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Increased CCL24/Eotaxin-2 with Postnatal Ozone Exposure in Allergen-Sensitized Infant Monkeys Is Not Associated with Recruitment of Eosinophils to Airway Mucosa

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Abstract

Epidemiology supports a causal link between air pollutant exposure and childhood asthma, but the mechanisms are unknown. We have previously reported that ozone exposure can alter the anatomic distribution of CD25+ lymphocytes in airways of allergen-sensitized infant rhesus monkeys. Here, we hypothesized that ozone may also affect eosinophil trafficking to allergen-sensitized infant airways. To test this hypothesis, we measured blood, lavage, and airway mucosa eosinophils in 3-month old monkeys following cyclical ozone and house dust mite (HDM) aerosol exposures. We also determined if eotaxin family members (CCL11, CCL24, CCL26) are associated with eosinophil location in response to exposures. In lavage, eosinophil numbers increased in animals exposed to ozone and/or HDM. Ozone + HDM animals showed significantly increased CCL24 and CCL26 protein in lavage, but the concentration of CCL11, CCL24, and CCL26 was independent of eosinophil number for all exposure groups. In airway mucosa, eosinophils increased with exposure to HDM alone; comparatively, ozone and ozone + HDM resulted in reduced eosinophils. CCL26 mRNA and immunofluorescence staining increased in airway mucosa of HDM alone animals and correlated with eosinophil volume. In ozone + HDM animal groups, CCL24 mRNA and immunofluorescence increased along with CCR3 mRNA, but did not correlate with airway mucosa eosinophils. Cumulatively, our data indicate that ozone exposure results in a profile of airway eosinophil migration that is distinct from HDM mediated pathways. CCL24 was found to be induced only by combined ozone and HDM exposure, however expression was not associated with the presence of eosinophils within the airway mucosa.

Keywords

ozone; allergen; infant; lung; eosinophil; chemokine

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CONFLICTS OF INTEREST STATEMENT

The authors of this manuscript declare that there are no conflicts of interest.

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INTRODUCTION

Ozone is a major contributor to lung morbidity, particularly when exposure takes place within the first six to eight years after birth, during which human lung growth continues to progress. Numerous studies have reported on the detrimental effects of ambient ozone on lung function in young children, demonstrating both acute and long-term consequences of living in communities with high concentrations (Spektor *et al.*, 1988; Kinney *et al.*, 1989; Higgins *et al.*, 1990; Spektor *et al.*, 1991; Frischer *et al.*, 1999; Burnett *et al.*, 2001). In addition, ozone exposure can exacerbate pre-existing lung disease in children, such as allergic asthma (Castillejos *et al.*, 1992; Romieu *et al.*, 1996; Romieu *et al.*, 1997; Mortimer *et al.*, 2002). Although it is often considered to be an additive inflammatory stimulus, several studies provide evidence to support a more direct role for ozone in the etiology of airways disease. Schoolchildren have an approximate three fold increased risk of developing asthma if they play outdoor sports (versus no sports) in Southern California communities with high ambient ozone levels (McConnell *et al.*, 2002). Infants of asthmatic mothers are at increased risk of developing respiratory symptoms when living in areas where ozone is near or just below federal standard levels, suggesting that certain sensitive populations are vulnerable to even low-level concentrations of airborne pollutants (Triche *et al.*, 2006). More recently, children with genetic variants of arginase and tumor necrosis factor genes were found to have a reduced risk of asthma or symptoms associated with asthma; however these associations were dependent upon living in either a low ozone or high ozone environment (Lee *et al.*, 2009; Salam *et al.*, 2009).

In adults, ozone-induced airways inflammation is characterized predominantly by a neutrophilic influx, a finding that is replicated in several animal models (Campos *et al.*, 1992; Hyde *et al.*, 1992; Krishna *et al.*, 1998; Holz *et al.*, 1999; Jorres *et al.*, 2000). The inflammatory effects of ozone within conducting airways of children are less understood, largely due to ethical concerns regarding the use of invasive sampling techniques. In a small population of healthy schoolchildren, it has been reported that neutrophil numbers in nasal passages significantly correlate with environmental ozone levels (Frischer *et al.*, 1993). In the same study, circulating and nasal eosinophil numbers were not significantly elevated in response to ozone, yet concentration of eosinophil cationic protein was increased in nasal lavage. A contribution of the eosinophil in ozone-mediated airways inflammation is further implicated by Kopp and colleagues, where a dose dependent increase in ambient ozone exposure correlated with an increase in nasal eosinophil cationic protein concentration in a cohort of 144 schoolchildren (Kopp *et al.*, 1999). Independent of atopy, concentration of urinary eosinophil protein X also significantly correlates with ozone exposure in schoolchildren (Frischer *et al.*, 2001). The consistent observation of eosinophil activation markers in conjunction with ozone exposure in young children suggests a prominent role for the eosinophil as an inflammatory leukocyte in early life.

Within the lung, a number of chemokines have been identified as potential mediators of eosinophil trafficking into the airways. CCL5/RANTES, CCL7/MCP-3, CCL13/MCP-4, and CCL3/MIP-1 α are all elevated in human asthmatics and associated with airways eosinophilia or eosinophil effector functions (Powell *et al.*, 1996; Stellato *et al.*, 1997; Harrison *et al.*, 1999). Each member of this group of chemokines can induce intracellular signaling by binding to more than one chemokine receptor (CCR1, -2, 3, and -5), suggesting a diversity of functions in addition to eosinophil chemotaxis (Lilly and Daugherty, 2001). In contrast, the three members of the eotaxin family of chemokines (CCL11/eotaxin, CCL24/eotaxin-2, CCL26/eotaxin-3) are unique because they exclusively signal via the receptor, CCR3. The association of CCL11 and CCL24 mRNA positive cells with activated eosinophils in bronchial biopsies from asthmatic subjects suggests a role in persistence of eosinophilia in the airway wall (Ying *et al.*, 1999). Expression of CCL26 is restricted to a

24–48 hour window following allergen challenge, and may be more important for new recruitment of eosinophils into the airways (Berkman *et al.*, 2001; Ravensberg *et al.*, 2005).

