

Modeling asthma in macaques: longitudinal changes in cellular and molecular markers

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Abstract

Question: Does systemic sensitization and chronic aeroallergen challenge in macaques replicate classical and emerging immunology and molecular pathology of human asthma?

Methods: Macaques were immunized and periodically challenged over 2 years with house dust mite allergen (HDM). At key times, serum, bronchoalveolar lavage (BAL) and bronchial biopsies were assayed for genes, proteins, and lymphocyte subpopulations relevant to clinical asthma.

Results: Immunization and periodic airway challenge induced changes in IgE, airway physiology, and eosinophilia consistent with chronic, dual phase asthma. Immunization increased IL-1 β and IL-6 in serum, and IL-13 expression in BAL. Airway challenge increased early expression of IL-5, -6, -13, -19, and eotaxin; and variable late phase expression of IL-4, -5, -13 and TARC. CD4⁺ lymphocytes comprised 30% of the CD3⁺ cells in BAL, increasing to 50% in the late phase. NKT cells represented less than 2% of the CD3⁺ cells. Corticosteroid treatment reduced serum histamine levels, percentage of CD4⁺ cells, and MDC expression; while increasing CD3⁺ and CD8⁺ cells in BAL.

Conclusion: Sensitization and periodic aeroallergen challenge of cynomolgus macaques results in physiological, cellular, molecular and protein phenotypes and therapeutic responses observed in human asthma, providing a model system useful in target and biomarker discovery and translational asthma research.

Key words: *asthma, primate model, NKT lymphocyte, corticosteroid treatment*

Introduction

Asthma remains a significant unmet medical need¹ and the search for effective asthma drugs with novel mechanisms of action continues. Non-human primate models most closely replicate the genetics, physiology, immunology and pathology of the human disease.²⁻⁴ Availability of these models uniquely allows examination of both induction of chronic allergy-driven airway inflammation and bronchoconstriction in a controlled setting, as well as identification of key mediators for the maintenance of chronic asthma. Additionally, these models provide a platform to assess preclinical safety and proof of activity/efficacy for novel asthma therapeutics under conditions that are observed in human patients.

Models of asthma have been developed in a variety of common laboratory animals including guinea pigs, rabbits, rats, mice, sheep, cats, dogs, and macaques (See review by Bice and colleagues).⁵ However, there are significant differences in airway architecture and immune responses to chronic allergen between species. Few species develop spontaneous allergic sensitivity to allergens, chronic/prolonged immune and inflammatory responses following pulmonary allergen exposure, progressive worsening of airway responses with repeated antigen challenge, and significant accumulation of lymphoid cells outside of bronchial-associated lymphoid tissue (BALT). Murine models have been instrumental in elucidating and dissecting pathways involved in immune responses, but translation of findings in the mouse to development of therapeutics in humans has been largely unsuccessful. Nonhuman primates are more similar to humans in their genetics, anatomy, development, and responses to pulmonary allergen challenge than other species. The animals are long lived, and can manifest symptoms of relapsing/remitting allergic asthma for years^{2,3,13}, thereby providing a penultimate model for

assessing safety and efficacy of novel therapeutics before clinical trials, as well as dissociating pathogenic factors involved in the induction and maintenance phases of the disease.

Previous reports have described findings in monkeys sensitive to *Ascaris suum*^{2, 6-9}. A small number of primate studies have used clinically meaningful aeroallergens, such as ragweed¹⁰ or house dust mite (HDM), delivered in aerosol form.¹¹⁻¹³ Plopper and colleagues have addressed validity of these models at anatomical and cellular levels,³ and Zou and colleagues documented changes in gene expression by micro-array profiling following acute challenge with allergen.¹⁴ While these studies have provided information on differential gene and protein expression before and after acute exacerbations (i.e., early and late phase responses), asthma is a chronic disease expressed over a lifetime, and little has been reported on longitudinal changes in gene and protein expression over longer periods of time under well-controlled conditions. The present study defines the chronic effects of HDM exposure on immune and inflammatory parameters relevant to human asthma. Starting with the HDM-naïve state, a small group of Cynomolgus macaques were observed longitudinally through systemic immunization with major mite antigens, *D. pteronyssinus* (*Dp*) and *D. farinae* (*Df*), followed by 12 months of periodic inhalation of HDM allergen. Effects of corticosteroid treatment were then defined in a larger group of HDM sensitive animals that had been periodically challenged with aerosolized HDM over 18 months. Whole blood, bronchoalveolar lavage, and bronchial biopsies were obtained and assayed broadly across multiple analytic techniques – including examination of novel pro-inflammatory chemokine and cytokine ligands/receptors, as well as lymphocyte subsets isolated from the airways following allergen challenge. Highly sensitive real-time RT qPCR methods and multiplex protein analysis identified differentially expressed, canonical and novel mediators of Th2 responses and lung pathology. CD3⁺, CD4⁺, CD8⁺, and NKT cells were enumerated during

early and late phases of the allergic response (EAR and LAR), and following corticosteroid treatment.

METHODS

Cynomolgus fascicularis, 30 to 42 months of age, were purchased from Alphagenesis, Yemassee, SC. Animal husbandry was conducted under USDA guidelines. Ten animals were sensitized to HDM allergen as described below, and 5 additional animals served as naïve controls. All protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University.

Study Design

The protocol consisted of an exploration phase in which a small number of allergic and HDM-naïve animals were studied to identify traditional and novel biomarkers of asthma, and a validation phase in which observations made during the exploration phase were confirmed in a larger number of allergic animals (Figure 1). Animals were sensitized to HDM allergen over a 7.5 month period by subcutaneous injections of allergen adsorbed to Alum [312 AU of Dp extract, Greer Laboratories, Lenoir, NC; Imject Alum, Pierce, Rockford, IL] administered at 2 week intervals until HDM-specific IgE titers approached the level of allergic control serum, and then at 4 week intervals until the first aeroallergen challenge. Following sensitization, the animals were challenged with nebulized HDM (1 to 2500 AU/mL Dp+Df x 4 min) at a concentration that induced 100% increase in lung resistance (R_L), 40% decrease in dynamic compliance (C_{dyn}), or decline in SpO_2 to $\leq 70\%$. Airway inflammation and responses to

nebulized histamine and methacholine 24 hours after allergen challenge were measured periodically to assess development of LAR.

Exploration Phase: Proteogenomic analyses were conducted before and after induction of the asthmatic phenotype in 3 HDM-sensitized animals (2 female, 1 male) exhibiting high HDM-specific IgE titers. Samples from 5 non-allergic animals (all female) were evaluated in parallel. As discussed in detail in the Results section, one of the non-allergic control animals, DL8A, was found to be intrinsically sensitive to HDM and was later classified and studied as a passively sensitized subject. Blood, serum, bronchoalveolar lavage, and bronchoscopic biopsy samples were analyzed to assess traditional and novel asthma disease biomarkers.

