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Early life allergen and air pollutant exposures alter longitudinal blood immune profiles in infant rhesus monkeys

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ABSTRACT

Early life is a critical period for the progressive establishment of immunity in response to environmental stimuli; the impact of airborne challenges on this process is not well defined. In a longitudinal fashion, we determined the effect of episodic house dust mite (HDM) aerosol and ozone inhalation, both separately and combined, on peripheral blood immune cell phenotypes and cytokine expression from 4 to 25 weeks of age in an infant rhesus monkey model of childhood development. Immune profiles in peripheral blood were compared with lung lavage at 25 weeks of age. Independent of exposure, peripheral blood cell counts fluctuated with chronologic age of animals, while IFNγ and IL-4 mRNA levels increased over time in a linear fashion. At 12 weeks of age, total WBC, lymphocyte numbers, FoxP3 mRNA and IL-12 mRNA were dramatically reduced relative to earlier time points, but increased to a steady state with age. Exposure effects were observed for monocyte numbers, as well as CCR3, FoxP3, and IL-12 mRNA levels in peripheral blood. Significant differences in cell surface marker and cytokine expression were detected following *in vitro* HDM or PMA/ionomycin stimulation of PBMC isolated from animals exposed to either HDM or ozone. Lavage revealed a mixed immune phenotype of FoxP3, IFNγ and eosinophilia in association with combined HDM plus ozone exposure, which was not observed in blood. Collectively, our findings show that airborne challenges during postnatal development elicit measureable cell and cytokine changes in peripheral blood over time, but exposure-induced immune profiles are not mirrored in the lung.

1. Introduction

The neonatal growth period in primate species is characterized by extensive maturation of both the immune system and the lung, representing a significant developmental window of susceptibility to environmental perturbation (Holt and Jones, 2000; Holt et al., 2005). Early life insults may influence individual health outcomes, a notion based upon the "Barker hypothesis," or "fetal origins of adult disease" theory, which was established from observational studies on fetal nutrient deficiencies found to be associated with adult conditions such as coronary artery disease, diabetes, and hypertension (Barker, 1997). Progressive tracking of cellular trajectories in neonates to assess the influence of inhaled environmental challenges to the immune system elicited by non-infectious antigens (allergens), toxicants, and microbes may provide critical clues on disease mechanisms that persist throughout an individual's lifetime. Understanding the chronology of immune cell development in the context of environmental perturbation could also lead to functional diagnostics at key checkpoints during neonatal maturation.

During fetal and neonatal development, the repertoire of immune cells localized within systemic and mucosal compartments of the body may be considered moveable landscapes. Even with steady progress in the understanding of immunity in the human neonate, substantial knowledge gaps remain. Human longitudinal studies have focused on cord and neonatal blood, describing the evolution of immune cell populations through the first year of life and demonstrating unique functionality that may contribute towards enhanced neonatal susceptibility to disease (Erkeller-Yuksel et al., 1992; Beck and Lam-Po-Tang, 1994; Comans-Bitter et al., 1997; Chipeta et al., 1998; de Vries et al., 2000; Gasparoni et al., 2003; Takahata et al., 2004; Hartel et al., 2005). Neonatal adaptive immunity is compromised, in part, by immature dendritic cell populations that limit establishment of robust T cell memory responses and differentiation of Th1 cell populations (Upham et al., 2006; Naderi et al., 2009). Variations in microRNA expression in neonates relative to adults also appear to play a role in age-dependent differences in T cell cytokine production and activation-dependent signaling (Weitzel et al., 2009; Palin et al., 2013). The functional transition of lymphocyte populations during infancy is thought to mediated

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by a layering process, consisting of overlapping fetal and adult cell populations originating from distinct hematopoietic precursors (Mold et al., 2010). Despite the known immune limitations in infants that increase vulnerability to infectious disease, adult-level T cell responses can develop under certain conditions, such as Bacillus Calmette–Guérin vaccination and cytomegalovirus infection (Vekemans et al., 2001; Hussey et al., 2002; Marchant et al., 2003). Collectively, cellular and molecular features of the immature neonatal immune system may contribute towards a unique response to environmental exposures.

It is not well understood how and when specific environmental exposures can alter immune cell constituents during early life in humans. We hypothesized that airborne exposures during the neonatal growth period can result in detectable alterations in the peripheral blood immune cell and cytokine profile that are dependent upon chronological age. To test this hypothesis within the context of disease development, we utilized a previously reported infant rhesus monkey model of childhood allergic airways disease, in which episodic exposure to a combination of house dust mite (HDM) aerosol and ozone produces multiple hallmark features of human asthma, including airways hyperresponsiveness, airways remodeling, and airways eosinophilia (Schelegle et al., 2001; Miller et al., 2003). The combined allergen and air pollutant model in infant monkeys was based upon multiple human epidemiologic studies demonstrating increased prevalence of childhood asthma in association with early life air pollutant exposures (recently reviewed in (Milligan et al., 2016)). In this current study, we progressively measured the impact of episodic exposure to HDM aerosol and ozone, either individually or in combination, on the peripheral blood immune cell and cytokine profile during the first 6 months of life in the rhesus monkey. We also addressed whether peripheral blood profiles elicited by HDM and/or ozone exposures were predictive of immune cell and cytokine profiles in the rhesus monkey lung at 6 months of age.