All three eotaxin family member peptides are synthesized by airway epithelial cells and can be differentially expressed by immunomodulatory cytokines, suggesting a complex regulatory mechanism. (Ying *et al.*, 1997; Ying *et al.*, 1999; Komiya *et al.*, 2003; Heiman *et al.*, 2005; van Wetering *et al.*, 2007). Because a number of different cell types express CCR3 in addition to eosinophils, it is likely that eotaxins have additional important biological functions beyond promoting the activation and migration of eosinophils. CCL11, but not CCL24 or CCL26, is expressed during normal human fetal lung development, primarily by airway epithelium and may be important for proliferation via autocrine signaling through epithelial cell associated CCR3 (Haley *et al.*, 2008). Human fibroblasts express CCR3 and can be induced to proliferate and synthesize collagen via CCL11 or CCL24 treatment (Puxeddu *et al.*, 2006; Kohan *et al.*, 2010). Eotaxins and CCR3 have also been recently been reported to play a key role in angiogenic processes associated with age-related macular degeneration, which is an eosinophil and mast cell-independent process (Takeda *et al.*, 2009).

To date, we have a very limited understanding of the adjuvant mechanisms for air pollutants and how exposure results in a persistent asthma phenotype. Using the rhesus monkey as an animal model for infant lung development, we have reported that episodic ozone exposure during the postnatal growth period can alter the frequency and anatomic distribution of CD25+ lymphocytes in allergen-sensitized airways (Miller *et al.*, 2009). In this current study, we hypothesized that the eosinophilic inflammatory response to allergic sensitization can be modulated in similar fashion by postnatal ozone exposure. To test our hypothesis, we measured circulating, airway lumen and airway mucosa eosinophils in 3-month old infant rhesus monkeys following combined ozone and house dust mite (HDM) exposure. We also determined if expression and localization of eosinophilic chemokines were affected by exposure, focusing on the three members of the eotaxin family.

MATERIALS AND METHODS

Animals and exposure

At 1–2 days of age, newborn male rhesus macaque (*Macaca mulatta*) monkeys were housed under filtered air conditions in 4.2 m³ capacity exposure chambers at the California Regional Primate Research Center, UC Davis. Care and housing of animals before, during and after treatment complied with the provisions of the Institute of Laboratory Animal Resources and conformed to practices of the American Association for Accreditation of Laboratory Animal Care. All animals at 2 weeks of age were sensitized to 10 µg HDM (*Dermatophagoides farinae*, Greer Laboratories, Lenoir, NC) in 10 mg aluminum hydroxide by subcutaneous injection. To mimic childhood vaccination, HDM sensitized animals also received heat-killed *Bordetella pertussis* intramuscularly as an adjuvant. At 4 weeks of age, all animals were given an additional subcutaneous injection of 10 µg HDM in 10 mg aluminum hydroxide. Starting at 30 days of age, animals were exposed to 5 cycles of filtered air (n= 6), HDM allergen (n= 6), ozone (n= 6), or ozone + HDM (n= 6). 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. On days of combined ozone and HDM aerosol, exposures were conducted in sequence. For each day of combined ozone and HDM, animals were first exposed to 8 hours of ozone, followed by a 2 hour delay to allow for animal feeding, then exposure to 2 hours of HDM aerosol. A summary of the exposure protocol is presented in Figure 1. HDM aerosols were generated with a lyophilized extract of *Dermatophagoides farinae* diluted in

PBS and nebulized with a high-flow rate nebulizer as previously described (Schelegle *et al.*, 2001). HDM total protein concentration in each chamber was approximately 300 $\mu\text{g}/\text{m}^3$ for each exposure day. All animals were necropsied at approximately 90 days of age. Necropsies of HDM-exposed animals occurred between 4 to 5 days following the last allergen or ozone exposure.

Airway lavage and leukocyte counts

At necropsy, the right caudal lobe from each animal was lavaged with 35 ml of endotoxin-free PBS. Lavage samples were cyto-centrifuged and stained with a modified Wright's stain (Diff-Quik, Sigma-Aldrich, St. Louis, MO). Differential leukocyte counts were determined by evaluating 300–340 cells using light microscopy.

Immunofluorescence staining for major basic protein

At necropsy, the left caudal lobe from each animal was inflated with a 50% v/v mixture of O.C.T (Sakura Finetek, Torrance, CA) and PBS, then sliced perpendicular to the long axis of the main intrapulmonary conducting airway into even blocks at 7–8 mm thickness per block. Each block from the left caudal lobe was numbered in sequence before embedding for cryosectioning. Cryosections of 5 μm thickness collected from the trachea and alternating numbered blocks were used for immunostaining. To detect eosinophils, cryosections were incubated with mouse anti-human major basic protein (MBP) monoclonal antibody (clone BMK13, BIODSIGN International, Saco, ME) at 0.5 $\mu\text{g}/\text{ml}$, followed by donkey anti-mouse ALEXA 488 (Molecular Probes, Eugene, OR) at 1:2000 dilution. Purified mouse IgG was used as a negative control. Immunofluorescence images were visualized on an Olympus (Ballerup, Denmark) PROVIS fluorescence microscope at 60x magnification. At least 10 random fields were imaged around one airway per section using a Zeiss camera (Zeiss, Oberkochen, Germany). Each image contained a region of epithelium and interstitium internal to the cartilaginous ring (if present). Four cryosections were sampled per animal, representing the most proximal portion (trachea) through the most distal portion of the intrapulmonary conducting airway (respiratory bronchiole). The volume of MBP+ cells within either epithelium or interstitium relative to surface area of basement membrane was measured as previously described (Miller *et al.*, 2003).

RT-PCR analysis

Immediately following necropsy, conducting airways were isolated from the right caudal lobe by fresh tissue microdissection as previously described (Miller *et al.*, 2003). RNA was isolated in TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was generated using random hexamers and MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA). Relative mRNA quantitation was analyzed using the comparative Ct method (Relative Quantitation of Gene Expression: ABI PRISM 7700 Sequence Detection System: User Bulletin #2: Rev B, Applied Biosystems).

Oligonucleotide primers for CCL11, CCL24, and CCL26 were designed for the TaqMan assay using the Primer Express™ Software (Applied Biosystems) based on human sequences obtained from the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>); sequence details used in this study were previously reported (Chou *et al.*, 2003). cDNA samples were amplified with the GeneAmp®7900HT Sequence Detection System using SYBR® Green PCR Master Mix (Applied Biosystems) as the detection fluorochrome. Commercially available Taqman primer/probe sets for rhesus CCR3 and rhesus carboxypeptidase 3 (CPA3) mRNAs (Applied Biosystems) were also used in this study. To control for loading variability, samples were normalized with values for 18s ribosomal RNA (CCL11, CCL24, CCL26) or GAPDH (CCR3, CPA3) according to the manufacturer's instructions (Applied Biosystems).

