

Immune and airway effects of house dust mite aeroallergen exposures during postnatal development of the infant rhesus monkey

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Summary

Background The effect of chronic environmental aeroallergen exposure on the immune system and airways has not been experimentally defined in very young children.

Objective The purpose of this study was to determine the immunophenotype of peripheral blood and airway leucocytes in the newborn rhesus macaque monkey, following recurrent aerosol exposure to house dust mite (HDM) (*Dermatophagoides farinae*).

Methods A regimen of HDM aerosolization was initiated for 2 h per day, three times per week, starting when rhesus macaque monkeys were 1 week of age. All monkeys were inoculated with diphtheria, tetanus, and acellular pertussis vaccine at 5 weeks of age to simulate human infant vaccination schedules.

Results Following 8 weeks of HDM aeroallergen exposure, infant monkeys exhibited a significant reduction in the total peripheral blood lymphocyte numbers and a decreased frequency of peripheral blood CD4⁺ T lymphocytes with a CD45RA[−] 'memory' immunophenotype. Lavage CD4⁺ T lymphocytes from HDM-exposed monkeys showed elevated expression of CD25, as well as an increase in CD45RA[−]/CD62L[−]/CD11a^{high} immunophenotype. Eosinophils were more abundant within airways of HDM-exposed monkeys, accumulating maximally within the trachea.

Conclusion These data demonstrate the development of immunological responses following chronic inhalation of a common environmental allergen during postnatal maturation in the non-human primate.

Keywords dermatophagoides farinae, eosinophils, immunophenotyping, infant, lung, primate, T lymphocyte

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Introduction

Although the newborn immune system is not naïve, both innate and adaptive arms undergo functional maturation throughout childhood (reviewed in [1]). In the human, newborn blood lymphocytes differ from their adult counterparts both in terms of the expression of cell surface markers and overall number within the circulation [2]. It has been known for over 65 years that the total leucocyte and lymphocyte counts in humans are highest at birth and decline in number with maturation [2–5]. A peak of blood T lymphocytes (counts and frequency) occurs at 1 week of age, followed by a peak of blood B lymphocytes at 2–3 months of age [4]. The pronounced changes in T and B lymphocyte populations during the first 2 months of life suggest that this is a significant window by which immune cell populations expand as a result of environmental stimuli. At

birth, the majority of peripheral blood T lymphocytes are CD45RA⁺ and do not express CD45RO (the low-molecular-weight isoform of CD45R); this cell population likely represents a 'naïve' or antigen-inexperienced phenotype. Because CD45RA⁺ T cells are reduced and CD45RO⁺ T cells are increased in frequency during maturation from infancy to adulthood, it has been established that an antigen-experienced or 'memory' phenotype can be defined by cell surface expression patterns for these markers. Although these findings would suggest that humans are immunologically naïve at birth, lymphoproliferation and cytokine studies have indicated that newborns come in contact with antigens during gestation, most likely from the transplacental transfer [6–12].

In parallel with the immune system, the respiratory tract at birth undergoes a period of rapid growth and differentiation that continues to 8 years of age in humans. Airways of healthy newborn infants contain a few leucocytes. It has been suggested that 'seeding' of leucocytes within the airways must be initiated following birth and exposure to environmental stimuli; it is likely that this process continues through the first 2 years of life [13]. The reference values for bronchoalveolar lavage from healthy children are generally similar to those

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reported for adults [14–17]. There are no significant changes in lavage cell profile in children between the age groups of 3–8 and 8–14 years, suggesting that the accumulation of resident immune/inflammatory cells within airways is essentially complete after the age of 3 years [16]. Available data on the immunophenotype of lavage lymphocyte subsets in normal children are limited. Ratjen et al. [18] have shown that the overall frequency of B lymphocytes, T lymphocytes, and NK cells in lavage from children (3–16 years of age) is similar to that obtained from adults, although the limited number of subjects and wide range of age groups in this study may mask significant changes that might be found in younger subjects.

Of the many airborne allergens, house dust mite (HDM) is distinguished in its prevalence, particularly in warm, humid environments such as indoor air. The presence of high concentrations of HDM has been correlated with increased sensitization and asthma in children [19, 20]. This is of particular significance for newborn infants, where restricted movement within the surrounding environment can result in chronically high aeroallergen concentrations. At present, the effect of recurrent aeroallergen inhalation on systemic and airway immune responses during postnatal maturation has not been experimentally defined in a primate species. The purpose of this study was to evaluate the early immune responses to chronic allergen aerosols by immunophenotypic assessment of peripheral blood and airway leucocytes in newborn rhesus monkeys following 8 weeks of exposure to HDM (*Dermatophagoides farinae*).

Materials and methods

Experimental protocol

Male rhesus monkeys (*Macaca mulatta*) were housed in 4.2 m³ capacity exposure chambers within the California Regional Primate Research Center, starting at 1–2 days of age. Chambers were ventilated at a rate of 30 changes per hour with filtered air. Each exposure chamber housed three animals throughout the study. Animals were selected for the study based on physical examination and negative intradermal skin test reactivity to HDM. Body weight between in control and HDM-exposed animal groups at 2 days of age was comparable (control: mean = 0.508 ± 0.027 kg; HDM: mean = 0.510 ± 0.018 kg). Intradermal skin testing was performed as previously described [21].

