25+ cells in lavage was increased only with ozone and HDM co-exposure, CD25+ cells were significantly increased within both epithelial and interstitial compartments in HDM exposed animals (Fig 5). In the epithelium, ozone exposure with HDM resulted in reduced volume of CD25+ cells relative to HDM alone. This effect is comparable to that observed with eosinophils, whereby an increased frequency of this cell type in lavage corresponded to a depletion from the epithelium, suggesting recent trafficking of this cell type into the airway lumen (Schelegle *et al.*, 2003). Interstitial abundance of CD25+ cells in HDM animals was not affected by ozone, but did result in redistribution of this cell type to the trachea and most proximal intrapulmonary airways (as compared with HDM alone). As yet, we cannot confirm the immune cell phenotype responsible for the predominating shift in CD25+ cell populations to the larger airways with ozone + HDM; a number of different leukocytes can express CD25, including B cells, NK cells, and monocytes. However, double immunofluorescence staining of a representative ozone + HDM exposed monkey airway suggests that most of the CD25+ cells are CD4+ (Fig 6).

In conclusion, our study shows that ozone exposure can immunomodulate both the systemic and pulmonary lymphocyte response to HDM aeroallergen exposure during postnatal development. Along with our previous findings of airways hyperresponsiveness associated with ozone and HDM co-exposure, these results point to microenvironment-specific changes in the airways, as opposed to magnitude of inflammation and immune responses, as important early life events that may support a physiologic reaction to allergen challenge in the lung.

Acknowledgements

The authors of this paper would like to acknowledge the expert technical assistance of Brian Tarkington, Jodie Usachenko, Lei Putney, and Sarah Davis during the course of this study.

Funding for this study is provided by NIH P01 ES00628, NIH P01 ES11617, NIH R01 HL081286, and NCR R00169.

References

- Abel K, Alegria-Hartman MJ, Zanutto K, McChesney MB, Marthas ML, Miller CJ. Anatomic site and immune function correlate with relative cytokine mRNA expression levels in lymphoid tissues of normal rhesus macaques. *Cytokine* 2001;16:191–204. [PubMed: 11814315]
- Abel K, Compton L, Rourke T, Montefiori D, Lu D, Rothausler K, Fritts L, Bost K, Miller CJ. Simian-human immunodeficiency virus shiv89.6-induced protection against intravaginal challenge with pathogenic sivmac239 is independent of the route of immunization and is associated with a combination of cytotoxic t-lymphocyte and alpha interferon responses. *J Virol* 2003;77:3099–3118. [PubMed: 12584336]
- Abel K, La Franco-Scheuch L, Rourke T, Ma ZM, De Silva V, Fallert B, Beckett L, Reinhart TA, Miller CJ. Gamma interferon-mediated inflammation is associated with lack of protection from intravaginal simian immunodeficiency virus sivmac239 challenge in simian-human immunodeficiency virus 89.6-immunized rhesus macaques. *J Virol* 2004;78:841–854. [PubMed: 14694116]
- Antunez C, Torres MJ, Mayorga C, Corzo JL, Jurado A, Santamaría-Babi LF, Vera A, Blanca M. Cytokine production, activation marker, and skin homing receptor in children with atopic dermatitis and bronchial asthma. *Pediatr Allergy Immunol* 2006;17:166–174. [PubMed: 16672002]
- Bottcher MF, Jenmalm MC, Voor T, Julge K, Holt PG, Björkstén B. Cytokine responses to allergens during the first 2 years of life in Estonian and Swedish children. *Clin Exp Allergy* 2006;36:619–628. [PubMed: 16650047]
- Brown V, Warke TJ, Shields MD, Ennis M. T cell cytokine profiles in childhood asthma. *Thorax* 2003;58:311–316. [PubMed: 12668793]
- Cupedo T, Nagasawa M, Weijer K, Blom B, Spits H. Development and activation of regulatory t cells in the human fetus. *Eur J Immunol* 2005;35:383–390. [PubMed: 15682453]
- de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Hop WC, Boerma GJ, Lotgering FK, van Dongen JJ. Longitudinal survey of lymphocyte subpopulations in the first year of life. *Pediatr Res* 2000;47:528–537. [PubMed: 10759162]
- Gauderman WJ, Gilliland GF, Vora H, Avol E, Stram D, McConnell R, Thomas D, Lurmann F, Margolis HG, Rappaport EB, Berhane K, Peters JM. Association between air pollution and lung function growth in southern California children: Results from a second cohort. *Am J Respir Crit Care Med* 2002;166:76–84. [PubMed: 12091175]
- Gemou-Engesaeth V, Fagerhol MK, Toda M, Hamid Q, Halvorsen S, Groegaard JB, Corrigan CJ. Expression of activation markers and cytokine mRNA by peripheral blood CD4 and CD8 T cells in atopic and nonatopic childhood asthma: Effect of inhaled glucocorticoid therapy. *Pediatrics* 2002;109:e24. [PubMed: 11826234]
- Gemou-Engesaeth V, Kay AB, Bush A, Corrigan CJ. Activated peripheral blood CD4 and CD8 T-lymphocytes in child asthma: Correlation with eosinophilia and disease severity. *Pediatr Allergy Immunol* 1994;5:170–177. [PubMed: 7951759]
- Gent JF, Triche EW, Holford TR, Belanger K, Bracken MB, Beckett WS, Leaderer BP. Association of low-level ozone and fine particles with respiratory symptoms in children with asthma. *JAMA* 2003;290:1859–1867. [PubMed: 14532314]
- Hartigan-O'Connor DJ, Abel K, McCune JM. Suppression of siv-specific CD4+ T cells by infant but not adult macaque regulatory T cells: Implications for siv disease progression. *J Exp Med* 2007;204:2679–2692. [PubMed: 17954571]
- Hartl D, Koller B, Mehlhorn AT, Reinhardt DTN, Schendel DJ, Griesse M, Krauss-Etschmann S. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007;119:1258–1266. [PubMed: 17412402]
- Heaton T, Rowe J, Turner S, Aalberse RC, de Klerk N, Suriyaarachchi D, Serralha M, Holt BJ, Hollams E, Yerkovich S, Holt K, Sly PD, Goldblatt J, Le Souef P, Holt PG. An immunoepidemiological approach to asthma: Identification of in-vitro T-cell response patterns associated with different wheezing phenotypes in children. *Lancet* 2005;365:142–149. [PubMed: 15639296]
- Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy* 2008;38:872–897. [PubMed: 18498538]

