

Airway House Dust Extract Exposures Modify Allergen-Induced Airway Hypersensitivity Responses by TLR4-Dependent and Independent Pathways¹

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TLR ligands and other allergen-nonspecific immunostimulatory molecules are ubiquitous in ambient air and have profound modulatory activities in animal models of allergic asthma. However, several of these molecules have been shown to promote exaggerated Th2-biased airway hypersensitivity responses (AHRs), whereas others attenuate the asthmatic phenotype. Therefore, it has proven difficult to extrapolate experimental results with purified molecules toward a more general understanding of the allergen-nonspecific immunomodulatory influence of living environments on the natural history of allergic asthma. These investigations determined how regular and intermittent airway exposures to an unpurified, but sterile house dust extract standard (HDEst) affected the OVA-specific AHR and immune status of previously Th2-sensitized mice. Low-dose daily and high-dose intermittent HDEst exposures modulated ongoing AHRs considerably, reducing eosinophil recruitment and methacholine responsiveness, while increasing neutrophilic inflammation. However, only daily airway delivery of low-dose HDEst attenuated OVA-specific Th2 cytokine production and Th2-biased AHRs to allergen challenge 1 mo later. Finally, whereas LPS mimicked many of the immunomodulatory characteristics of HDEst in this murine asthma model, daily airway HDEst delivery was highly effective in attenuating the AHR of OVA/alum-sensitized TLR4-deficient mice. Taken together, these investigations provide direct evidence that living environments contain allergen-nonspecific immunostimulatory molecules that influence the airway hypersensitivity status of allergen-sensitized mice by TLR4-dependent and independent mechanisms. *The Journal of Immunology*, 2008, 181: 2925–2932.

Children raised in industrialized countries are far more likely to develop asthma and other atopic diseases than children living in underdeveloped parts of the world. Moreover, prevalence rates have increased dramatically over the last half-century in affected countries, a time span too brief to be accounted for by genetic drift alone (1–3). Therefore, although unproven, it is generally believed that environmental changes associated with the modern life style increase a child's risk of becoming allergic (4–6). Consistent with this view, the hygiene hypothesis proposes that children of affluent countries suffer from a deficiency in environmental contact with microbes due to modern public health practices (i.e., clean water supplies, sterilized and processed foods, and the routine use of antibiotics and vaccines), rendering them at increased risk for dysregulated immunity to allergens ubiquitous in their living environments (3, 7, 8).

Microbes produce a wide variety of molecules that directly activate receptors expressed on cellular constituents of the innate immune system (9). These microbe-associated molecular patterns include (TLR) ligands, which can dramatically influence Ag-specific immunity. Mice and humans immunized with Ag and immunostimulatory sequence oligodeoxynucleotide (ISS-ODN,³ TLR9) develop robust Th1-biased adaptive responses and are protected

from Th2-biased airway hypersensitivities (3). In contrast, several laboratories have found that mice immunized with Ag and TLR2 ligands develop Th2-biased adaptive responses and experimental asthma upon Ag challenge (10, 11). Likewise, mice intranasally (i.n.) immunized weekly with Ag and appropriate doses of LPS (TLR4) develop Th2-biased airway hypersensitivities (12, 13). However, if Ag delivery remains weekly while LPS is delivered daily, at one-seventh the adjuvant dose, mice develop short-term (13) and long-term Ag-specific tolerance (our unpublished observation).

In addition to their study in allergen naive mice, TLR ligands have been investigated as immunomodulatory agents given to previously Th2-sensitized mice at the time of airway allergen challenge. A single dose of ISS-ODN delivered within 24 h of allergen challenge has been shown to protect mice from developing Th2-biased hypersensitivity responses in murine models of asthma, allergic rhinitis, and allergic conjunctivitis (14–16). In contrast, several laboratories have found that peptidoglycan (TLR2) and LPS exacerbate experimental asthma when codelivered to sensitized mice during the allergen challenge period (17–20). However, study results have not always been consistent. For example, Velasco et al. (21) reported that airway peptidoglycan or lipid A (TLR4) administration to Th2-sensitized mice reduced the percentage of eosinophils seen in their airways after allergen challenge. Such inconsistencies in the reported effects of TLR2 and TLR4 ligands on the airway hypersensitivity response (AHR) have yet to be reconciled.