One-week-old animals ($n = 6$) were exposed to HDM aerosols for 2 h per day, 3 days per week (MWF), for a total of 8 weeks (Table 1). Aerosols were generated with an extract of *D. farinae* (Greer Laboratories, Lenoir, NC, USA) diluted in phosphate-buffered saline (PBS), and nebulized with a high-flow-rate nebulizer as previously described [21]. The protein concentration of the HDM aerosols consisted of 506 ± 38 µg/m³ per day ($n = 6$), a concentration comparable to that previously used to induce symptoms of allergic asthma in adult rhesus monkeys [21]. Control animals ($n = 5$) remained in filtered air for the duration of the study period. Blood samples were collected at birth and at 5, 8, and 9 weeks of age for complete blood counts (CBC) and immunophenotyping by flow cytometry (5 and 9 weeks only). Blood leucocyte values were determined by a Beckman Coulter analyzer

Table 1. HDM exposure and procedure schedule for infant monkeys

Age (weeks)	Procedure
0 (birth)	Physical evaluation, intradermal skin testing, CBC
1	HDM aerosol
2	HDM aerosol
3	HDM aerosol
4	HDM aerosol
5	HDM aerosol, DtaP inoculation, CBC, flow cytometry
6	HDM aerosol
7	HDM aerosol
8	HDM aerosol, intradermal skin testing, pulmonary function testing, CBC
9	HDM aerosol, necropsy, flow cytometry, histology

HDM, house dust mite; CBC, complete blood counts.

(Beckman Coulter Inc., Miami, FL, USA), and differential counts were obtained from blood smears. All the animals were vaccinated with diphtheria, tetanus, and acellular pertussis (DTaP; Darby Drug Company, Westbury, NY, USA) at 5 weeks of age. Because human infants in the US are routinely inoculated with DtaP vaccine at 2 months of age, animals in this study were immunized to obtain a comparable stimulation of systemic immunity. Animals were necropsied at 9 weeks of age. Lavage was obtained at necropsy by flushing the right caudal lobe with PBS. Leucocyte differentials from lavage were determined as previously described [21].

Immunophenotyping of lymphocytes by FACS

Peripheral blood mononuclear cells (PBMC) and lavage cells were collected and prepared for immunofluorescence staining as described in Schelegle et al. [21]. Cells were stained with monoclonal antibodies in a stepwise manner as described previously, using purified goat IgG (10 µg/mL; Sigma, St Louis, MO, USA) to block the non-specific binding of antibodies [22]. The reagents used for immunostaining were as follows: (1) mouse anti-human CD2 fluorescein isothiocyanate (FITC), CD4 phycoerythrin (PE), CD8 PE, CD25, and HLA-DR monoclonal antibodies (DAKO, Carpinteria, CA, USA); (2) mouse anti-human CD45RA FITC/allophycocyanin, CD20, CD11a monoclonal antibodies, and PE-Cy⁷-conjugated streptavidin (Caltag, Burlingame, CA, USA); (3) mouse anti-human CD62L (unconjugated and PE) and CD4 biotin (Becton Dickinson, San Jose, CA, USA) monoclonal antibodies; (4) PE-Cy⁵-conjugated goat F(ab')₂ anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA); and (5) mouse anti-human CD3 FITC and CD11a FITC monoclonal antibodies (Pharmingen, San Diego, CA, USA). All monoclonal antibodies used for this study have been confirmed to produce immunoprofiles by FACS analysis identical to that of human peripheral blood leucocytes (data not shown). Two- and three-colour analysis was performed on a FACScan, while four-colour analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson). In all, 30 000–50 000 events per sample were acquired and analysed with CELLQuest software (Becton

Dickinson). Lymphocyte gates for PBMC and lavage were defined by forward and side-light scatter properties.

Eosinophil quantitation

At necropsy, the left caudal lobe was inflated with a 50% v/v mixture of OCT (Sakura Finetek, Torrance, CA, USA) and PBS, sliced perpendicular to the long axis of the main intrapulmonary conducting airway into even slices, and then individually embedded for cryosectioning. The trachea was also embedded for cryosectioning. Acetone-fixed 5 µm cryosections were stained with mouse anti-human major basic protein (BIODESIGN International, Sako, ME, USA) monoclonal antibody, followed by goat anti-mouse IgG conjugated with ALEXA 488 (Molecular Probes, Eugene, OR, USA). Immunostained images were visualized on an Olympus PROVIS fluorescence microscope. At least 10 fields were randomly sampled around one airway per section. Each field contained a region of the epithelium and interstitium internal to the cartilaginous ring (if present). Four cryosections were sampled per animal, representing the most proximal portion (lobar bronchus) through the most distal portion of the intrapulmonary conducting airway (respiratory bronchiole). Point counts for the epithelium, interstitium, and positive fluorescence were determined on each field using Stereology Toolbox software (Allegient Technologies, Davis, CA, USA). The amount of basement membrane surface area relative to the volume of each compartment (epithelium vs. interstitium) was determined using the CastGrid software and sampling system (Olympus, Denmark).

Data analysis

Unless indicated, all data are reported as mean ± SE. Groups were compared using either the Student's *t*-test or two-way ANOVA.

Results

Overall health of animals

To determine the effect of aeroallergens on postnatal maturation of the immune system and airways, we evaluated newborn rhesus monkeys following 8 weeks of recurrent HDM exposures during the first 2 months of life. Throughout the 9-week study period, filtered air control and HDM experimental animal groups maintained comparable weight gain (filtered air at 65 days: 0.898 ± 0.163 kg, $n = 5$; HDM at 65 days: 0.874 ± 0.098 kg, $n = 6$). The systemic immune response to aeroallergen exposures at approximately 1 and 2 months of age was assessed from peripheral blood samples collected at the end of 5 and 8 weeks, immediately following 3 alternating days of HDM exposures. There were no significant differences in absolute white blood cell (WBC) counts between filtered air and HDM animal groups at 0, 5, and 8 weeks of age (Table 2). Peripheral blood lymphocytes increased (both percentage and absolute counts) with postnatal maturation for both animal groups ($P = 0.0001$ as compared with week 0). In comparison with control animal values, the percentage and absolute number of peripheral blood lymphocytes at weeks 5 and 8 were significantly