- Holt PG, Upham JW, Sly PD. Contemporaneous maturation of immunologic and respiratory functions during early childhood: Implications for development of asthma prevention strategies. *J Allergy Clin Immunol* 2005;116:16–24. [PubMed: 15990766]
- Hubeau C, Puchelle E, Gaillard D. Distinct pattern of immune cell population in the lung of human fetuses with cystic fibrosis. *J Allergy Clin Immunol* 2001;108:524–529. [PubMed: 11590376]
- Huh JC, Strickland DH, Jahnsen FL, Turner DJ, Thomas JA, Napoli S, Tobagus I, Stumbles PA, Sly PD, Holt PG. Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (dcs) and t cells precede the late phase reaction in experimental asthma: Dc activation occurs in the airway mucosa but not in the lung parenchyma. *J Exp Med* 2003;198:19–30. [PubMed: 12835476]
- Jartti T, Burmeister KA, Seroogy CM, Jennens-Clough ML, Tisler CJ, Salazar LP, DaSilva DF, Evans MD, Vrtis RF, Wallace PK, Ruiz-Perez B, Gangnon RE, Lemanske RF, Gern JE. Association between cd4+cd25high cells and atopy in children. *J Allergy Clin Immunol* 2007;120:177–183. [PubMed: 17433828]
- Johnston CJ, Holm BA, Finkelstein JN. Sequential exposures to ozone and lipopolysaccharide in postnatal lung enhance or inhibit cytokine responses. *Exp Lung Res* 2005;31:431–447. [PubMed: 16025923]
- Johnston CJ, Holm BA, Gelein R, Finkelstein JN. Postnatal lung development: Immediate-early gene responses post ozone and lps exposure. *Inhalation Toxicol* 2006;18:875–883.
- Julia V, Hessel EM, Malherbe L, Glaichenhaus N, O'Garra A, Coffman RL. A restricted subset of dendritic cells captures airborne antigens and remains able to activate specific t cells long after antigen exposure. *Immunity* 2002;16:271–283. [PubMed: 11869687]
- Kruschinski C, Skripuletz T, Bedoui S, Raber K, Straub RH, Hoffmann T, Grote K, Jacobs R, Stephan M, Pabst R, von Horsten S. Postnatal life events affect the severity of asthmatic airway inflammation in the adult rat. *J Immunol* 2008;180:3919–3925. [PubMed: 18322200]
- Macaubas C, de Klerk NH, Holt BJ, Wee C, Kendall G, Firth M, Sly PD, Holt PG. Association between antenatal cytokine production and the development of atopy and asthma at age 6 years. *Lancet* 2003;362:1192–1197. [PubMed: 14568741]
- McConnell R, Berhane K, Gilliland F, London SJ, Islam T, Gauderman WJ, Avol E, Margolis HG, Peters JM. Asthma in exercising children exposed to ozone: A cohort study. *Lancet* 2002;359:386–391. [PubMed: 11844508]
- Miller LA, Hurst SD, Coffman RL, Tyler NK, Stovall MY, Chou DL, Gershwin LJ, Schelegle ES, Plopper CG, Hyde DM. Airway generation-specific differences in the spatial distribution of immune cells and cytokines in allergen-challenged rhesus monkeys. *Clin Exp Allergy* 2005;35:894–906. [PubMed: 16008676]
- Miller LA, Plopper CG, Hyde DM, Gerriets JE, Pieczarka E, Tyler N, Gershwin LJ, Schelegle ES, Van Winkle LS. Immune and airway effects of house dust mite aeroallergen exposures during postnatal development of the infant rhesus monkey. *Clin Exp Allergy* 2003;33:1686–1694. [PubMed: 14656356]
- 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]
- Neaville WA, Tisler C, Bhattacharya A, Anklam K, Gilbertson-White S, Hamilton R, Adler K, DaSilva DF, Roberg KA, Carlson-Dakes KT, Anderson E, Yoshihara D, Gangnon R, Mikus LD, Rosenthal LA, Gern JE, Lemanske RF. Developmental cytokine response profiles and the clinical and immunologic expression of atopy during the first year of life. *J Allergy Clin Immunol* 2003;112:740–746. [PubMed: 14564354]
- Neuhaus-Steinmetz U, Ulffhausen F, Herz U, Renz H. Priming of allergic immune responses by repeated ozone exposure in mice. *Am J Respir Cell Mol Biol* 2000;23:228–233. [PubMed: 10919990]
- Osebold JW, Zee YC, Gershwin LJ. Enhancement of allergic lung sensitization in mice by ozone inhalation. *Proc Soc Exp Biol Med* 1988;188:259–264. [PubMed: 3393542]
- Pham-Thi N, de Blic J, Le Bourgeois M, Dy M, Scheinmann P, Leite-de-Moraes MC. Enhanced frequency of immunoregulatory invariant natural killer t cells in the airways of children with asthma. *J Allergy Clin Immunol* 2006;117:217–218. [PubMed: 16387611]
- Rosenstreich DL, Eggleston P, Kattan M, Baker D, Slavin RG, Gergen P, Mitchell H, McNiff-Mortimer K, Lynn H, Ownby D, Malveaux F. The role of cockroach allergy and exposure to cockroach allergen