Immunofluorescence staining for CCL11, CCL24, CCL26 and CCR3

Frozen cryosections for chemokine and chemokine receptor protein immunostaining were prepared in the same manner as those for major basic protein. Cryosections of midlevel airways (block 3 from the left caudal lobe) from each experimental group were stained with the following primary antibodies purchased from R&D Systems, Inc., (Minneapolis, MN): biotinylated purified polyclonal goat anti-human CCL11, biotinylated purified polyclonal goat anti-human CCL24, and biotinylated purified polyclonal goat anti-human CCL26. Antibody binding was detected with streptavidin ALEXA 488 (Molecular Probes, Eugene, OR) at 1:2000 dilution or goat anti-mouse ALEXA 488 (Molecular Probes) at 1 µg/ml. Mouse anti-rhesus macaque CCR3 antibody (clone 5B9) was used as previously reported (Zhang *et al.*, 2002). All primary antibody concentrations were used at 1 µg/ml in 1% BSA in PBS. Purified MOPC 21 mouse IgG₁ (ATCC, Manassas, VA) and total goat IgG (Sigma-Aldrich®, St. Louis, Missouri) were used as a negative control antibodies. Immunofluorescent images for CCL11, CCL24, and CCL26 were visualized on an Olympus (Ballerup, Denmark) PROVIS fluorescence microscope at 60x magnification. Immunofluorescent images for CCR3 were visualized on an Olympus BX61 fluorescence microscope at 60x magnification.

CCL11, CCL24, and CCL26 ELISA

DuoSet ELISA Development Systems kits (R&D Systems, Inc., Minneapolis, MN) for the detection of human CCL11, CCL24, and CCL26 protein were used to detect the concentration of chemokine protein in lavage samples, according to the manufacturer's instructions. Lavage samples and standard curves using recombinant human CCL11, CCL24, and CCL26 were quantified with the VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA) using the Softmax® Pro Software (version 4.7.1).

Statistical analysis

All data are reported as mean ± SEM (standard error of the mean) unless indicated. Graphing and statistical analysis for all data were conducted using Prism® 5 and InStat statistical analysis software (GraphPad Software, San Diego, CA). Treatment groups and airway levels were compared using either one- or two- way ANOVA, where $p < 0.05$ was considered significant. The Tukey-Kramer Multiple Comparison Test was used for a post-test for one-way ANOVA. Pearson's R was utilized for correlation analysis.

RESULTS

Eosinophils and eotaxins in the airway lumen

At 3 months of age, monkeys were evaluated at 4–5 days following the last ozone and/or allergen exposure cycle. As shown in Figure 2A, combined ozone and HDM exposure resulted in a significant reduction in peripheral blood WBC numbers, with no effect of ozone or HDM alone in comparison with filtered air control animals. Blood eosinophil numbers were also significantly reduced at 3 months of age in the combined ozone and HDM exposure animal group in comparison with filtered air or HDM alone groups (Fig. 2B). Lavage eosinophil numbers were significantly increased in HDM, ozone, and ozone + HDM exposure groups, relative to filtered air control animals (Fig. 3). There was no effect of exposure regimen on lavage macrophage, lymphocyte, and neutrophil numbers.

To determine if expression of eotaxins may contribute to recruitment of eosinophils into the airway lumen following ozone and/or HDM exposure, we measured CCL11, CCL24, and CCL26 protein concentration in lavage fluid (Fig. 4). There was a trend towards increased CCL11 protein concentration as a result of combined ozone and HDM exposure, relative to filtered air controls, HDM alone or ozone alone ($p = 0.0611$) (Fig. 4A). CCL24 protein

concentration in lavage was increased with combined ozone and HDM exposure, relative to filtered air controls or HDM alone (Fig. 4B). CCL26 protein concentration in lavage was also increased in response to combined ozone and HDM exposure, relative to filtered air controls and ozone alone (Fig. 4C). There was a trend towards increased CCL26 protein in lavage samples from the HDM alone animal group, but this did not reach statistical significance ($p=0.0564$). There were no significant correlations between lavage protein concentration of CCL11, CCL24 or CCL26 and lavage eosinophil number within exposure groups.

Eosinophils and eotaxins in the airway mucosa

To determine if ozone and HDM exposure had a differential effect on eosinophil accumulation in the airway mucosa of infant monkeys, eosinophil volume was measured in four different conducting airway generations using stereological methods (Fig. 5). Overall, eosinophil volume was higher within the interstitium versus the epithelial compartment in all animal groups evaluated. Eosinophil distribution in airway epithelium correlated with the eosinophil distribution in airway interstitium (Pearson's $R=0.9986$, $p=0.0014$). Eosinophil volume was most abundant in airway epithelium (Fig. 5A) and interstitium (Fig. 5B) in response to HDM alone, relative to filtered air controls, ozone alone, or ozone + HDM groups. There were no significant differences in epithelial and interstitial eosinophil volume between filtered air controls and ozone-exposed groups, although there was a trend towards increased interstitial eosinophil volume in combined ozone and HDM animals as compared to filtered air controls ($p=0.06$). There was no preferential accumulation of eosinophils in large versus small airways within exposure groups.

Because ozone and HDM exposure (alone and in combination) resulted in differential anatomic compartmentalization of eosinophils in the lung, we next investigated if airway mucosal expression of eotaxins associated with exposure regimen. Cryosections obtained from midlevel airways of each of the four animal exposure groups were evaluated for CCL11, CCL24, and CCL26 protein by immunofluorescence staining (Fig. 6). Airways from filtered air control animals showed little CCL11, CCL24, or CCL26-associated immunofluorescence staining (Fig. 6b–d). CCL11 immunofluorescence staining in ozone and/or HDM animal groups was comparable to filtered air control animals (Fig. 6b, f, j, n). CCL24 immunofluorescence staining was detectable only in the ozone + HDM animal group; positive signal was primarily localized to the airway epithelium (Fig. 6o). CCL26 was highly expressed in airway epithelium in response to HDM alone (Fig. 6h), with comparatively reduced immunofluorescence staining in combination with ozone exposure (Fig. 6p). In addition to epithelial cells, we observed CCL26 immunofluorescence in association with leukocytes within the interstitium of airway mucosa from animals exposed to HDM alone (arrow in Fig. 6h).