Table 2. Changes in peripheral blood leucocyte counts as a function of monkey age (weeks)

	Week 0	Week 5	Week 8
WBC absolute count ($\times 10^3/\text{mm}^3$)			
Filtered air	9.9 ± 2.8	9.4 ± 1.6	10.6 ± 2.5
HDM	12.7 ± 4.0	9.1 ± 1.1	9.4 ± 3.2
Lymphocyte percentage (%)			
Filtered air	31.6 ± 4.7	61.2 ± 4.2	66.8 ± 11.4
HDM*	31.3 ± 15.0	54.3 ± 5.0	50.5 ± 18.2
Lymphocyte absolute count ($\times 10^3/\text{mm}^3$)			
Filtered air	3.0 ± 0.5	5.7 ± 0.8	6.8 ± 1.0
HDM**	3.2 ± 1.0	5.0 ± 1.0	4.3 ± 1.0

WBC, white blood cell; HDM, house dust mite. Values are the mean ($\pm$ SE) results from WBC counts obtained during weeks 0, 5, and 8 of the study period. For weeks 5 and 8, blood samples were collected at the end of the weekly exposure regimen, following three HDM aerosol or filtered air treatments. * $P = 0.0586$ as compared with filtered air control animals at weeks 5 and 8. ** $P = 0.0095$ as compared with filtered air control animals at weeks 5 and 8.

reduced in HDM-exposed animals. HDM had no effect on the frequency of peripheral blood eosinophils throughout the study period (data not shown). Animals were also evaluated for immediate cutaneous hypersensitivity at week 8 of the study period. Filtered air control animals did not develop a positive intradermal reaction to HDM. One out of six animals in the HDM group developed a positive intradermal reaction to HDM.

Immunophenotype of peripheral blood T lymphocytes at 5 weeks of age

The effect of 4 weeks of HDM aerosol exposure on circulating T lymphocyte phenotypes was evaluated in 5-week-old monkeys by flow cytometry. The percentage of CD4^+ T helper and CD8^+ T cytotoxic lymphocytes within CD2^+ (T and natural killer lymphocyte) cell populations was not significantly different between filtered air control and HDM animal groups (Fig. 1a). The activation status of circulating T lymphocytes was measured by cell surface expression of CD25; HDM-exposed animals exhibited a reduced percentage of CD25^+ cells within both CD4^+ and CD8^+ T lymphocyte subsets (Fig. 1b). For both control and

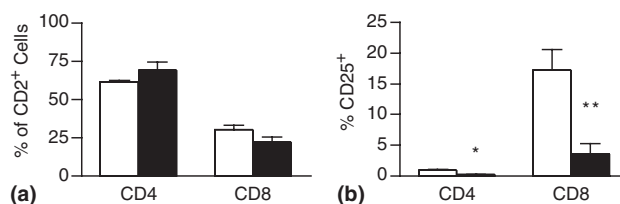


Fig. 1. Effect of house dust mite (HDM) exposure on peripheral blood T lymphocyte subsets at 5 weeks of age. Mononuclear cell isolates from peripheral blood collected at the end of week 5 of the study period were stained with monoclonal antibodies for CD2, CD4, CD8, and CD25, followed by three-colour FACS analysis. Data represent the mean $\pm$ SE percentage of fluorescence-positive cells from filtered air control (open bars) or HDM (dark bars) animal groups. (a) Distribution of CD4^+ and CD8^+ cells. Values are expressed as a percentage of CD2^+ cell subsets within peripheral blood mononuclear cells, defined by a lymphocyte gate. (b) Distribution of CD25^+ cells. Values are expressed as a percentage of either CD4^+ or CD8^+ T lymphocyte subsets. * $P < 0.0001$ as compared with control $\text{CD4}^+/\text{CD25}^+$ T lymphocytes. ** $P = 0.0043$ as compared with control $\text{CD8}^+/\text{CD25}^+$ T lymphocytes.

Table 3. Frequency of memory/naïve cell surface markers on CD4⁺ T-helper cells in response to HDM

Week/sample	Group	CD45RA-	CD45RA ⁺ /CD62L ⁺	CD45RA ⁺ /CD62L-	CD45RA-/CD62L ⁺	CD45RA-/CD62L-
Week 5 PBMC	FA	31.4 ± 2.2	53.2 ± 2.8	15.4 ± 2.5	15.4 ± 1.3	16.0 ± 2.6
	HDM	45.4 ± 8.1	49.6 ± 7.5	5.0 ± 1.0*	29.2 ± 6.5**	16.2 ± 2.5
Week 9 PBMC	FA	60.9 ± 6.2	25.7 ± 9.0	13.4 ± 5.6	36.7 ± 11.8	24.2 ± 7.3
	HDM	36.2 ± 7.4***	49.1 ± 10.5	14.7 ± 5.6	21.7 ± 6.6	14.5 ± 4.5
Week 9 Lavage	FA	94.8 ± 1.7	4.7 ± 1.4	1.6 ± 0.8	4.1 ± 0.6	89.6 ± 2.1
	HDM	96.7 ± 1.2	2.3 ± 0.3	1.6 ± 0.6	4.2 ± 1.5	92.0 ± 2.0

Data are the mean ± SE percentage of cells that are either fluorescence positive or negative for the combination of CD45RA and CD62L markers. Values are expressed as percentages of CD4⁺ T-helper cells, defined by a lymphocyte gate based on forward and side-light scatter properties.