2. Materials and methods

2.1. Animals

Male rhesus macaque (*Macaca mulatta*) monkeys were housed indoors at the California National Primate Research Center under high efficiency particulate air (HEPA) filtered air conditions within three days following birth. Animals were reared under nursery conditions without dams, and were fed a standardized diet of infant formula, Purina monkey chow and supplemental produce. All animals were inoculated with diphtheria, tetanus, and acellular pertussis vaccine (Infanrix; Darby Drug Co, Westbury, NY) at 2 weeks of age to mimic childhood vaccination schedules. All animal procedures were approved by the University of California, Davis, Institutional Animal Care and Use Committee.

2.2. Exposure protocol

The study design is illustrated in Fig. 1. Starting at 4 weeks of age, twenty-four monkeys were exposed to 11 cycles of filtered air, HDM aerosol, ozone, or HDM aerosol + ozone (Table 1). For ozone animal groups, each cycle consisted of ozone exposure for 5 days (0.5 ppm at 8 h/day), followed by 9 days of filtered air. For HDM animal groups, cycle consisted of HDM aerosol on days 3–5 of each cycle, following by 9 days of filtered air (Greer Laboratories, Inc. Lenoir, NC). Animals groups not exposed to ozone remained in HEPA filtered air throughout each cycle. Details of HDM and ozone exposure methodology for this study were previously reported (Schelegle et al., 2003; Moore et al., 2012). Ozone was generated using a Sanders model 25 ozonizer (Eltze, Germany) and the concentration was monitored using a Dasibi 1003-AH ozone analyzer (Dasibi Environmental Corporation, Glendale, CA).

Table 1
Animal group treatments.

Treatment group	HDM SQ	HDM IN	HDM aerosol	Ozone
Filtered air (n = 6)	x			
HDM (n = 6)	x	x	x	
Ozone (n = 6)	x			x
HDM + ozone (n = 6)	x	x	x	x

At 2, 4, and 8 weeks of age, all animals received subcutaneous injections of 0.5 μg Der p1 and 0.25 μg Der p2 purified from *Dermatophagoides peteronyssinus* (Indoor Biotechnologies, Inc., Charlottesville, VA) in Imject aluminum hydroxide adjuvant (10 mg, Thermo Fisher Scientific, Rockford, IL). At 16 weeks of age, all animals also received 1 μg Der p1 and 0.5 μg Der p2 in Imject adjuvant. HDM aerosol animal groups were also treated with intranasal HDM at weeks 3, 5, and 9 (whole HDM extract, 49 μg per dose, Greer Laboratories, Inc. Lenoir, NC).

Blood samples were obtained at alternate cycles, with collection taking place immediately after completion of HDM aerosol on the third day (or equivalent time point for filtered air or ozone alone animal groups). Complete blood counts for whole blood samples were measured using a Beckman Coulter analyzer (Beckman Coulter Inc., Miami, FL). All animals were necropsied at 25 weeks of age, 72–96 h following the last HDM aerosol or ozone exposure. Animals were euthanized by an intravenous overdose of sodium pentobarbital.

2.3. Flow cytometry analysis

Whole blood samples were immunostained with mouse anti-human CD2 fluorescein isothiocyanate (FITC; clone RPA-2.1, Beckman Coulter), CD45 Peridinin chlorophyll protein (PerCP; clone TU116, Beckman Coulter); CD3 Allophycocyanin (APC; clone SP34, BD Biosciences, San Jose, CA) and CD19 Phycoerythrin (PE; clone J4.119, NIH NHP Reagent Resource (http://www.nhpreagents.org/NHP/default.aspx)). Four-color analysis was performed on a FACSCalibur (BD Biosciences), acquiring 30,000–50,000 events per sample and analyzed with CELLQuest software (BD Biosciences).

2.4. Quantitative RT-PCR analysis

RNA was isolated from whole blood using the RiboPure-Blood kit (Applied Biosystems, Foster city, CA). RNA was extracted from lavage

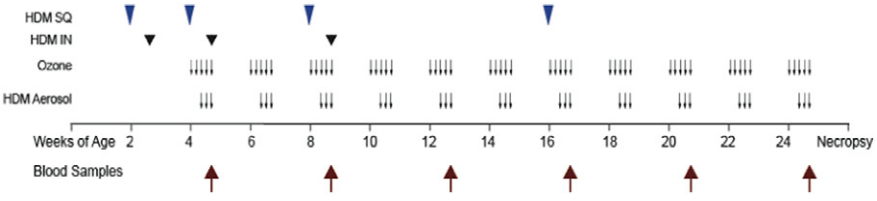

Fig. 1. House dust mite (HDM) and ozone exposure protocol. Infant monkeys received Der p1 and Der p2 antigens *via* subcutaneous (SQ) injection at 2, 4, 8, and 16 weeks of age. Intranasal (IN) HDM was administered at 3, 5, and 9 weeks of age for HDM aerosol groups only. Beginning at 4 weeks of age, animals were exposed to 11 cycles of HDM aerosol, ozone, or HDM aerosol + ozone as indicated by arrows. Animals were necropsied at 25 weeks of age, 3–4 days following the last HDM aerosol or ozone exposure. Blood collection time points are indicated below the exposure regimen.