The modulatory influence of repeated TLR ligand exposures on ongoing AHRs has not been adequately assessed. However, one study with endotoxin-contaminated OVA addressed this issue indirectly (22). In this model, OVA/alum-sensitized mice received

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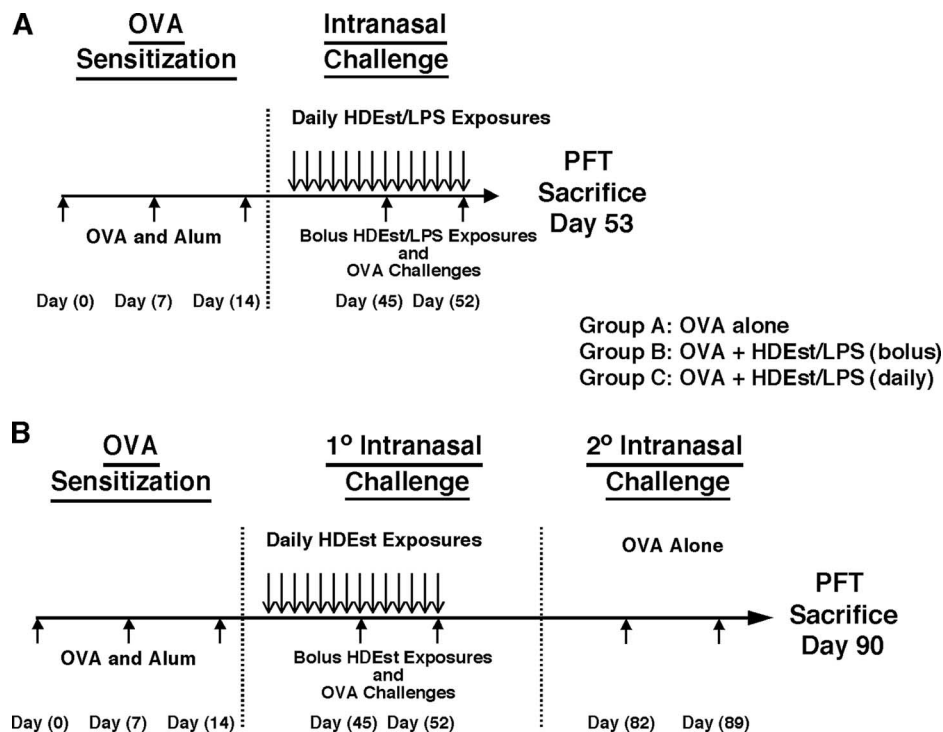
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³ Abbreviations used in this paper: ISS-ODN, immunostimulatory sequence oligodeoxynucleotide; AHR, airway hypersensitivity response; BALF, bronchoalveolar la-

vage fluid; BLN, bronchial lymph node; HDE, house dust extract; HDEst, HDE standard; i.n., intranasal; ko, knockout; Mch, methacholine; WT, wild type.

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FIGURE 1. Th2 sensitization, allergen challenge, and HDEst/LPS delivery schedules. Naive mice ($n = 4$ per group) were Th2 sensitized by three weekly i.p. injections of OVA and alum. Airway OVA challenges were initiated 1 mo after the final OVA/alum injection. Seven days before the first of two weekly i.n. OVA challenges, one group of mice began receiving low-dose i.n. HDEst (3 μ l) or LPS (10 ng) on a daily basis, the last dose being delivered with the final OVA challenge. Another group of Th2-sensitized mice received a high-dose HDEst (21 μ l) or LPS (70 ng) bolus concurrently with each i.n. OVA challenge. *A*, For experiments presented in Figs. 2, 4, and 5, airway hypersensitivity and BLN cytokine responses were assessed 24 h after the second OVA challenge. *B*, For experiments described in Fig. 3, mice were observed for an additional month before receiving a second series of OVA challenges (2° challenge), and outcome parameters were assessed 24 h after the final i.n. OVA challenge.



daily airway challenges with purified (endotoxin-free) or commercial (endotoxin-contaminated) OVA for 9 days before AHRs were assessed. The investigators found that Th2-sensitized mice airway exposed to purified OVA developed exaggerated Th2-biased AHRs, compared with mice challenged with endotoxin-contaminated OVA. This observation was unexpected, because endotoxin exposures are known to induce airway inflammation.