* $P = 0.0024$ as compared with CD45RA⁺/CD62L- values for filtered air (FA) control animals at 5 weeks.

** $P = 0.0922$ as compared with CD45RA-/CD62L+ values for filtered air control animals at 5 weeks.

*** $P = 0.0345$ as compared with CD45RA- values for filtered air control animals at 9 weeks.

HDM animals, there was an approximately 18-fold increase in the frequency of CD8⁺ cells that express CD25, as compared with CD4⁺ cell populations (control = 17.84 and HDM = 18.2 for CD8 vs. CD4). Memory T helper cell populations were evaluated by cell surface expression of CD45RA and CD62L (L-selectin). The percentage of total CD45RA⁻ (predominantly memory) T helper cells was elevated in response to HDM; this was primarily due to an increase in the CD45RA⁻/CD62L⁺ cell phenotype (Table 3). The percentage of total CD62L⁻ T helper cells was significantly reduced in response to HDM exposure (control (31.4%) vs. HDM (21.2%); $P = 0.0192$); this decrease is reflected in a reduction of CD45RA⁺/CD62L⁻ T helper cells in HDM animals.

Immunophenotype of peripheral blood lymphocytes at 9 weeks of age

The immunophenotype of peripheral blood lymphocytes was further assessed in 9-week-old monkeys following 8 weeks of HDM aerosol exposure. There were no differences in the percentage of CD2⁺, CD3⁺ (T cell), and CD20⁺ (B cell) peripheral blood lymphocytes between the control and HDM animal groups (Fig. 2a). The percentage of CD3⁺ cells within peripheral blood for both control and HDM animals was nearly identical to that of CD2⁺ cells, indicating that the majority of the CD2⁺ cell population consisted of T lymphocytes and not CD3⁻ natural killer cells. As with 5-week-old animals, HDM exposure had no effect on the percentage of CD4⁺ and CD8⁺ cells within CD2⁺ populations at 9 weeks of age (Fig. 2b). The ratio of CD8⁺ vs. CD4⁺ cells that express CD25 was reduced from approximately 18-fold at 5 weeks of age to two-fold at 9 weeks (Fig. 2c; control = 2.0 and HDM = 2.2 for CD8 vs. CD4). In contrast with 5-week-old animals, the percentage of CD25⁺ cells within CD4⁺ and CD8⁺ T lymphocyte subsets was not reduced in the HDM animal group; CD25⁺ lymphocytes were slightly elevated in response to HDM exposure (Figs 1b and 2c). As control animals matured from 5 to 9 weeks of age, the percentage of total CD45RA⁻ cells within CD4⁺ populations increased approximately two-fold (Table 3; $P = 0.0021$, 5 vs. 9 weeks). The increase in CD45RA⁻ cell populations from control animals is reflected in a significant reduction in CD45RA⁺/CD62L⁺ T helper cells, a population of which 95–98% are 'naïve' ($P = 0.0188$, 5 vs. 9 weeks) [23]. In contrast to their control counterparts, no significant

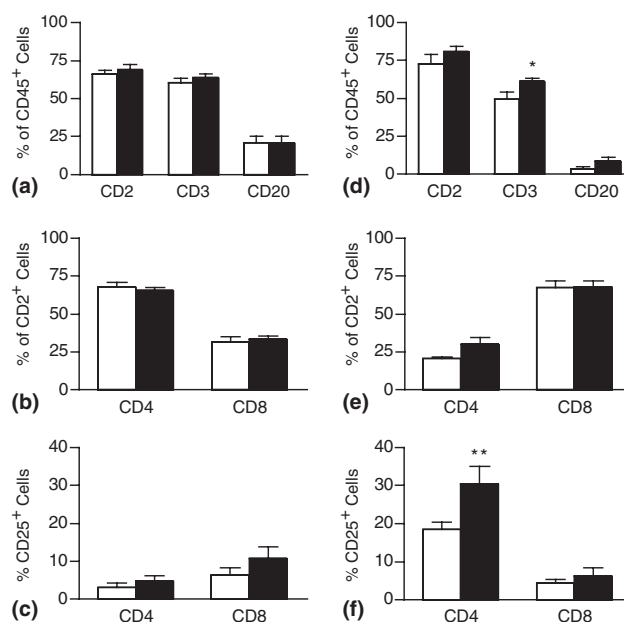


Fig. 2. Effect of house dust mite (HDM) exposure on peripheral blood and lavage T lymphocyte subsets at 9 weeks of age. Peripheral blood mononuclear cell (PBMC) isolates or lavage cells collected during week 9 of the study period were stained with monoclonal antibodies for CD45, CD2, CD3, CD20, CD4, CD8, and CD25, followed by three- or four-colour FACS. Data represent the mean ± SE percentage of fluorescence-positive cells from control (open bars) vs. HDM (dark bars) animals. (a–c) represent data from PBMC and (d–f) represent data from lavage cells. (a, d) Distribution of CD2⁺, CD3⁺, and CD20⁺ cells. Values are expressed as a percentage of CD45⁺ cell subsets within PBMC, defined by a lymphocyte gate. (b, e) Distribution of CD4⁺ and CD8⁺ cells. Values are expressed as a percentage of CD2⁺ cells subsets, defined by a lymphocyte gate. (c, f) Distribution of CD25⁺ cells with respect to CD4⁺ and CD8⁺ T lymphocyte subsets. * $P = 0.0367$ as compared with control animals. ** $P = 0.0464$ as compared with control animals.

changes in the percentage of CD45RA⁻ cells within CD4⁺ populations were observed for HDM animals at 5 vs. 9 weeks. Further, the percentage of CD45RA⁻ T helper cells from HDM animals was significantly reduced in comparison with control animals at 9 weeks.