Validation Phase: Seven animals (2 female, 5 male) exhibiting high HDM-specific IgE titers were chronically challenged with nebulized HDM over 17 months. During the last months, the animals were challenged at 4 week intervals, and every 8 weeks - 24 hours after allergen aerosol administration - the animals were challenged with Mch and the lungs lavaged. Following the last LAR sample collection, the animals were treated with methylprednisolone sodium acetate (Depo-Medrol, 4.5 mg/kg i.m.; 1/week x 2 weeks) followed by methylprednisolone sodium succinate (Solu-Medrol, 10 mg/kg i.v.). One hour later, the animals were challenged with HDM, and EAR and LAR were assessed.

Pulmonary function testing

Subjects were anesthetized with telazol (2.5 mg/kg i.m.) and propofol (10 to 15 µg/kg/hr i.v.). An endotracheal tube and esophageal balloon were inserted. C_{dyn} and R_L were measured by standard computer analysis of the flow and pressure signals (MuMed PR800, MuMed, London, England). Baseline parameters were recorded, saline was delivered via a Devilbiss

ultrasonic nebulizer for 4 min prior to HDM provocation and for 2 minutes prior to histamine and methacholine provocation, the endotracheal tube was aspirated, and pulmonary function was monitored for one minute.

Increasing concentrations of HDM allergen (1, 10, 100, 500, and 2500 AU/mL) were delivered at 5 minute intervals until a 100% increase in R_L was observed. Pulmonary function was then monitored at 5 minute intervals for 15 minutes. Bronchoconstriction was reversed with aerosolized albuterol (0.083% for 4 minutes). SpO_2 , blood pressure, and heart rate were monitored continuously. Supplemental O_2 was administered as need to maintain SpO_2 above 70%. Provocation was discontinued if SpO_2 , blood pressure, or heart rate were not maintained.

To assess AHR, histamine or methacholine were nebulized for 2 minutes in doubling doses between 0.01 and 10.24 mg/ml. The provocative concentrations that induced a 100% increase in R_L ($PC_{100} R_L$) and 40% decrease in C_{dyn} ($PC_{40} C_{dyn}$) were determined for each animal.

Endotracheal bronchoalveolar lavage (BAL) and bronchial biopsy was performed on anesthetized and intubated subjects (*see Online Supplemental Materials and Methods*).

Immunoassays

HDM-specific IgE was measured as previously reported.¹⁵ Briefly, HDM-specific IgE levels in serum were reported as a percent of the levels in historical allergic and naïve control serum samples. IgE levels in the control serum samples were quantified using a commercially available kit (Kit # 7070, Alpha Diagnostics International, San Antonio, TX). The HDM positive and HDM negative control serum had similar levels of total IgE (87 U/ml and 114 U/ml, respectively, with the units being calibrated to the International Units for human IgE as established by the World Health Organization). The amount of HDM-specific IgE in the

control serum was quantified by repeating the assay on HDM-coated, serum blocked plates using the Alpha Diagnostics reagents. HDM-specific IgE in the allergic control serum was 13 U/ml compared to a non-detectable level in naïve control serum (i.e. no difference from background in uncoated wells).

Serum and BALF histamine levels were determined using the Histamine ELISA Kit from MP Biomedicals, LLC (Santa Ana, CA). IL-1 β , IL-4, IL-5, IL-6, IL-13 and IL-8 were measured by Luminex Multiplex Kit (Millipore Corporation, Billerica, MA). Eotaxin and MDC were determined by an electrochemiluminescence biosensor assay [Meso Scale Discovery (MSD); Gaithersburg, MD].

Gene expression analysis

Total RNA was isolated from whole blood using the PAXgene System (PreAnalytiX, Valencia, CA) and from bronchoalveolar lavage (BAL) cells using the RNeasy method (Qiagen, Valencia, CA), according to the manufacturers' protocols. Total RNA was isolated from lung biopsy tissue by the VersageneTM RNA purification system (Gentra Systems, Minneapolis, MN).

Total RNA from blood and BAL cells was reverse-transcribed using Quantitect^R reverse transcription method (Qiagen, Valencia, CA). Total RNA from lung biopsy tissue was reverse-transcribed using WT-OvationTM Pico System (NuGen Technologies, San Carlos CA). Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA) or obtained commercially from Applied Biosystems (ABI). Real-time quantitative PCR was performed on 10 ng of cDNA from each sample using either of two methods as previously reported.¹⁶ Human primers for 153 different genes were used. Cross-reactivity of primer sets to non-human primate

samples was tested on archived samples and was deemed acceptable for comparing between samples, not for absolute quantitative measurements.

Flow cytometric analysis

BAL cells were enumerated, checked for viability (Vi-Cell Cell Viability Analyzer, Beckman Coulter, Fullerton, CA), blocked with human IgG (Sigma, St Louis, MO) and normal mouse serum (Jackson Immuno-Research Laboratories, West Grove, PA), and stained with fluorophore-conjugated monoclonal antibodies against CD3, CD4, CD8 and CD11b (BD Biosciences, San Jose, CA). NKT cells were defined by staining with dimeric human CD1d-Ig (BD Biosciences, San Jose, CA) loaded with the glycolipid α -galactosyl ceramide (α -GalCer).¹⁷ Propidium iodide was added at a concentration of 2 μ g/ml to each cell sample 10 min prior to flow cytometry to distinguish live from dead cells (*Online Supplement Figure E1: Representative FACS Plots*). Data was acquired using BD FACS Canto II flow cytometer equipped with FACS DiVa acquisition software for FACS Canto II, version 6.0 (Becton Dickinson, San Jose, CA). A total of 1,000,000 events were recorded per sample.

Statistical analysis

Differences between groups were evaluated using Students T-test or ANOVA and Tukey's post-hoc analysis was used as appropriate. Data for biomarkers were analyzed in JMP Software (SAS Institute, Cary, NC) using the Wilcoxon test. Level of significant was $P < 0.05$.

RESULTS

Pulmonary physiologic responses

In the Exploratory Phase, 3 HDM-naïve subjects and 2 animals that were injected and challenged with HDM antigen but did not develop HDM sensitivity were compared to 3 allergic-asthmatic subjects (1436, 5136, and 6447). One of the naïve controls, DL8A, exhibited a high serum anti-HDM IgE titer (76% of allergic control serum), which was equivalent to the actively-sensitized animals (Table I). Following collection of the initial biological samples, DL8A was intentionally exposed to HDM antigen, and developed robust EAR and LAR. DL8A was therefore reclassified as a passively-sensitized allergic asthmatic subject. The remaining 4 nonallergic control animals exhibited low HDM-specific IgE titers ($24 \pm 12\%$ of allergic control serum), few inflammatory cells in BALF (6×10^6 total cells, 0.2×10^6 eosinophils in the compartment), and high PC₁₀₀ to Mch (1.2 ± 0.5 mg/mL) (*See Supplemental Material Table E1. Characteristics of Control Cohort*). The 2 negative control animals that were exposed to HDM exhibited no appreciable bronchoconstriction (*Supplemental Material Table E1*).