- in causing morbidity among inner-city children with asthma. *N Engl J Med* 1997;336:1356–1363. [PubMed: 9134876]
- Schelegle ES, Gershwin LJ, Miller LA, Fanucchi MV, Van Winkle LS, Gerriets JP, Walby WF, Omlor AM, Buckpitt AR, Tarkington BK, Wong VJ, Joad JP, Pinkerton KB, Wu R, Evans MJ, Hyde DM, Plopper CG. Allergic asthma induced in rhesus monkeys by house dust mite (*dermatophadoides farinae*). *Am J Pathol* 2001;158:333–341. [PubMed: 11141508]
- Schelegle ES, Miller LA, Gershwin LJ, Fanucchi MV, Van Winkle LS, Gerriets JE, Walby WF, Mitchell V, Tarkington BK, Wong VJ, Baker GL, Pantle LM, Joad JP, Pinkerton KE, Wu R, Evans MJ, Hyde DM, Plopper CG. 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]
- Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, Cowan JO, Herbison GP, Silva PA, Poulton R. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med* 2003;349:1414–1422. [PubMed: 14534334]
- Wilson DW, Plopper CG, Dungworth DL. The response of the macaque tracheobronchial epithelium to acute ozone injury. A quantitative ultrastructural and autoradiographic study. *Am J Pathol* 1984;116:193–206. [PubMed: 6540523]
- 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;146:888–894. [PubMed: 1416415]

Figure 1. Experimental timeline for ozone and allergen exposure during postnatal development Infant rhesus monkeys were sensitized to HDM via subcutaneous (SQ) injection with adjuvant at day 14 and day 28. Starting at 30 days of age, monkeys were exposed to 11 cycles of ozone and/or HDM aerosol. Each cycle consisted of ozone exposure for 5 days, followed by 9 days of filtered air (0.5 ppm at 8h/day). HDM aerosol was delivered during the last 3 days of the ozone exposure period. Lavage and tissue specimens were collected at 175 days of age.