Immunophenotype of lavage lymphocytes at 9 weeks of age

Within monkey lavage samples collected at 9 weeks of age, the percentage of CD3⁺ T lymphocytes significantly increased

in response to 8 weeks of HDM exposure (Fig. 2d). CD20⁺ cells were also slightly elevated within lavage from HDM animals ($P = 0.0969$ as compared with control animals). There were no significant differences in the percentage of CD2⁺ cells for both the animal groups. In contrast to peripheral blood at 9 weeks of age, the frequency of CD3⁺ cells within lavage for both control and HDM animals was approximately 70% of CD2⁺ cells, indicating that 30% of the CD2⁺ population consisted of CD3⁻ natural killer cells. The percentage of CD4⁺ cells within CD2⁺ lavage cell populations was elevated in response to HDM exposure ($P = 0.0614$), and the overall ratio of CD4⁺/CD8⁺ cells was inverted as compared with peripheral blood samples collected at the same time-point. The percentage of CD25⁺ cells was significantly increased within lavage CD4⁺, but not CD8⁺ lymphocyte populations (Figs 2e and f). The distribution of CD25⁺ cells within T lymphocyte subsets was inverted (CD8 vs. CD4) as compared with peripheral blood; the ratio of CD8⁺ vs. CD4⁺ cells that also express CD25 was consistently 0.2 for both the control and HDM animal groups. The majority ($\geq 90\%$) of CD4⁺ lavage cells were of a CD45RA⁻/CD62L⁻ immunophenotype for both animal groups (Table 3).

Expression of CD11a on memory T lymphocyte subsets at 9 weeks of age

To further study the effects of HDM exposure on postnatal maturation of the immune response, we assessed peripheral blood and lavage cells for the expression of CD11a, which, in conjunction with CD45RA and CD62L, serves as an additional marker for 'memory' T lymphocytes. CD11a^{high} and CD11a^{low} cells were defined by the establishment of a FACS gate based on bimodal CD11a expression within the total lymphocyte population (Fig. 3a) [24]. CD45RA⁻ peripheral blood T helper cells from 9-week-old monkeys expressed low levels of CD11a; there were no significant differences in expression between the control and HDM groups (Fig. 3b). In lavage, HDM exposure resulted in an approximate three-fold increase in the percentage of CD45RA⁻ T helper cells that expressed high levels of the CD11a (as compared with control animals). Within a smaller group of monkeys, four-colour FACS analysis for a combination of CD62L and CD11a markers revealed an

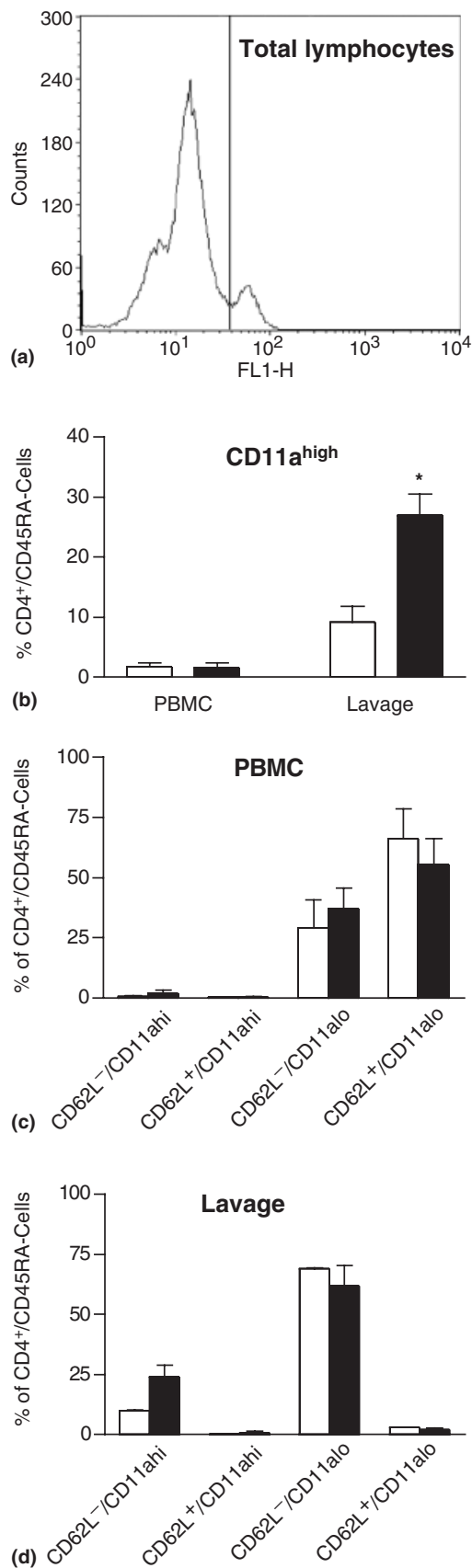


Fig. 3. Effect of house dust mite (HDM) exposure on CD11a expression by memory T lymphocyte subsets. Peripheral blood mononuclear cell (PBMC) isolates or lavage cells collected during week 9 of the study period were stained with monoclonal antibodies for CD4, CD45RA, CD62L, and CD11a, followed by three- or four-colour FACS analysis. Data represent the mean $\pm$ SE percentage of cells from control (open bars) vs. HDM (dark bars) animals. (a) Discrimination of CD11a fluorescence high or low cells using bimodal histogram analysis of total lymphocytes. CD11a^{high} or CD11a^{low} populations are distinguished by a gate set to the histogram minimum between two peaks (black line). One representative PBMC isolate is shown. (b) Frequency of CD4⁺/CD45RA⁻ cell populations from PBMC or lavage cells that are fluorescence high for CD11a (control: $n = 5$; HDM: $n = 6$). (c) Distribution of CD4⁺/CD45RA⁻ cell populations from PBMC that are stained for a combination of CD62L (fluorescence positive or negative) and CD11a (fluorescence high or low) markers (control: $n = 2$; HDM: $n = 3$). (d) Distribution of CD4⁺/CD45RA⁻ cell populations from lavage that are stained for a combination of CD62L (fluorescence positive or negative) and CD11a (fluorescence high or low) markers (control: $n = 2$; HDM: $n = 3$). * $P = 0.0039$ as compared with control values.