The HDM-responders exhibited progressive increases in HDM-specific IgE titers during sensitization with HDM (*Online Supplemental Figure E2. IgE time course*). Compared to the nonallergic controls, they exhibited high serum HDM-specific IgE titers, HDM-induced bronchoconstriction, hyper-responsiveness to Mch 24 hours after HDM exposure, and late phase increases in BAL lymphocytes and eosinophils (Table I).

In the Validation Phase, HDM- sensitized animals were challenged with aero-HDM at 4 week intervals for 17 months. During the last 5 months, BAL samples were collected every 8

weeks, 24 hours after HDM challenge. Following the last challenge and lavage, the animals were treated with methylprednisolone for 2 weeks. Steroid treatment decreased HDM-induced decreases in C_{dyn} (Table I). PC_{100} to Mch was increased, and the number of eosinophils and lymphocytes in BAL fluid was reduced.

Gene expression analysis

Animals were sensitized to HDM, and specimens were collected. Animals were then challenged with aero-HDM every 4 weeks. LAR and EAR samples were collected after 9 months of periodic exposure to HDM. One subject, 6447, did not exhibit robust acute bronchoconstriction to HDM aeroallergen leading up the LAR time point, so LAR samples were not collected for gene expression analysis.

Samples were screened on a selected set of 153 genes (*Online Supplemental Table E2: Exploratory Gene Expression Profile*). Blood and mucosal biopsy samples failed to show consistent changes in gene expression and the data are therefore not reported. BAL cells collected during EAR exhibited altered expression of numerous cytokines including IL-4, -5, -13, -6, -8, and -19 (Figure 2A). IL-4, IL-19, and CCL17 (TARC) were up-regulated during LAR (Figure 2B).

Animal-to-animal variation was observed, but individual subjects exhibited consistent patterns of IL-4, -5, -13 and TARC expression (Figure 3A-D). Prior to aeroallergen challenge low levels of IL-4 and IL-5 were expressed, similar to the naïve controls. In contrast, IL-13 was elevated. The passively-sensitized animal, DL8A, exhibited the highest expression of IL-13 before intentional airway challenge (Figure 3B). All of the actively sensitized animals (1436, 5136, and 6447) exhibited higher levels of TARC expression than the non-allergic controls,

whereas DL8A exhibited a level of expression equal to controls (Figure 3D). Airway challenge increased expression of IL-5 and IL-13 during EAR. Expression of IL-4 increased in 2 of 3 animals. The animal that did not exhibit an increase in IL-4 during EAR was 6447, who exhibited the lowest physiological response to aeroallergen challenge (Table I). Two of 2 animals exhibited decreases in IL-5 and IL-13 expressions between EAR and LAR, and increases in expression of TARC (Figure 3D).

In the Validation Phase, 28 genes were selected for expression analysis. BAL samples were collected 24 hours after an aero-HDM challenge, the animals were treated with systemic steroids for 2 weeks, rechallenged, and samples were collected 24 hours later. As in the Exploratory Phase, gene expression was variable during LAR, and no clear trends were observed in response to steroid treatment (*Online Supplemental Table E3 Normalized Values of Validation Data*).

Protein levels in serum and BALF

In the Exploratory Phase, protein levels of IL-1 β , -4, -5, -6, -8, -13, MDC, and eotaxin were determined in serum and BAL samples. IL-4, -5, and -13 were below threshold values for the assays (< 80, 16, and 16 pg/mL, respectively). Serum IL-1 β and IL-6 were elevated following sensitization, did not change during EAR, and decreased during LAR (Figure 4A,B). In contrast, IL-1 β and IL-6 in BAL were low, similar to non-allergic controls prior to intentional airway challenge. Within 60 minutes of aerosol HDM challenge, levels of IL-6, IL-8, and eotaxin in BAL increased in 3 of 4 animals (Figure 5B,D). As reported for gene expression, the subject with the lowest sensitive and responsiveness to HDM, 6447, was the outlier. In LAR samples, cytokine levels were variable.

In the Validation Phase, steroid treatment decreased EAR serum histamine levels (Figure 6A). MDC levels were highly variable and often high before steroid treatment, but consistently low following steroid treatment (Figure 6B). Two animals exhibiting the highest eotaxin levels prior to steroid treatment showed decreases in eotaxin following treatment, but no consistent change was observed across the cohort (Figure 6C).

Flow cytometric analysis of BAL cells

Multi-parameter flow cytometric analysis was used to identify lymphocyte subpopulations in BAL during EAR and LAR. Representative data are shown in *Supplemental Material Figure E1*. Lymphocytes constituted 2-5% of total BAL cells (Table I). CD3⁺ cells represented up to 55% of the live cells in the lymphocyte scatter gate. The percentage of CD4⁺ BAL cells were higher in LAR than EAR (Figure 7A). The number of CD4⁺ cells was not different (data not shown).

Of the CD3+ cells, NKT cells represented a small sub-population (< 5%) during EAR (Figure 7A), and the percentage of NKT cells decreased during LAR. CD3+ and CD8+ T lymphocytes (Figure 7B) were significantly increased during LAR following steroid treatment (Figure 7B). Concurrently, the percentage of CD4+ cells decreased following steroid treatment. NKT cells did not change.

DISCUSSION

Previous reports have addressed developmental³, genetic¹⁴, and functional¹²⁻¹⁵ attributes of nonhuman primate models of asthma. This follow-up report systematically addresses molecular, cellular and protein changes longitudinally at different times in model development, documents corticosteroid effects following establishment of chronic disease, and defines changes in NKT lymphocyte subset numbers in the chronic allergic asthma state.

One goal of this project was to provide predictive measures for manifestation of allergic asthma symptoms in *Cynomolgus* macaques and thereby facilitate assignment of subjects to experimental groups in prospective studies. Similar to humans and outbred animals, it is difficult to predict which subjects will develop allergic asthma symptoms, and to predict the timecourse of disease manifestations in a given subject. We monitored levels of serum HDM-specific IgE to identify animals for assessment of gene and protein expression early in disease pathogenesis. Yet, one of the animals with the highest HDM-specific IgE concentrations, 6447, was slow in developing early phase symptoms. In addition, one of the animals assigned as a naïve control, DL8A, appeared to be predisposed to developing allergic asthma symptoms. While the findings of this study, particularly the finding that IL-13 gene expression is an early predictor, may facilitate *a priori* selection of animals for sensitization, the model remains most appropriate for

studies of established disease, where the animals can be fully characterized prior to testing therapeutic candidates.. With the induction of a robust allergic asthmatic response, the disease symptoms remain remarkably stable provided that the environment remains constant and exposure to allergen (both concentration and periodicity) is controlled.