Eotaxins and CCR3 gene expression in the airway mucosa

We next determined if CCL11, CCL24, and CCL26 mRNA expression corresponded with airway mucosa immunofluorescence staining for these chemokines. Infant monkey conducting airways were isolated and separated from parenchyma at necropsy, then evaluated for CCL11, CCL24, and CCL26 mRNA expression. As shown in Figure 7A, there were no differences in CCL11 mRNA expression for ozone and/or HDM animals. Ozone or HDM alone also had no effect on CCL24 mRNA expression within airways as compared with filtered air controls (Fig. 7b). In contrast, there was an approximate 5-fold increase in airway CCL24 mRNA expression in response to combined ozone and HDM exposure, relative to filtered air, HDM alone, or ozone alone. CCL26 mRNA expression was approximately 10-fold higher in response to HDM exposure versus filtered air controls (Fig. 7c). Exposure to ozone alone did not alter CCL26 mRNA expression compared to filtered

air controls. Ozone and HDM combined exposure showed an approximate 3-fold decrease in CCL26 mRNA expression in airway tissue as compared to HDM alone.

In parallel with lavage eotaxin concentration, we compared mucosal eotaxin mRNA levels with mucosal eosinophil volume. CCL26 mRNA expression significantly correlated with eosinophil volume in airway epithelium and interstitium for all animal exposure groups (Table 1). There was no association of CCL11 mRNA with eosinophil volume in airway mucosa, regardless of animal exposure group. Although CCL24 mRNA levels also did not correspond to eosinophil volume, it did correlate with CCL24 protein concentration in lavage (Table II). Because we observed significant CCL24 protein and mRNA exclusively in the combined ozone and HDM exposure group but did not find a corresponding association with eosinophil accumulation, we evaluated airway tissues for CCR3 mRNA expression to determine if other lung cells may be responding to this eotaxin family member. As shown in Figure 8a, CCR3 mRNA was significantly increased in combined ozone and HDM exposure as compared to filtered air controls. There was a modest, but not statistically significant increase in CCR3 mRNA for HDM alone and ozone alone groups. Immunofluorescence staining for CCR3 in midlevel airways of HDM alone animals showed positive signal associated with bilobed leukocytes (Fig. 8b). In comparison, CCR3 immunofluorescence positive cells in ozone + HDM airways are larger in size and have a morphologically distinct appearance (8d). Airway tissues were also evaluated for the chymase positive mast cell marker, CPA3; mRNA expression was detected at low levels and there was no significant effect of exposure (data not shown).

DISCUSSION

Although much has been described regarding the immunological impact of allergen exposures in the lung, little is known about the biological mechanisms for air pollutant effects on developing pulmonary and immune cells during early childhood. To directly assess how these environmental challenges can affect the lung during infancy, we conducted this study in the rhesus macaque monkey as a non-human primate model of childhood development. We have previously reported that cyclic ozone exposure alters the frequency and anatomic distribution of CD25+ lymphocytes in allergen-sensitized infant monkeys (Miller *et al.*, 2009). The purpose of this present study was to determine if eosinophils, which are an important parameter of the asthma phenotype, also exhibit differential recruitment patterns to the lung in response to ozone and allergen exposures. Using a cyclic regimen to mimic the episodic nature of air pollutant exposure, we found that inhalation of ozone, HDM, or a combination of ozone + HDM starting at 30 days of age resulted in recruitment of airway lumen eosinophils in infant monkeys (Fig. 3). In contrast, significant accumulation of eosinophils in airway mucosa was observed only with HDM exposure; ozone alone or in combination with HDM significantly reduced eosinophils in airway mucosa (Fig. 5). Concurrent evaluation of peripheral blood showed that combined exposure decreased total WBC and eosinophils (Fig. 2). By directly comparing eosinophil abundance in airway lavage and tissue with expression of eotaxin family members, we have been able to demonstrate that ozone does not uniformly function as an additive or inhibitory factor in conjunction with the inflammatory effects of allergen. In addition, by distinguishing the expression of eotaxins at specific sites within the lung, we have also found expression of CCL24 is uniquely induced by combined ozone and allergen exposure. Finally, the observed reduction in circulating WBC and eosinophils with combined exposure demonstrates that the effect of ozone is not limited to sites of ozone deposition in the lung; systemic responses were also elicited.

We have shown that HDM treatment of 2-month old rhesus monkeys (aerosol only) and 6-month old rhesus monkeys (systemically sensitized and aerosol) result in airway mucosa

eosinophilia (Miller *et al.*, 2003; Schelegle *et al.*, 2003). Findings from this current study with HDM alone (systemically sensitized and aerosol) in 3-month old rhesus monkeys are consistent with our published data on tissue eosinophils, thereby establishing that eosinophil recruitment to the airway wall is a predictable outcome of aeroallergen exposure in the very young. In 2-month old monkeys, HDM aerosol exposure resulted in the induction of CCL26 mRNA and protein expression in airway mucosa, primarily by epithelial cells and airway nerves (Chou *et al.*, 2005). Here, we found similar results in 3-month old monkeys with HDM alone, with a trend towards increased lavage CCL26 protein. These data support a prominent role for CCL26, but not CCL11 or CCL24, as a mediator of eosinophil trafficking to the airway wall in response to HDM exposure. In a study with human adult asthmatics, CCL11 and CCL24 mRNA expression in bronchial biopsies was shown to be constitutively elevated in comparison with control subjects, whereas CCL26 mRNA was enhanced at 24 hours after allergen challenge (Berkman *et al.*, 2001). This suggests that CCL26, but not CCL11 or CCL24, may be responsible for new eosinophil recruitment into the airway wall following allergen challenge. In a separate study with mild adult asthmatics, CCL24 and CCL26, but not CCL11 was found to be responsible for ongoing airway eosinophilia 48 hours after allergen challenge (Ravensberg *et al.*, 2005). These data from human subjects reinforce the notion that CCL26 plays a key role in recruitment of airway eosinophils following an allergen challenge. The lack of correlation between CCL11 and CCL24 mRNA expression with tissue eosinophilia is in contrast to rodent studies, where only double and not single knock out mice for CCL11 and CCL24 significantly decreased peribronchial eosinophilia following ovalbumin challenge (Pope *et al.*, 2005b). In light of a lack of a defined function for CCL26 in rodents (Pope *et al.*, 2005a), these disparities may be due to species and age related differences with regards to expression of chemokines within the lung.