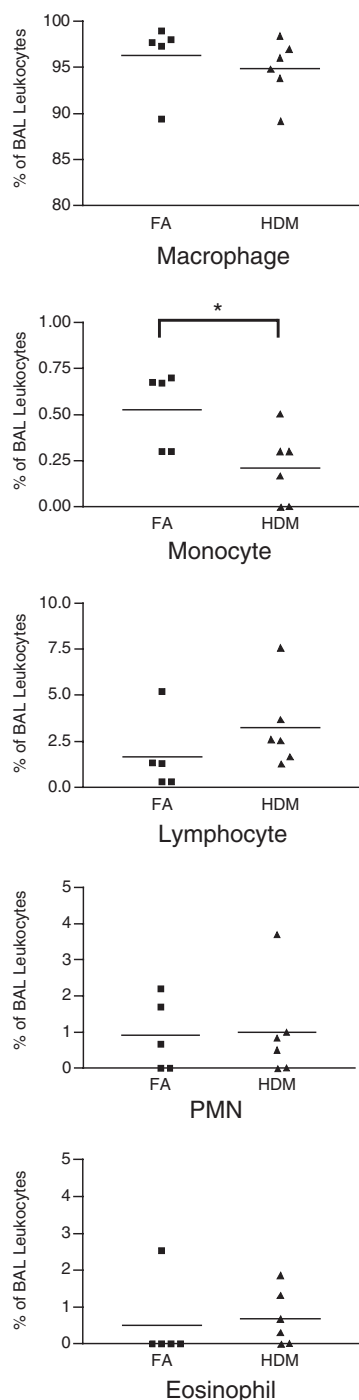


Fig. 4. Effect of house dust mite (HDM) exposure on lavage leucocyte differentials at 9 weeks of age. Lavage samples were collected during week 9 of the study period. Data points represent individual values obtained from filtered air control (FA; squares) vs. HDM experimental (triangles) animals for percentage of the leucocyte indicated. Horizontal bars represent the mean value for each set of counts. * $P = 0.0291$ as compared with control animals.

approximate two-fold greater frequency of peripheral blood $CD45RA^-$ T helper cell populations that were $CD62L^+/CD11a^{low}$ vs. $CD62L^-/CD11a^{low}$ for both the control and HDM groups (Fig. 3c). In lavage, four-colour FACS analysis indicated that $>60\%$ of $CD45RA^-$ T helper cells in lavage

from both the control and HDM animal groups were $CD62L^-/CD11a^{low}$; $CD45RA^-/CD11a^{high}$ T helper cells were predominantly $CD62L^-$ and a minority population of T helper cells within airways (Fig. 3d).

Quantitation of eosinophils within airway compartments

By differential staining of monkey lavage collected at 9 weeks of age, airway eosinophils were identified from 4/6 HDM (67%) vs. 1/5 control (20%) animals (Fig. 4). Only monocytes were significantly affected by HDM exposure, reducing in population by over 50%.

In addition to lavage, lung eosinophils within different airway generations of 9-week-old monkeys were quantitated by morphometric analysis. The volume of tissue eosinophils (defined as cells that expressed major basic protein) was determined for both epithelial and interstitial compartments, comparing proximal vs. distal airways. Overall, HDM exposure resulted in a significantly greater abundance of eosinophils within both epithelial and interstitial compartments, as compared with filtered air control animals (Figs 5a and b). An airway generation (block)-dependent effect for the volume of eosinophils was also observed ($P = 0.0004$), accumulating at maximal volume within the most proximal portion of the airway tree sampled (trachea). With respect to localization of cells within individual airway levels, the interstitial compartment held a greater volume of eosinophils, as compared with the epithelium.

Discussion

In the current study, we found that recurrent aerosol exposures to HDM during the first 9 weeks of life resulted in the development of immunological changes within both the peripheral blood and the airways. To the best of our knowledge, the effects of environmental aeroallergen exposures during this window of immune and pulmonary system maturation have not been evaluated in a primate species.