Gene expression analysis of BAL cells revealed consistent patterns of induction of Th2-related signature genes including IL-4, IL-5, IL-13 and TARC. Due to the highly sensitive nature of real-time quantitative PCR, we were able to detect changes in these genes that were below the limit of detection in BALF by protein assays, in the case of IL-4, IL-5 and IL-13, and were not assayable in nonhuman primates (NHP) by protein analysis due to lack of reagents, in the case of TARC. Whole blood gene expression analysis from these animals did not show significant or consistent changes in response to challenge, and many genes were below the limit of detection in whole blood, in contrast to BAL cells. Lung biopsies from these animals showed highly variable expression patterns probably due to sampling differences, tissue heterogeneity and variability introduced by linear amplification. This study shows that measuring gene expression from BAL cells is a sensitive and reproducible method to assess Th2 gene signatures in this model.

Following immunization but before intentional airway exposure to allergen, IL-1 β and IL-6 proteins were elevated in serum. These two general inflammatory, acute phase cytokines are elevated in human allergy and reduced following immunotherapy.^{18,19} Expression of Th2-specific cytokines was increased in the airways within 60 minutes of allergen exposure. The early increase in Th2 cytokine expression was followed by an increase in eotaxin expression and eosinophils in the airways 23 hours later. In human BAL fluid, Th2 cytokine concentrations

increase over 24 to 42 hours following segmental allergen challenge.²⁰ However, as in this study, variability of Th2 cytokine expression increases with time after allergen challenge in humans.²⁰

It has been proposed that NKT cells mediate Th2 inflammatory responses and may be a viable target in treating asthma.^{22,23} Vijayanand et. al.²¹ has reported low numbers of NKT cells in human asthmatic airways, whereas Akbari reported that the majority of CD4⁺ cells in the airway lumen are NKT cells.²² We found that the sub-population of invariant NKT cells in BAL fluid is small, indicating that large numbers of these cells are not required to sustain an allergic asthmatic phenotype.

CD4⁺ and CD8⁺ lymphocytes are elevated in blood and BALF of patients with allergic asthma.²⁴⁻²⁶ Likewise, the nonhuman primates in this study exhibited increases in CD4⁺ cells in BALF during LAR. Interestingly, Robinson et al.²⁷ reported activation of CD4⁺ T cells in humans with allergic asthma, and postulated that cytokines produced by activated Th2-type CD4⁺ T cells may contribute to LAR. Th1 and Th2 responses, including cytokine and chemokine mediated responses, are well documented in allergic asthma.²⁸⁻³⁰ Our observations indicate that activation state might be more critical than absolute numbers of T cell subpopulations in establishing chronic disease.

Corticosteroids are the most effective anti-inflammatory therapy in asthma, broadly inhibiting effector function of Th2 cells and eosinophils. In this study, corticosteroid treatment inhibited LAR > EAR, reducing acute bronchoconstriction serum histamine, PC100 to Mch, airway inflammation, and MDC concentration in BAL fluid – all mirroring the efficacy of corticosteroid treatment in asthmatic subjects. Interaction of MDC with CCR4 is thought to recruit Th2 lymphocytes to target tissues.³¹ MDC has been reported to be elevated in allergic rhinitis³² and severe asthma.³³ However, not all investigators have observed an elevation in this

chemokine, and at least one study revealed a small increase in MDC concentration in exhaled breath condensates in patients treated with inhaled corticosteroid treatment.³⁴

Recent studies have shown that corticosteroids inhibit the development of Treg cells and tolerance to allergens in mice, thereby enhancing subsequent Th2 responses and aggravating the course of asthma and allergic diseases.³⁵ Other investigators have also reported an expansion in T cells after steroid treatment in asthma patients.³⁶ These findings are in agreement with our observations that CD3⁺ and CD8⁺ cells were higher after steroid treatment. Furthermore, inhibition of Treg cells may account for the increased variability in Th2 cytokine expression following steroid treatment in the present study.

Validated animal models that closely mirror human disease mitigate the risk intrinsic to translating basic research observations into clinical practice. The results of this study demonstrate that HDM-sensitive macaques periodically exposed to aerosolized allergen over extended periods of time exhibit allergen-induced changes in airway physiology and immunology that are similar to those seen in human asthmatics. Robust physiological and inflammatory responses are observed coincident with low numbers of NKT cells, indicating that large numbers of these cells are not required to express an asthmatic phenotype. In HDM sensitive animals whose airways have been primed with aerosolized allergen over time, Th2 cytokine levels in the airways increase within hours after an allergen challenge. However, the time course of expression is highly dependent on the individual and this source of variation should be accounted for when designing preclinical studies where cytokine expression is a primary readout. In conclusion, chronic periodic exposure of nonhuman primates to HDM closely models human atopic asthma and should be a valuable tool in discovery and translational research.

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Table I. Characteristics Before and After Allergen Provocation for HDM-Responsive Cohorts

	EAR				24 hr. Post-aeroHDM				
	Serum α-HDM-IgE (% Allergic Control)	Mch PC100 (mg/ml)	HDM (AU/ml)	ΔR _L (%)	ΔC _{dyn} (%)	BALF Differential Cell Counts ²			
						Total	Lymph.	EOS	PMN
HDM-Responsive									
Exploratory/1436	84	1.7	500	423	-78				
Exploratory/5136	114	0.8	2500	329	-65	20.0	0.7	5.3	0.3
Exploratory/6447	96	1	2500	209	-83	20.0	2.1	3.0	1.1
Validation Group (n=7)						16.0	1.0	2.5	0.2
Pre-steroid treatment ¹	74±6	0.9±0.4	762±327	94±15	-54±4	19.2±1.7	0.7±0.1	1.6±0.4	0.6±0.4
Post-steroid treatment			519±368	61±22	-34±8*	14.9±1.2*	0.3±0.06*	0.8±0.3*	0.3±0.07
Passively Sensitized/DL8A	71	0.76	500	300	-73	16.0	0.4	1.4	0.9

¹Average over 5 months prior to steroid treatment

²(cells/ml BALF returned) x ml of BALF instilled

*Effect of steroid treatment, P<0.05

ND:Not Done

Data on the negative control data are provided in the online supplement Table E1.

FIGURE LEGENDS

Figure 1. Study design. Animals were sensitized to HDM antigen for 6 months, then periodically challenged with aerosolized HDM. Four animals underwent proteogenomic characterization during the Exploratory Phase (3 actively sensitized + DL8A who was intrinsically sensitive); seven others underwent steroid treatment during the Validation Phase. EAR and LAR were measured beyond month 6 for following allergen provocation. Methacholine (Mch), bronchoalveolar lavage (BAL)