In contrast with HDM, eosinophil accumulation in 3 month-old animals that received ozone alone or in combination with HDM was limited to the airway lumen. This suggests that chemotactic factors would be predominantly secreted into the airway lumen with ozone exposure. Lavage eosinophil numbers were not significantly greater in ozone groups, which indicates that the overall quantity of eosinophils recruited to the lung by ozone (lumen and mucosa) was likely to be less than that for HDM alone. In this study, we focused on evaluation of conducting airways for the presence of eosinophils. As yet, we do not know the anatomic (airway) site for eosinophil migration from the peripheral blood into the airway lumen, particularly in response to ozone exposure. Eosinophils may have been recruited from the distal alveolar airways, however we did not observe substantial numbers of eosinophils within lung parenchyma specimens collected at 4–5 days post allergen challenge (data not shown). Interestingly, ozone exposure does not reduce HDM-induced lung eosinophilia at 6 months of age in the infant monkey; eosinophilia was observed in both airway lumen and mucosa (Schelegle *et al.*, 2003). It is possible that attenuation of airway tissue eosinophils by ozone exposure in younger animals is the result of enhanced emigration from the epithelial compartment, however we would expect that this would result in an additive effect with HDM in lavage eosinophil numbers. The attenuating effects of ozone may also be attributed to increased granulocyte apoptosis via repression of anti-apoptotic proteins transcription (Fievez *et al.*, 2001); but we would also concurrently expect a decline in eosinophil number in lavage. Determination of the mechanisms involved in site-specific distribution of eosinophils within the airways may be critical to understanding the initiation of inflammatory airway diseases. Rodents sensitized to allergen exhibit increased emigration of eosinophils from the airway tissue into the lumen; however, in rodents with pre-existing viral infections, eosinophils tend to remain in the airway wall after allergen challenge (Sorkness *et al.*, 2007). In humans, regional variations in eosinophil distribution within the airways are exclusive to asthmatics, but not cystic fibrosis patients (Haley *et al.*, 1998).

Distinct eosinophil trafficking patterns at different stages of postnatal development may be attributed to specific expression of cytokines and chemokines that are unique to the age of the animal (Johnston *et al.*, 2006). In 3-month old monkeys, CCL11 in airways was not significantly affected by HDM or ozone alone, but there was a trend towards increased lavage protein with combined ozone and HDM exposure. Because all chemokine expression parameters were evaluated 4–5 days after ozone or allergen exposure in our studies, it is possible that CCL11 mRNA expression in lung tissue and CCL11 protein in lavage fluid returned to baseline levels at the time of analysis. Kinetic studies in mice show that CCL11 mRNA levels decrease within 12 hours of ovalbumin challenge, whereas CCL11 protein levels in lavage decrease over 48 hours (Pope *et al.*, 2005b). The limited CCL11 expression at the time point evaluated in this current study is further supported by other laboratories which establish a role for eotaxin in the recruitment of eosinophils to asthmatic airways within the first 24 hours of the late phase allergic response (Brown *et al.*, 1998). We observed that CCL26 mRNA and protein in airway tissue from HDM-exposed monkeys was reduced by ozone, but found significant concentrations of CCL26 protein in lavage from combined HDM and ozone animal groups. This may explain the discordant finding of abundant eosinophils in lavage and few eosinophils in tissue with ozone + HDM, but the cellular source for CCL26 in lavage is unknown. The finding of CCL24 mRNA and protein expression in response to combined ozone and HDM exposure is unique; to the best of our knowledge this is the first report of a chemokine that is exclusively expressed in the context of combined exposures. It is also noteworthy that significant (or near significant) levels of CCL11, CCL24 and CCL26 protein in lavage were found only in the combined ozone and HDM animal group, yet there were no differences in lavage eosinophil numbers between animal groups. Although we did not evaluate eosinophils for parameters of activation in infant monkeys, CCL11, CCL24, and CCL26 have been reported to enhance degranulation and superoxide anion generation in this cell population (Badewa *et al.*, 2002).

Airway CCL24 mRNA and protein expression was significantly enhanced only in response to combined ozone and HDM exposure, and did not correlate with the presence of tissue eosinophils, CCL11, and CCL26. As yet we do not know what the physiologic role of CCL24 is within infant airways, but functions in addition to eosinophil chemotaxis may be considered. van Wetering and colleagues have reported that *in vitro* induction of CCL24 and CCL26 via Th2 cytokines is dependent upon the state of airway epithelial cell differentiation (van Wetering *et al.*, 2007). Specifically, CCL26 was preferentially released by mucociliary differentiated cultures, whereas CCL24 was preferentially released by squamous differentiated cultures. This study lends support to the idea that the differentiated state of the epithelium, either via environmental challenge, developmental delay, or a combination of both, may dictate the type of inflammatory response mediated by the lung. All eotaxin family members signal via CCR3, have autocrine capabilities, and can alter the expression of other eotaxins in airway epithelial cells (Saito *et al.*, 2000; Abonyo *et al.*, 2005). Because CCL24 was only increased in response to combined ozone and HDM exposure, it is tempting to speculate that CCL24 may inhibit HDM-induced CCL26. However, mRNA levels and lavage protein concentration for CCL24 were considerably less than that for CCL26. Binding affinity for human CCL24 for both macaque and human CCR3 is 2–3 fold higher than that for human CCL26, therefore we cannot discount the possibility of CCL24 and CCL26 interacting at the receptor level (Zhang *et al.*, 2002). Significantly increased levels of CCR3 mRNA expression in airway mucosa from ozone + HDM animals suggests that other cell types besides eosinophils may respond to CCL24 in the mucosa. CCR3 is highly expressed on chymase positive mast cells (Romagnani *et al.*, 1999), but we found little expression of the chymase positive mast cell marker, CPA3 (Irani *et al.*, 1991; Romagnani *et al.*, 1999); this finding is consistent with previously published data indicating that ozone and allergen exposed infant rhesus monkeys have very few mast cells within intrapulmonary airways (Van Winkle *et al.*, 2010). Immunofluorescence staining in infant

airways suggests that the phenotype of CCR3⁺ cells induced by combined ozone + HDM is similar to MHC class II⁺ CCR3⁺ dendritic cells that we have previously observed in conjunction with airway nerve fiber bundles (Figure 8d)(Chou *et al.*, 2005). Future studies will include immunophenotyping to fully characterize the CCR3⁺ cell type(s) within infant airways exposed to ozone and HDM.

In conclusion, our results suggest that the postnatal period of development is a dynamic period with regards to ability of the lung to respond to environment challenges. The inflammatory mechanisms of ozone that mediate eosinophil responses during infancy are distinct from those of HDM. Data from combined exposures do not support a uniformly additive or inhibitory effect of ozone with regards to eosinophils and eotaxins. Rather, the interaction of ozone effects on the allergen-sensitized lung results in a distinct profile of eosinophil trafficking in both peripheral blood and lung.