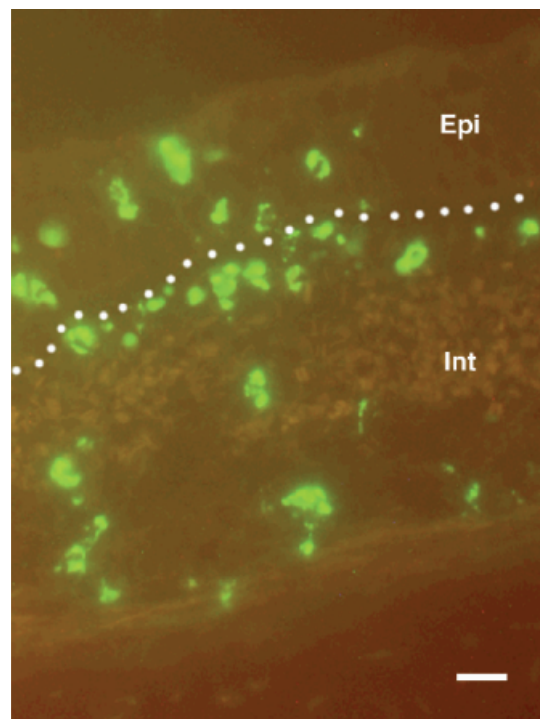
The overall health of animals following 8 weeks of HDM aerosol exposure was good, as evidenced by weight gain and WBC counts, which were comparable to controls. Control animal values for total WBC and lymphocyte counts are consistent with those reported by de Vries et al. [4] and DeMaria et al. [25], and correlate well with a recent longitudinal survey of human infants, showing a dramatic rise in circulating lymphocyte numbers from cord blood through 6 weeks of age. Following infancy, both humans and rhesus monkeys normally exhibit a progressive decline in peripheral blood lymphocyte numbers with an increase in age [25]. An increase in circulating lymphocytes during the first 2 months of life suggests that this window of neonatal development is a period of rapid growth and maturation for the immune system. In comparison with the values obtained at 1 week of age, the frequency and absolute numbers of peripheral blood lymphocytes were elevated in both 5- and 8-week-old HDM-exposed monkeys. However, in comparison with their age-matched control counterparts, peripheral blood lymphocytes were significantly reduced. Further, peripheral blood lymphocyte values appeared to decline past 5 weeks of age. The mechanism of this

phenomenon is unclear; a decline in circulating lymphocytes may be a result of recruitment to other organs. We did observe an increase in CD3⁺ T lymphocytes within lavage

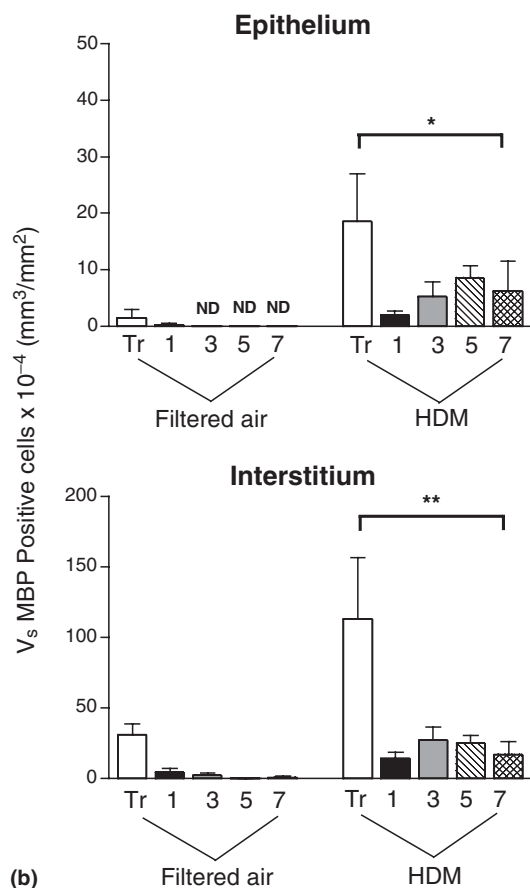
from HDM-exposed animals, suggesting recent local recruitment (and therefore depletion) from blood as a result of allergen inhalation. Alternatively, accelerated maturation of the immune system (and therefore a normal progressive decline) may also explain these findings.

Cell surface expression of CD2 defines a leucocyte population that consists of T lymphocytes and some natural killer cells; in peripheral blood from 9-week-old monkeys, nearly all CD2⁺ cells were CD3⁺ T lymphocytes. In lavage from 9-week-old monkeys, the frequency of CD3⁺ cells was approximately 70% of CD2⁺ cells, suggesting that CD2⁺ natural killer cells also contribute to the lymphocyte profile of infant airways. This relationship is reversed in adults, whereby the majority of CD2⁺ cells in lavage are also CD3⁺, and the frequency of peripheral blood CD3⁺ cells within CD2⁺ populations is approximately 80% [26]. Another distinction of monkey lavage lymphocyte populations at infancy that was observed in this study is the apparent inverse relationship of CD4⁺/CD8⁺ ratios as compared with peripheral blood. A similar finding has been reported for human children 3 months to 10 years of age, showing a CD4/CD8 ratio of 0.6 [14]. Data from several studies indicate that the CD4/CD8 ratio for human adult lavage is similar to that found in peripheral blood, with a range of 1.6–2.7 [26–28].

The frequency of CD2⁺, CD3⁺, or CD20⁺ peripheral blood lymphocyte populations did not substantially change in response to HDM aerosol exposures, both at 5 and 9 weeks of age. The percentage of CD3⁺ lavage lymphocytes was significantly increased in 9-week-old HDM monkeys, suggesting enhanced airway recruitment of T lymphocytes in response to aeroallergen exposure. At 5 weeks of age, there was a greater frequency of peripheral blood CD8⁺ T lymphocytes that expressed CD25 in comparison with CD4⁺ T lymphocytes, particularly within the control animal group. This relationship appears to reverse itself with maturity, as juvenile (1–3 years of age) rhesus monkeys exhibit a greater frequency of CD25⁺ cells within the CD4⁺ T lymphocyte population in comparison with CD8⁺ T lymphocytes (data not shown). A reduction in the frequency of peripheral blood CD25⁺-activated lymphocytes was observed for both CD4⁺ and CD8⁺ T cell subsets at 5 weeks of age in HDM animals. CD25 expression on peripheral blood lymphocytes for both children and adults with allergic airways disease is typically increased [29–31]; however, it has also been reported that cord blood from infants at high risk for developing allergy (born to at least one atopic parent)



(a)



(b)