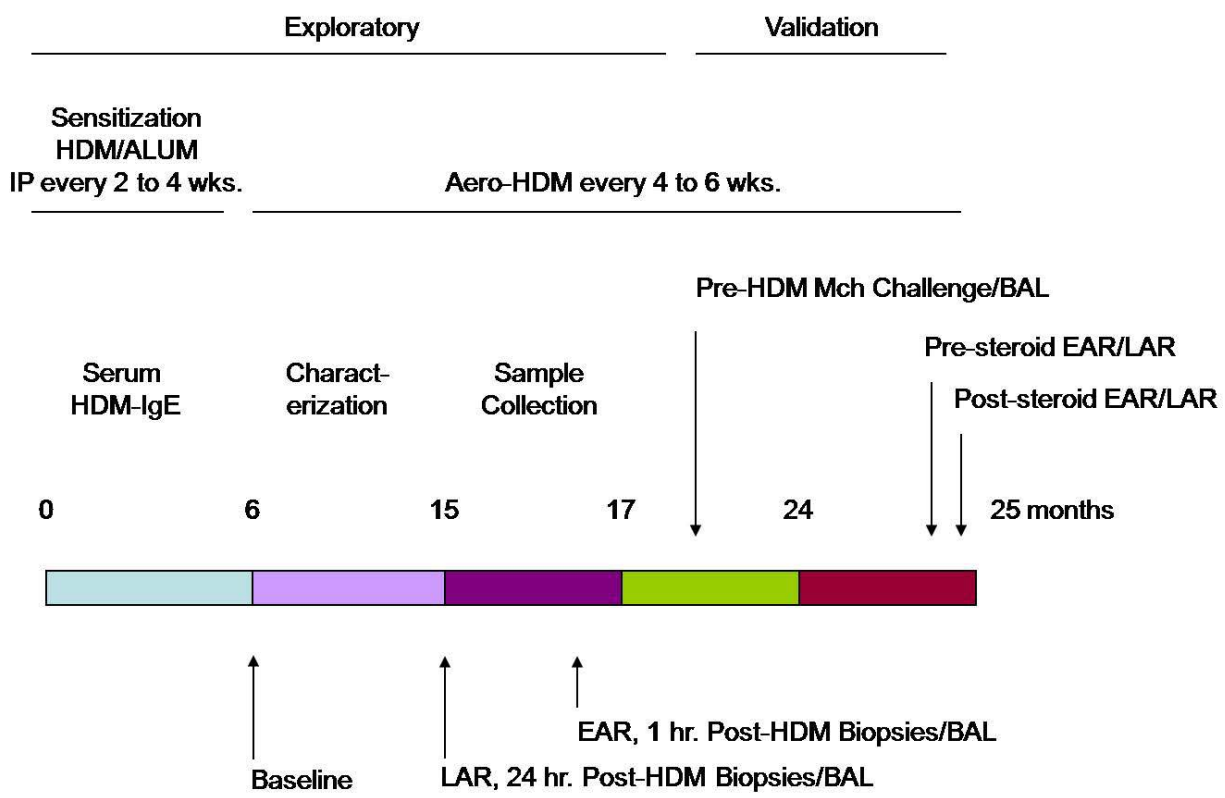


Figure 2. Gene expression profiles of BAL cells before and after aero-HDM challenge. Cells were collected from HDM-sensitized animals 60 minutes (EAR, Panel A) and 24 hours (LAR, Panel B) after aero-HDM challenge. Expression of 153 genes was analyzed by RT-qPCR, and

B.

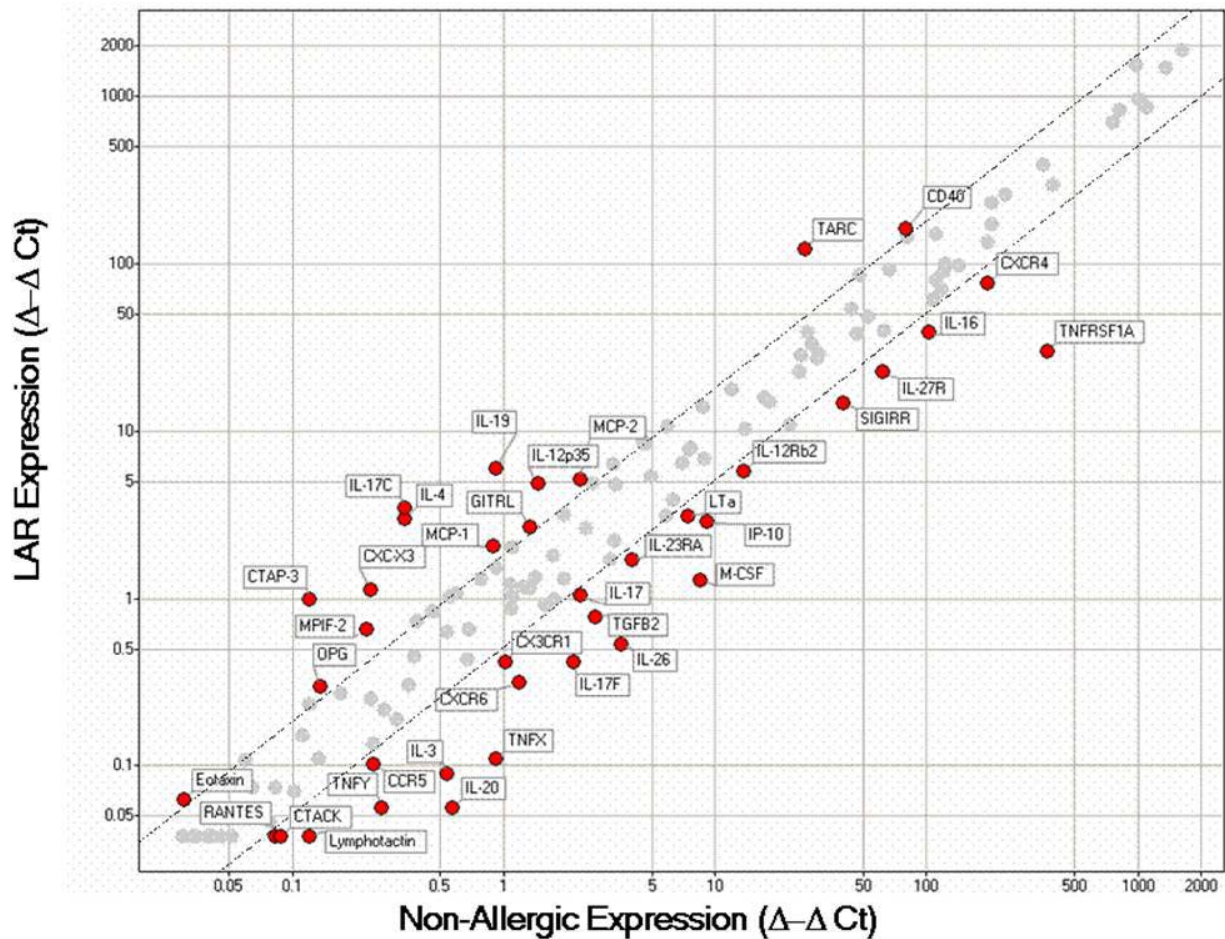


Figure 3. Gene expression in individual HDM challenged animals during the Exploratory Phase. IL-4, IL-5, IL-13, TARC expression shown in Figure 2 are replotted to show actual values for the allergic animals and mean \pm SE for the non-allergic control animals. Real-time quantitative PCR of RNA isolated from BAL cells shows induction of the Th2 cytokines/chemokines compared to an average of 4 control animals. *EAR different from Sensitized, $P < 0.05$