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Highlights

- Ozone can modulate the localization of eosinophils in infant allergic airways.
- Expression of eotaxins within the lung is affected by ozone and allergen exposure.
- CCL24 induction by ozone and allergen exposure is not linked to eosinophilia.

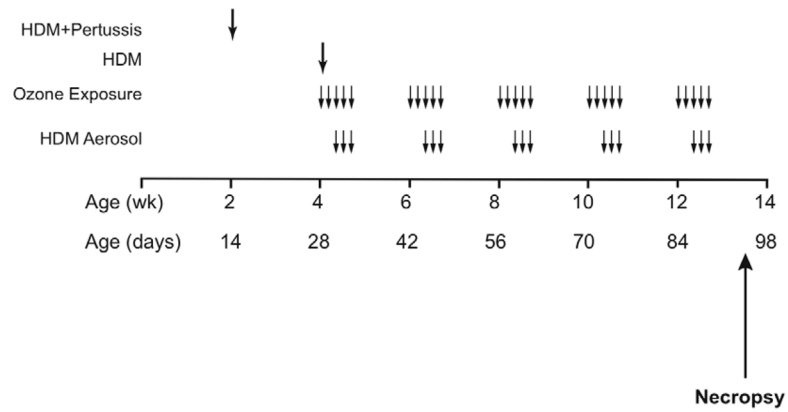


Figure 1. Experimental timeline for ozone and allergen exposure during postnatal development Infant rhesus monkeys were sensitized to HDM via subcutaneous (SQ) injection with adjuvant at day 14 and day 28. Starting at 30 days of age, monkeys were exposed to 5 cycles of ozone and/or HDM aerosol. Each 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 approximately 90 days of age.

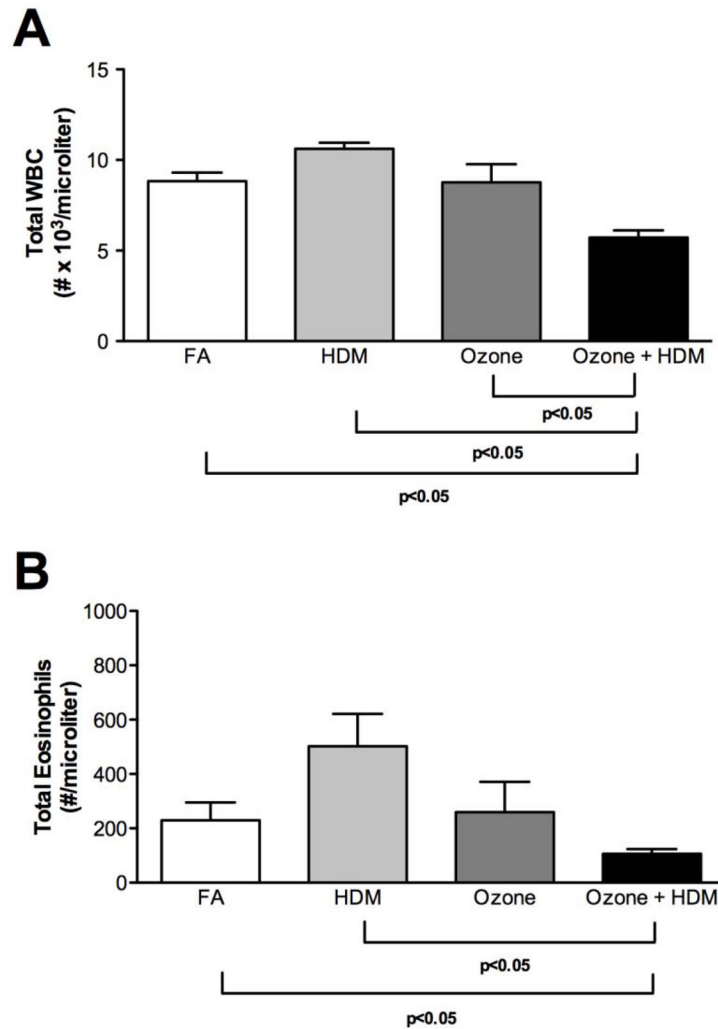


Figure 2. Effect of ozone and allergen exposure on peripheral blood WBC and eosinophil numbers at 3 months of age

Peripheral blood samples were collected from infant monkeys at necropsy, 4–5 days following the last allergen and/or ozone exposure. Samples obtained from both 4 and 5 day necropsy timepoints were pooled. Each column represents the mean \pm SE values for 6 animals, each group treated with filtered air (FA), house dust mite (HDM), ozone, or HDM + ozone as described in Materials and Methods. (A) WBC, (B) eosinophils.

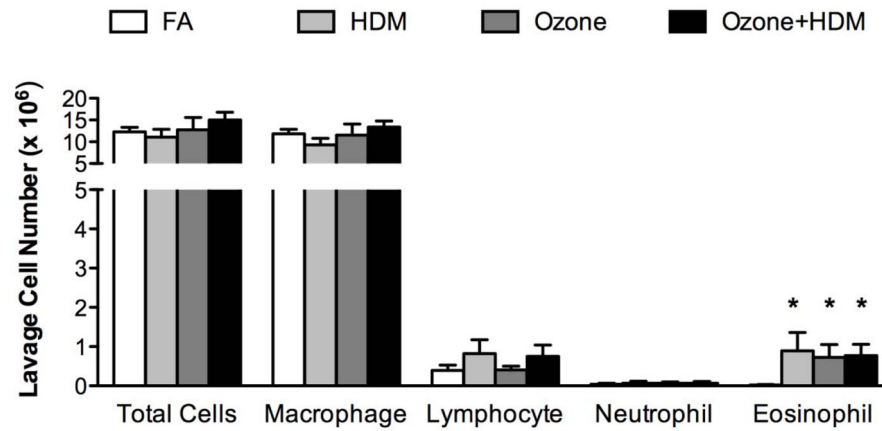


Figure 3. Effect of ozone and allergen exposure on lavage cell numbers at 3 months of age
 Lavage samples were collected from infant monkeys at necropsy, 4–5 days following the last allergen and/or ozone exposure. Samples obtained from both 4 and 5 day necropsy timepoints were pooled. Columns represent the mean \pm SE cell numbers for 6 animals, each group treated with filtered air (FA), house dust mite (HDM), ozone, or HDM + ozone as described in Materials and Methods. * $p < 0.05$ as compared with filtered air control animals.