Figure 2. Changes in peripheral blood cell counts and lymphocyte frequency as a function of monkey age (months)

Peripheral blood samples were collected from monkeys on a bi-monthly basis, starting at 2 weeks of age. Blood samples for each time point were collected on the fifth day of each cycle, immediately following the last ozone and/or allergen exposure. Each time point represents the mean $\pm$ SE values for 5–6 animals. **A**, Total WBC absolute counts from filtered air control (FA) animals compared with ozone (top graph), HDM (middle graph), and Ozone + HDM (bottom graph). **B**, Peripheral blood lymphocyte frequency from filtered air control (FA) animals compared with ozone (top graph), HDM (middle graph), and ozone + HDM (bottom

graph). Significant effects versus filtered air are indicated by * $p < 0.05$ and ** $p < 0.01$, as determined by Bonferonni post tests.

Figure 3. Time course of CD25 expression on lymphocyte populations in peripheral blood and lavage during postnatal development

Peripheral blood mononuclear cells were evaluated by flow cytometry at approximately 3 months (cycle 5), 4.5 months (cycle 8), and 6 months (necropsy) of age. Lavage cells were evaluated at necropsy only. Each column represents the mean $\pm$ SE of values obtained from 3 animals treated with filtered air, ozone, HDM, or ozone + HDM. **A**, Frequency of CD4+/CD25+ T lymphocytes **B**, Frequency of CD8+/CD25+ T lymphocytes. Significant effects are indicated by * p <0.05 vs. filtered air; + p <0.05 vs. ozone; # p <0.05 vs. HDM; ** p <0.01 vs. filtered air; ++ p <0.01 vs. ozone; ## p <0.001 vs. HDM, as determined by Bonferroni post tests.

Figure 4. Effect of ozone and allergen exposure on distribution of CD4+ cells within airway mucosa of 6 month old monkeys

CD4+ cells were quantitated by immunofluorescence staining of histological sections obtained from trachea and left caudal lobes. Columns represent the average volume $\pm$ SE of fluorescence positive staining cells (mm³) from 6 animals treated with filtered air, ozone, HDM or ozone + HDM. Volume of fluorescence positive cells were measured within the epithelial or interstitial compartment with respect to surface area of basal lamina (mm²). Tr, 1, 3, 5, 7 represent cryosections obtained from numbered tissue blocks of trachea and regions from the most proximal (1) to distal (7) intrapulmonary airways of the left caudal lobe. **A**, Overall volume (sum of five airway generations) of CD4+ cells within the epithelial and interstitial compartment of conducting airways. **B**, Volume of CD4+ cells within the epithelial and interstitial compartment of five airway generations.

Figure 5. Effect of ozone and allergen exposure on distribution of CD25+ cells within airway mucosa of 6 month old monkeys

CD25+ cells were quantitated by immunofluorescence staining of histological sections obtained from trachea and left caudal lobes, as described for Fig 4. Columns represent the average volume $\pm$ SE of fluorescence positive staining cells (mm³) from 6 animals treated with filtered air, ozone, HDM or ozone + HDM. **A**, Overall volume of CD25+ cells (sum of five airway generations) within the epithelial and interstitial compartment of conducting airways. **B**, Volume of CD25+ cells within the epithelial and interstitial compartment of five airway generations. nd=none detected. Significant effects are indicated by * $p < 0.05$ vs. HDM; + $p < 0.01$ vs. filtered air; # $p < 0.001$ vs. filtered air, as determined by Bonferroni post tests.

Figure 6. Double immunofluorescence staining for CD4+ and CD25+ cells in airway mucosa
Cryosections from a midlevel airway of a representative HDM-exposed infant monkey were double immunostained for CD4 (A, green fluorescence) and CD25 (B, red fluorescence). Overlap of green and red fluorescence shows two CD4+ cells that are not CD25 positive (C, arrows). The dotted line separates the epithelial and interstitial compartments. Scale bar = 20 μ m.