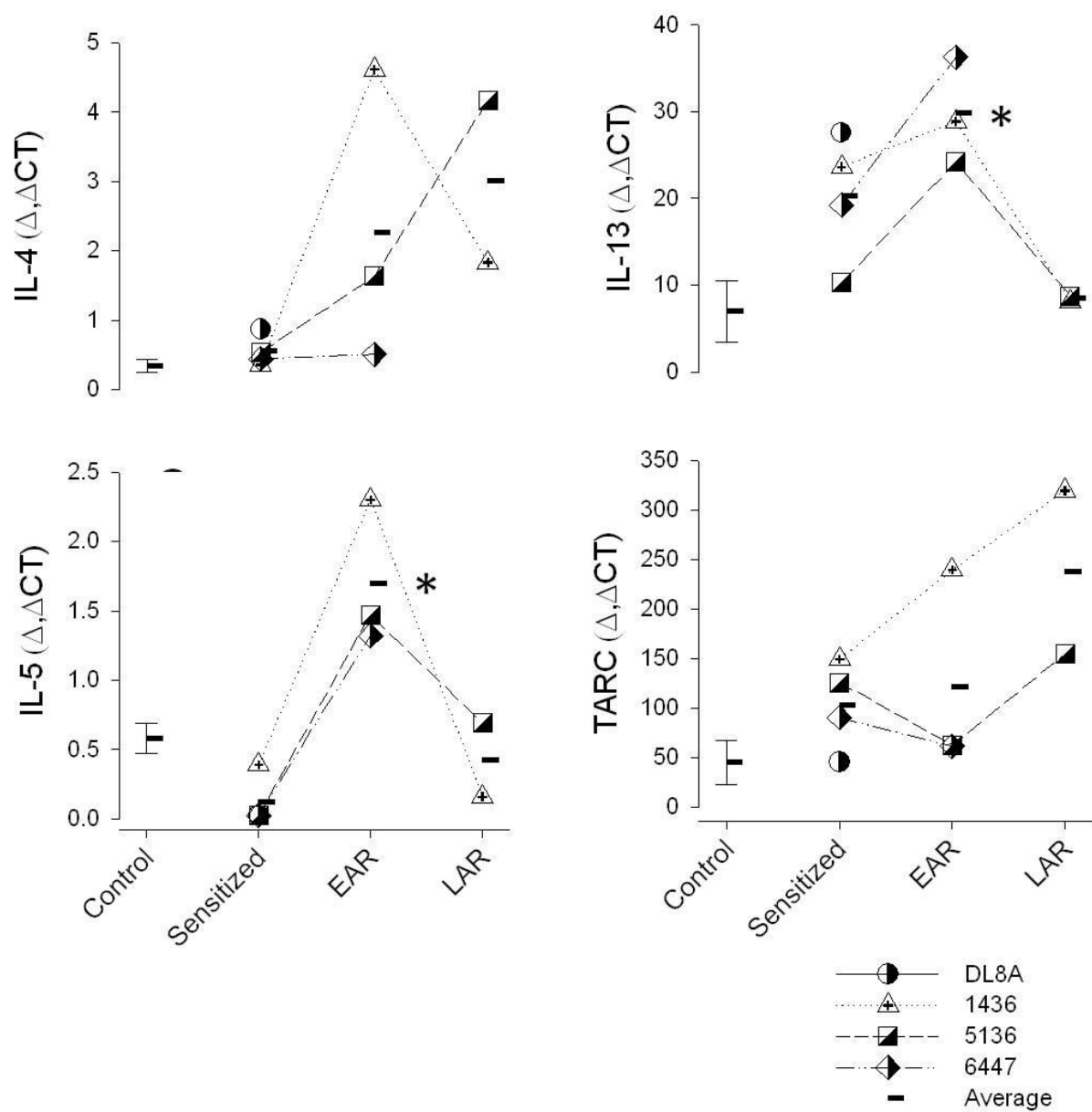


Figure 4. Serum protein levels measured in the Exploratory Phase. IL-1 β , -6, -8, eotaxin, and MDC were measured in naive animals and in HDM sensitive animals before (Sensitized), and after 9 months of periodic aeroallergen challenge. EAR and LAR samples were collected after

aeroallergen challenge. Values for non-allergic control animals are mean \pm SE, n=4. Sensitized animals, each symbol represents an individual animal. *Sensitized and EAR are different from Control, $P < 0.05$