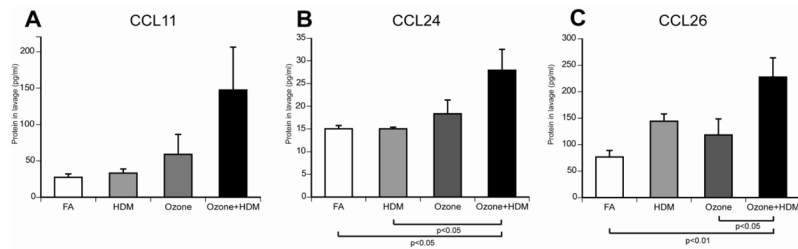


Figure 4. Effect of ozone and allergen exposure on lavage CCL11, CCL24, and CCL26 protein in infant monkeys

Lavage samples were collected as for Figure 3. Each column represents the average concentration (pg/ml) \pm SEM for CCL11 (A), CCL24 (B), and CCL26 (C) protein in lavage. n = 6 for each exposure group.

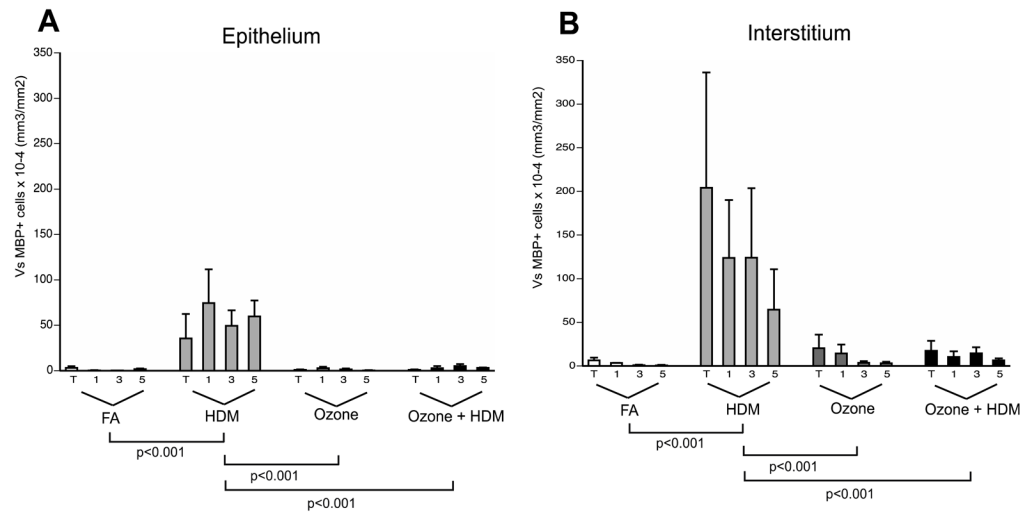


Figure 5. Effect of ozone and allergen exposure on airway mucosa eosinophils in infant monkeys
 The volume of eosinophils within the airway wall was determined by immunostaining of cryosections obtained from the left caudal lobe of each animal, tissues were collected at necropsy (3 months of age). Eosinophils were identified as immunofluorescence positive for major basic protein. Each group was treated with filtered air (FA), house dust mite (HDM), ozone, or HDM + ozone as described in Materials and Methods. Values for each column represent the average volume of eosinophils relative to the surface area of basement membrane \pm SEM. Blocks T, 1, 3, and 5 correspond to the trachea (T) and regions progressively sampled from the most proximal (1) to distal (5) airways of the lobe. For each airway generation, $n=6$ animals for each of the experimental groups. Eosinophil volume was separately determined for the airway epithelial compartment (A) and interstitial compartment (B).

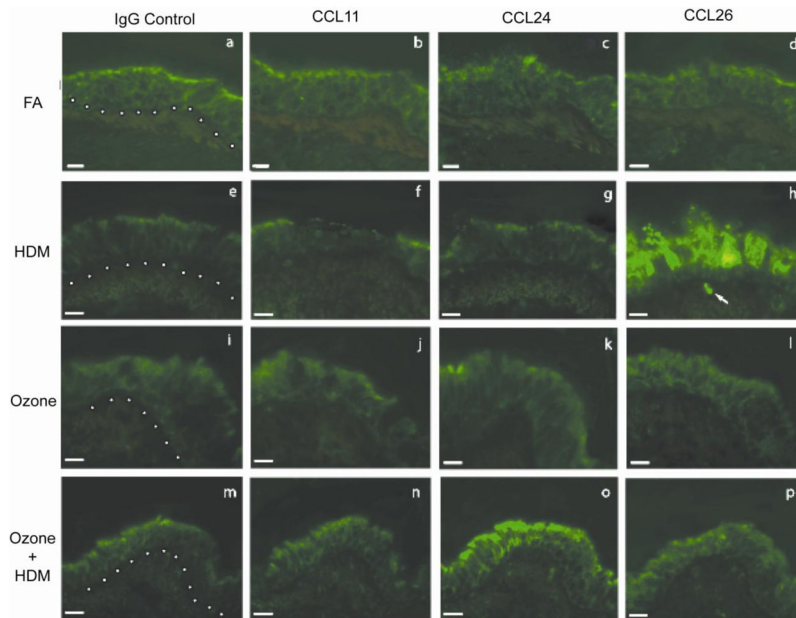


Figure 6. Effect of ozone and allergen exposure on CCL11, CCL24 and CCL26 protein expression in infant monkey airways

Immunofluorescence staining for CCL11, CCL24 and CCL26 was conducted on adjacent cryosections obtained from a midlevel intrapulmonary airway of the left caudal lobe from each animal at 3 months of age. One representative infant monkey from each FA (a–d), HDM (e–h), ozone (i–l), and ozone + HDM (m–p) exposure group is shown. Purified goat IgG was used as a negative control (a, e, i, m). Each image panel contains a portion of the airway lumen (top), airway epithelium (center), and airway interstitium (bottom). The dotted line represents the basement membrane zone in panels a, e, i and m. The arrow in (h) points to a CCL26+ cell within the airway interstitium (scale bar= 10 μ m).