Fig. 5. Effect of house dust mite (HDM) exposure on the abundance and distribution of eosinophils within airway compartments at 9 weeks of age. Eosinophils within the trachea and left caudal lobe sections were quantitated by immunofluorescence staining for major basic protein (MBP). (a) Immunostaining for MBP (green fluorescence) in a cryosection of trachea obtained from an HDM-exposed infant monkey. The epithelial (Epi) and interstitial (Int) compartments are demarcated by a dotted line. Bar = 20 μ m. (b) Quantitation of MBP within trachea and left caudal lobe sections obtained from control (open bars) or HDM (dark bars) animals. Blocks Tr, 1, 3, 5, and 7 correspond to the trachea (Tr) and regions from the most proximal (1) to distal (7) airways of the lobe. Values for each column represent the average volume $\pm$ SE of cells that are fluorescence positive for MBP within the epithelium (top graph) or interstitium (bottom graph) with respect to the surface area of basal lamina. * P = 0.0012 as compared with control animals. ** P = 0.0017 as compared with control animals. ND = none detected.

has significantly lower numbers of CD25⁺ T lymphocytes [32]. The reduction in circulating CD25⁺ T lymphocytes within HDM animals apparently resolves itself at 9 weeks of age. Elevated numbers and frequency of CD25⁺ lymphocytes within the airways is a consistent finding in allergic airways disease, and is indicative of recent antigenic stimulation [30, 31]. Our finding of increased frequency for CD25⁺ T helper cells within lavage from HDM-exposed infant monkeys suggests that the postnatal mucosal immune system of the lung is capable of specifically responding to antigenic stimulation via HDM inhalation.

In addition to lymphocyte activation, the effect of recurrent HDM aerosol exposures on the phenotype of CD4⁺ 'memory' cell populations was evaluated in monkeys at 9 weeks of age. Eight weeks of HDM exposure resulted in a decline in peripheral blood CD45RA⁻ 'memory' T helper cell populations. Within both the control and HDM animal groups, we found that the majority of CD45RA⁻ peripheral blood T helper cell populations were CD11a^{low}, consisting primarily of lymphocytes that were CD62L⁺. The CD45RA⁻/CD62L⁺/CD11a^{low} T helper cell population produces primarily IL-4, representing a Th2 phenotype; this cytokine profile is consistent with that observed in human infant peripheral blood [10, 24]. The finding of predominantly CD45RA⁻/CD62L⁻ T helper cells within airway lavage suggests that this subpopulation is preferentially recruited to the lung. The CD62L⁻ immunophenotype for memory T helper cells has also been reported for adult human lavage lymphocyte populations [33]. Although the frequency of CD45RA⁻/CD62L⁻ peripheral blood T helper cells was not significantly affected by HDM exposures, a slight depletion of this phenotype from the circulation may not be detectable. Alternatively, the predominant CD62L⁻ expression profile for lavage lymphocytes may be due to recent shedding of L-selectin from CD62L⁺ cells recruited into the airways. In addition to the CD62L⁻ phenotype, CD45RA⁻ T helper cells within lavage were primarily CD11a^{low}. Yet, in the minority CD11a^{high} population, HDM exposure increased the frequency of this cell phenotype almost three-fold. These findings might suggest that the small CD45RA⁻/CD62L⁻/CD11a^{high} T helper population is responding directly to antigenic stimulation via HDM inhalation. Alternatively, because this population was also observed in control animals (albeit at a reduced frequency), it may represent a more differentiated or 'mature' form of the memory T helper cell phenotype within postnatal airways. It is interesting to note that CD45RA⁻/CD62L⁻/CD11a^{high} T helper cells make the most IFN- γ and are considered the most Th1-like of all T helper subsets [24].

Airway eosinophilia is an important criterion in the diagnosis of allergy and asthma. In this study, we quantitated eosinophils from five airway generations of 9-week-old monkeys, using major basic protein as a unique marker for this leucocyte phenotype. To assess airway recruitment, we also evaluated lavage from 9-week-old monkeys for eosinophil frequency. Within airway walls of filtered air control monkeys, eosinophils were rare, and often not detected in the epithelial compartment. Consistent with this finding, eosinophils were detected in lavage from one monkey out of five in the control animal group. In HDM-exposed monkeys, the volume of eosinophils within both the epithelial and

interstitial compartments of the lung was significantly increased. The analytical approach used for this study also demonstrated that eosinophils are differentially distributed throughout the large (proximal) and small (distal) airways, preferentially accruing within the trachea in response to HDM. Although eosinophils were detected within four monkeys out of six in the HDM animal group, the overall frequency of lavage eosinophils did not reach statistical significance; this may be attributed to a limited capacity for eosinophil trafficking into the airway lumen. The finding of eosinophilia within airways of very young animals is supported by a recent study indicating that human infants with a familial history of atopy exhibit elevated levels of eosinophil cationic protein and eosinophil protein X in nasal lavages as early as 4 weeks of age [34].

It has been postulated that the development of allergy and asthma is preceded by allergen exposure during early childhood, initiating a progressive course of immune sensitization [35–38]. Of key interest are the mechanisms through which sensitization occurs during infancy, as this is a period of dynamic growth and differentiation for both the pulmonary and immune systems. Several studies have shown that human exposure to high concentrations of aeroallergens during infancy is associated with the onset of allergic sensitization and asthma later in childhood [39–41]. Given this, the identification of key indicators during infancy that are predictive for the future development of airway disease is of particular importance.

In conclusion, these findings show that chronic inhalation of environmental aeroallergens during postnatal maturation of the pulmonary and immune systems can result in the development of immune responses, including airways eosinophilia, which are suggestive of early steps towards allergic sensitization in the infant lung. Longitudinal studies to follow should indicate if recurrent aerosol exposure to high concentrations of HDM during this sensitive period of lung and immune system development can stimulate progression to airway disease.

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