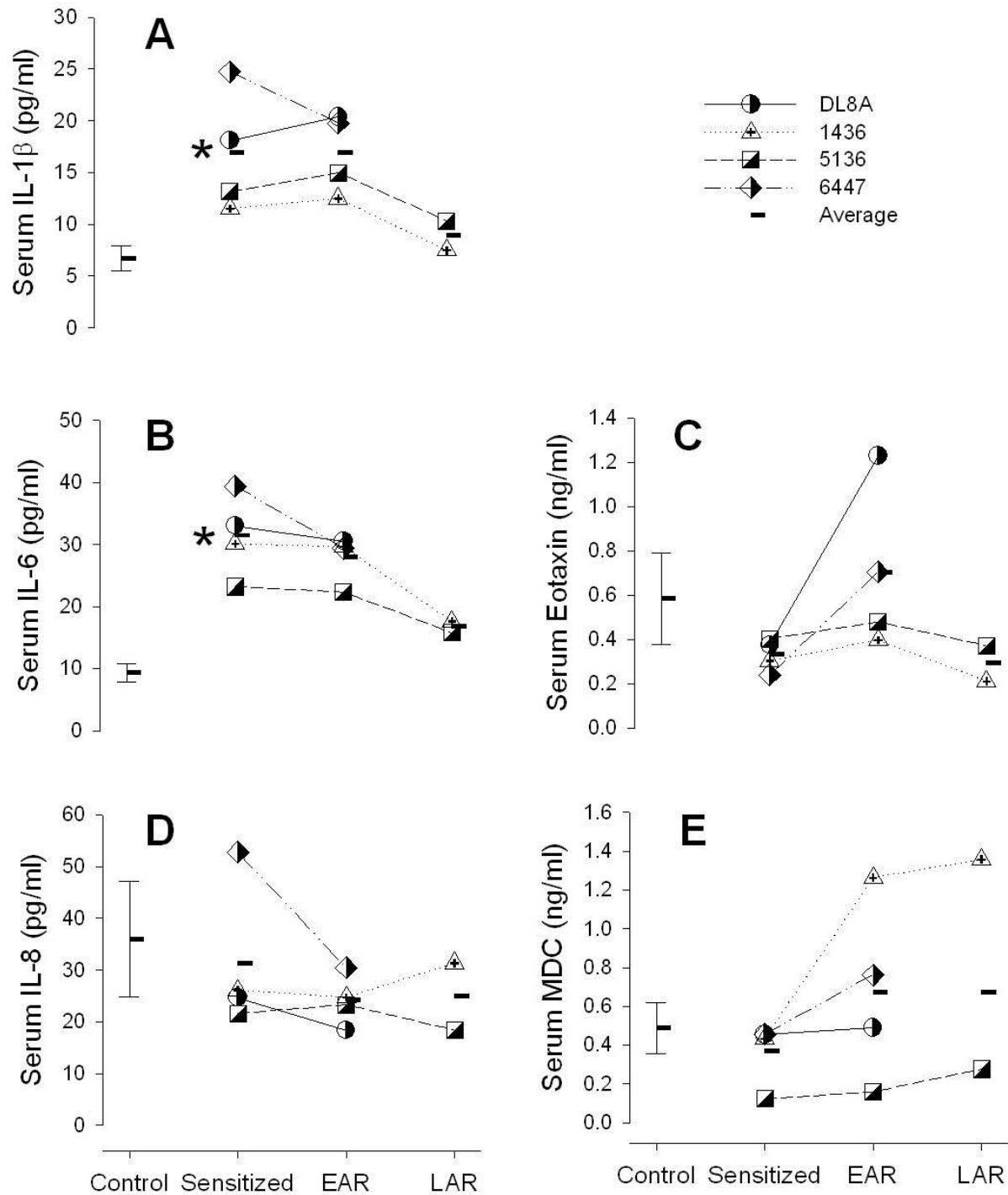


Figure 5. BAL proteins in the Exploratory Phase. IL-1 β , -6, -8, eotaxin, and MDC were measured in naive animals and HDM-sensitive animals before (Sensitized), and after 9 months of periodic aeroallergen challenge. EAR and LAR samples were collected after aeroallergen challenge. Values for non-allergic control animals are mean \pm SE, n=4. For Sensitized animals, each symbol represents an individual animal. *EAR different from Sensitized, P<0.05

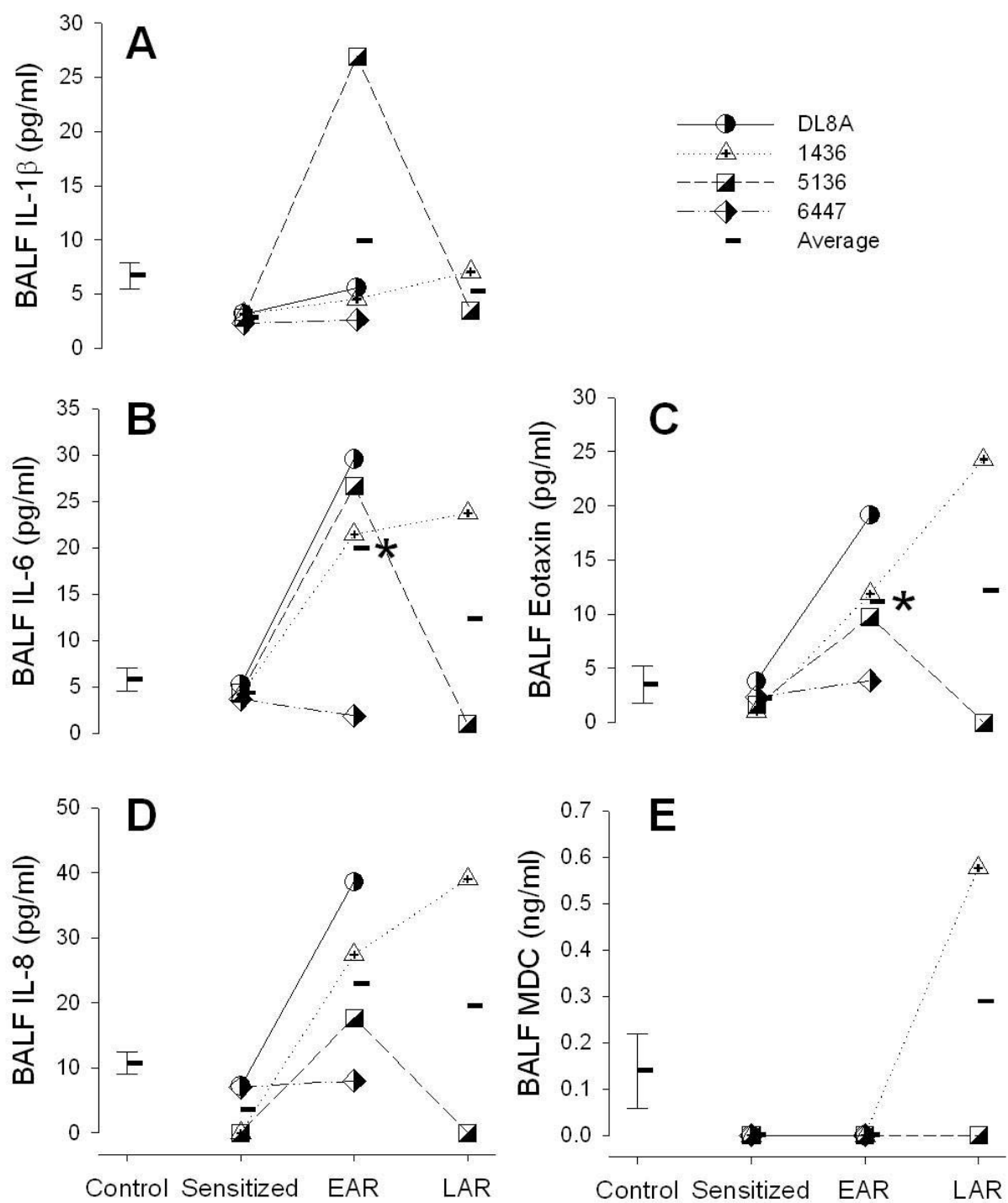


Figure 6. Effects of steroid treatment on serum histamine and BAL proteins. EAR and LAR readouts were recorded in HDM-sensitive animals and periodically-challenged animals before and after 2 week so steroid treatment. Serum histamine was measured 20 minutes after HDM challenge. MDC and eotaxin levels in BAL were measured 24 hours after challenge. *Post-steroid treatment value different from Pre-treatment, $P < 0.05$