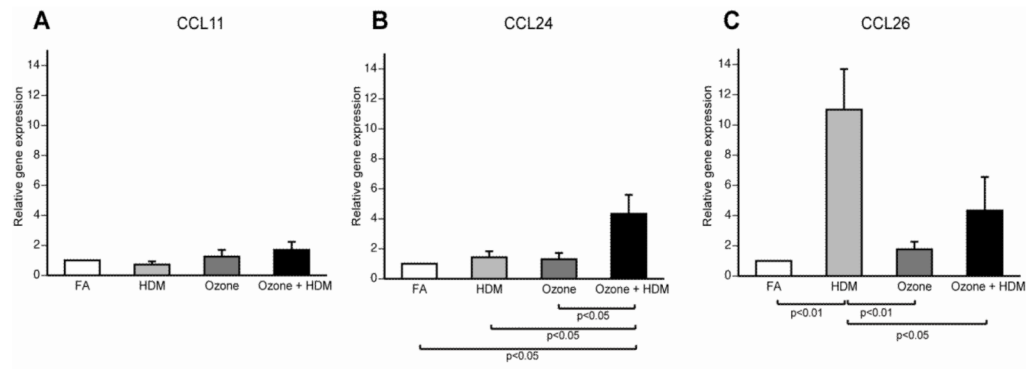
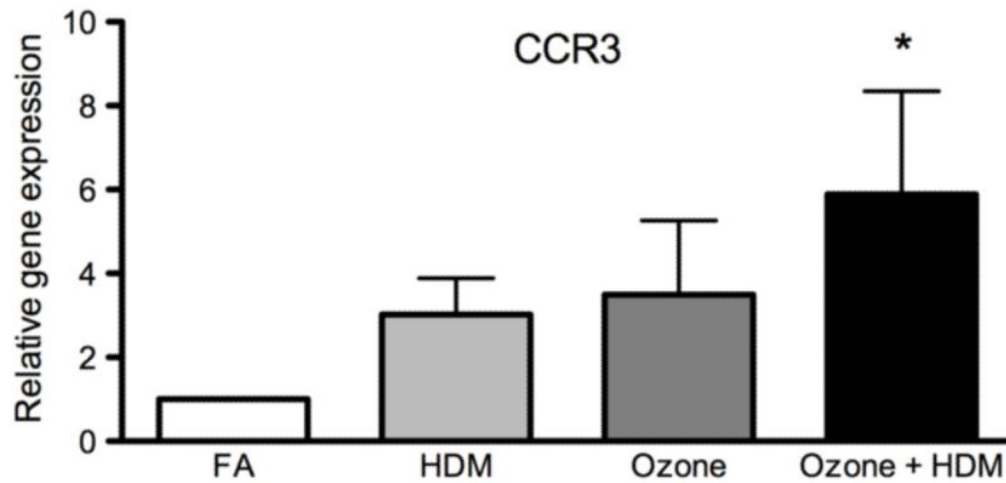


Figure 7. Effect of ozone and allergen exposure on CCL11, CCL24, and CCL26 mRNA expression in infant monkey airways

CCL11, CCL24 and CCL26 mRNA expression was determined by real-time RT-PCR analysis of microdissected conducting airways isolated from the right caudal lobe of each animal at 3 months of age. Each column represents the change in gene expression for CCL11 (A), CCL24 (B), and CCL26 (C) relative to filtered air control infant monkeys. Columns represent the mean \pm SE for 6 animals, each group treated with filtered air (FA), house dust mite (HDM), ozone, or ozone + HDM as described in Materials and Methods.



8A

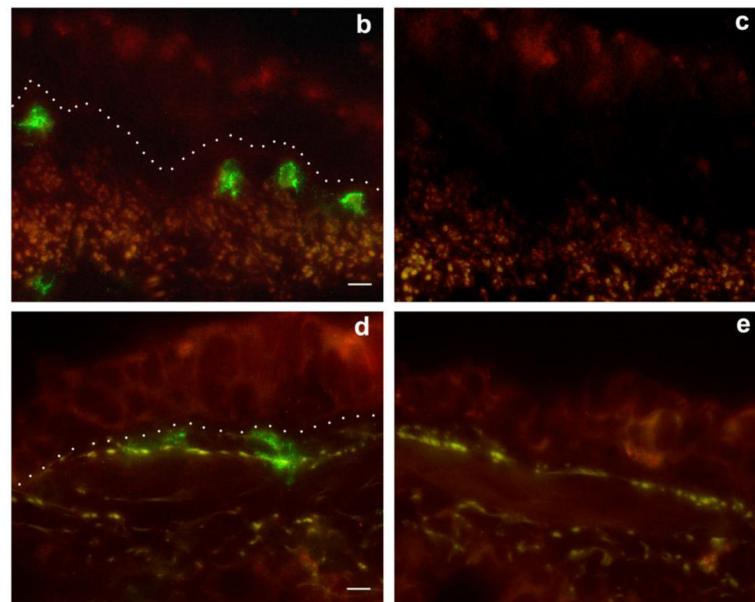


Figure 8. Effect of ozone and allergen exposure on CCR3 mRNA and protein expression in infant monkey airways

(A) CCR3 mRNA expression was determined by real-time RT-PCR analysis of microdissected conducting airways isolated from the right caudal lobe of each animal at 3 months of age. Each column represents the change in gene expression for CCR3 relative to filtered air control infant monkeys. Columns represent the mean \pm SE for 6 animals, each group treated with filtered air (FA), house dust mite (HDM), ozone, or ozone + HDM as described in Materials and Methods. (B–E) Immunofluorescence staining for CCR3 was conducted on adjacent cryosections as described for Figure 6. One representative infant monkey from HDM (b–c) and ozone + HDM (d–e) exposure group is shown. Mouse IgG₁ clone MOPC 21 was used as a negative control (c, e). The dotted line represents the basement membrane zone in panels b and d. (scale bar= 10 μ m).

Table I

Correlation Between CCL11, CCL24, and CCL26 mRNA Expression with Volume of Airway Mucosa Eosinophils.

		Pearson's R	p value
CCL11 mRNA vs.	eos(epithelium)	-0.6976	0.3024
	eos(interstitium)	- 0.6757	0.3243
CCL24 mRNA vs.	eos(epithelium)	- 0.2222	0.7778
	eos(interstitium)	- 0.2051	0.7949
CCL26 mRNA vs.	eos(epithelium)	0.9588	0.0412*
	eos(interstitium)	0.9646	0.0354*

Table II

Correlation Between Airway Mucosa mRNA with Lavage Protein for CCL11, CCL24, and CCL26.

	Pearson's R	p value
CCL11 mRNA in tissue vs. CCL11 protein in lavage	0.9234	0.0766
CCL24 mRNA in tissue vs. CCL24 protein in lavage	0.9667	0.0333*
CCL26 mRNA in tissue vs. CCL26 protein in lavage	0.3365	0.6635