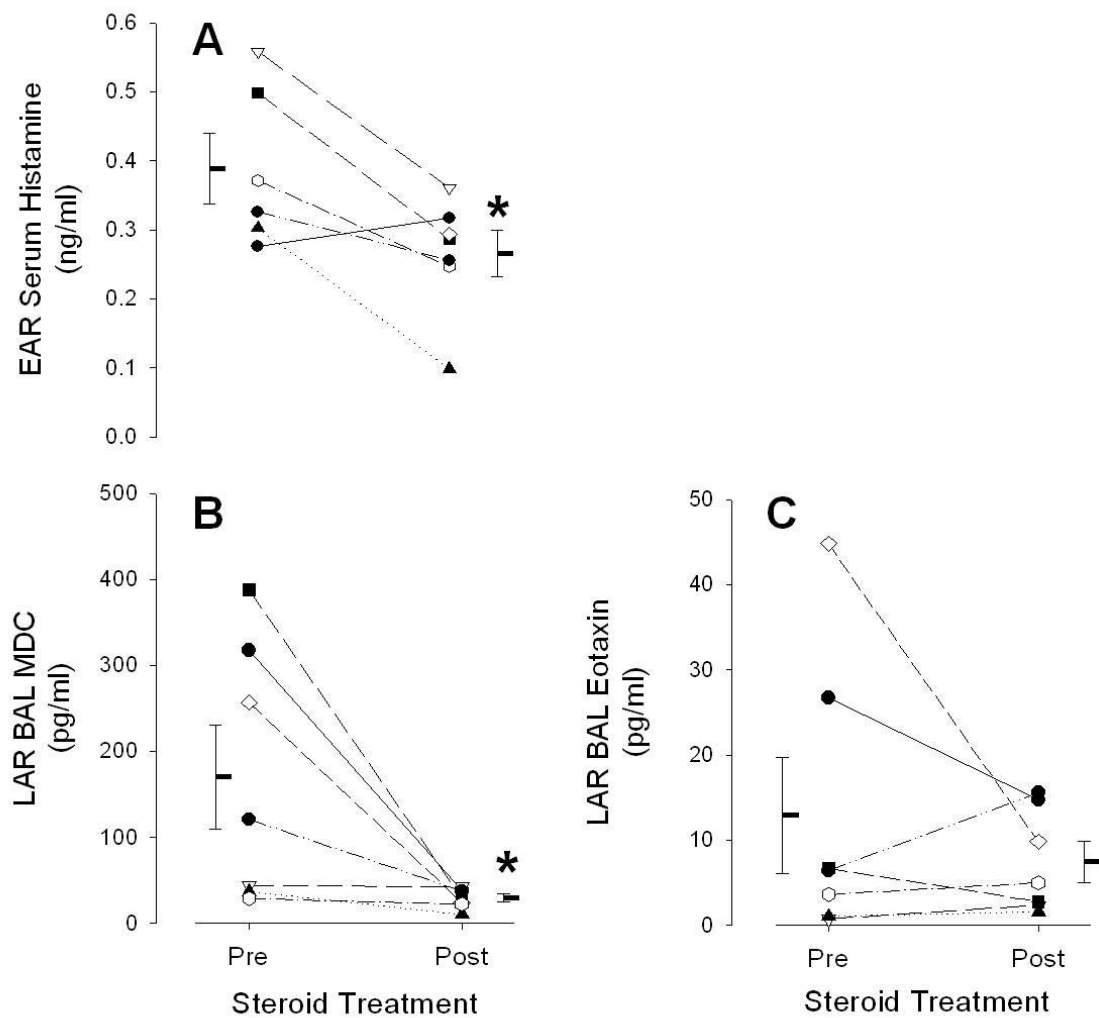
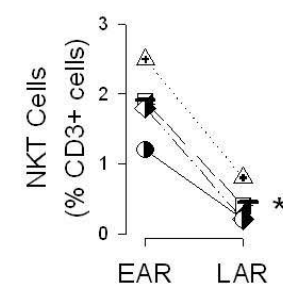
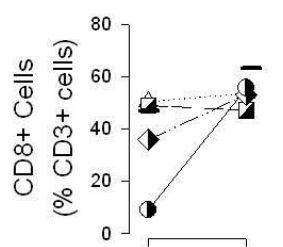
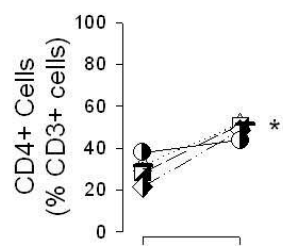
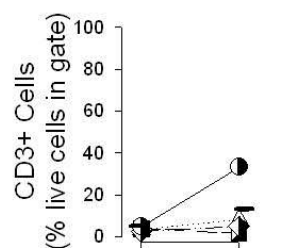
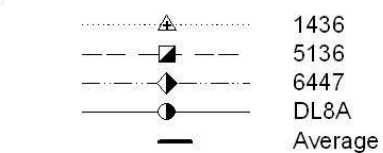
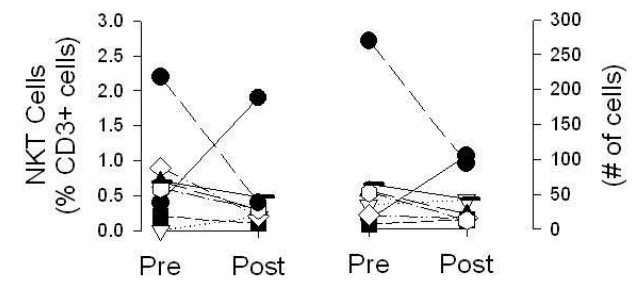
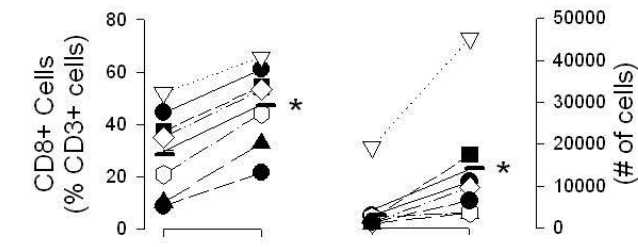
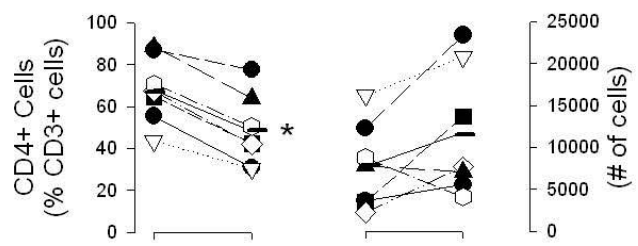
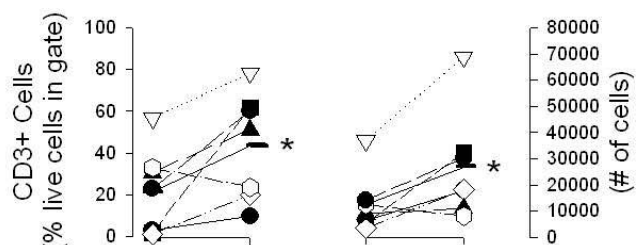
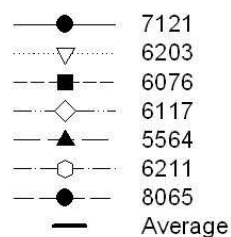


Figure 7. Lymphocyte subpopulation in BALF of HDM-sensitive animals. (A) Exploratory Phase: BALF was collected 60 minutes (EAR) or 24 hours (LAR) after aeroallergen challenge. (B) Validation Phase: BALF was collected 24 hours after allergen challenge, before (Pre) and after (Post) 2 weeks of steroid treatment. CD3+, CD4+, CD8+, and NKT cells were enumerated by flow cytometry. * Pre different from Post, $P < 0.05$.

A



B



Steroid