The fact that TLR2, TLR4, and TLR9 ligands can readily modify the allergic phenotype in experimental animals has clinical relevance, because these molecules are ubiquitous in homes and ambient air (23–26). Nonetheless, available evidence suggests that the multitude of TLR-dependent and independent immunostimulatory factors to which infants are daily exposed have both synergistic and antagonistic immunomodulatory effects on atopic status. This is a major impediment to extrapolating results of laboratory studies with purified molecules toward a more unifying and predictive model of how relevant ambient exposures influence the development and persistence of allergic respiratory diseases.

Recognizing the molecular complexity of the world in which we live and that ambient immunostimulatory particulates of clinical relevance collect in house dust, we have begun to characterize the immunostimulatory activities of unpurified, but sterile house dust extracts (HDEs) (13, 27, 28). Previous studies have found that HDE activation of bone marrow-derived dendritic cells is partially dependent on TLR2, TLR4, and TLR9, and almost completely dependent on MyD88, a molecule involved in signaling through all TLRs, except TLR3 (28). Moreover, as with LPS, weekly i.n. OVA vaccinations with HDEs derived from 10 homes consistently primed mice to develop Th2-biased hypersensitivities, whereas daily HDE delivery at one-seventh the adjuvant dose rendered mice resistant to OVA sensitization (13). These studies suggest that airway exposures to allergen-nonspecific immunostimulants ubiquitous in living environments have the potential to promote the development of either Th2-biased hypersensitivities or tolerance to ambient aeroallergens, depending on the level and frequency of exposures. Furthermore, because LPS and HDEs were found to have similar Th2 adjuvant and tolerogenic activities, these

investigations left open the possibility that LPS was responsible for a majority of immunomodulatory activities associated with HDEs and their homes of origin.

Although children with allergic asthma continually inspire air containing the allergen-nonspecific immunostimulatory constituents of HDEs, it remains to be determined whether these exposures modify pre-existing aeroallergen hypersensitivities and pulmonary responses to aeroallergen encounter. The current investigations characterized how intermittent and daily i.n. delivery of a HDE standard (HDEst) affected the AHRs and immune profiles of previously Th2-sensitized mice. Treatment with i.n. HDEst was found to have both rapid and long-lasting effects on pulmonary responses to allergen challenge. As in previous studies, dose and dosing intervals proved important variables in determining the modulatory influence of HDEst on pre-existing Th2-biased airway hypersensitivities. Finally, experiments with TLR4-deficient mice established that daily i.n. HDEst exposures attenuated AHRs, at least in part, by TLR4-independent mechanisms.

Materials and Methods

Mice, OVA, and purified LPS

Investigations received prior approval from our institution's animal welfare committee. Female mice aged 4–6 wk were used for all studies. BALB/c and C57BL/6 mice were purchased from Harlan Sprague-Dawley, and TLR4 knockout (ko) mice were bred in our animal facility. Except for experiments with TLR4 ko mice (C57BL/6 background), BALB/c mice were used in all investigations. OVA (grade VI; Sigma-Aldrich) and *Escherichia coli* 026-B6 LPS (Sigma-Aldrich; 10 EU = 1 ng) were purchased from commercial vendors.

Preparation of individual HDEs and the HDEst

With approval from our institution's human subjects committee, dust samples were obtained by vacuuming a single carpeted bedroom in each of 15 suburban homes in San Diego County, California. Methods used for the collection and processing of house dust have been described in detail previously (28). Briefly, study bedrooms were left unvacuumed for 1 wk before exposed carpeting was vacuumed with a Quick Broom (Hoover) for 5 min. Collected house dust was then run through a coarse sieve to remove large particulate matter and suspended in sterile PBS at 100 mg/ml. House

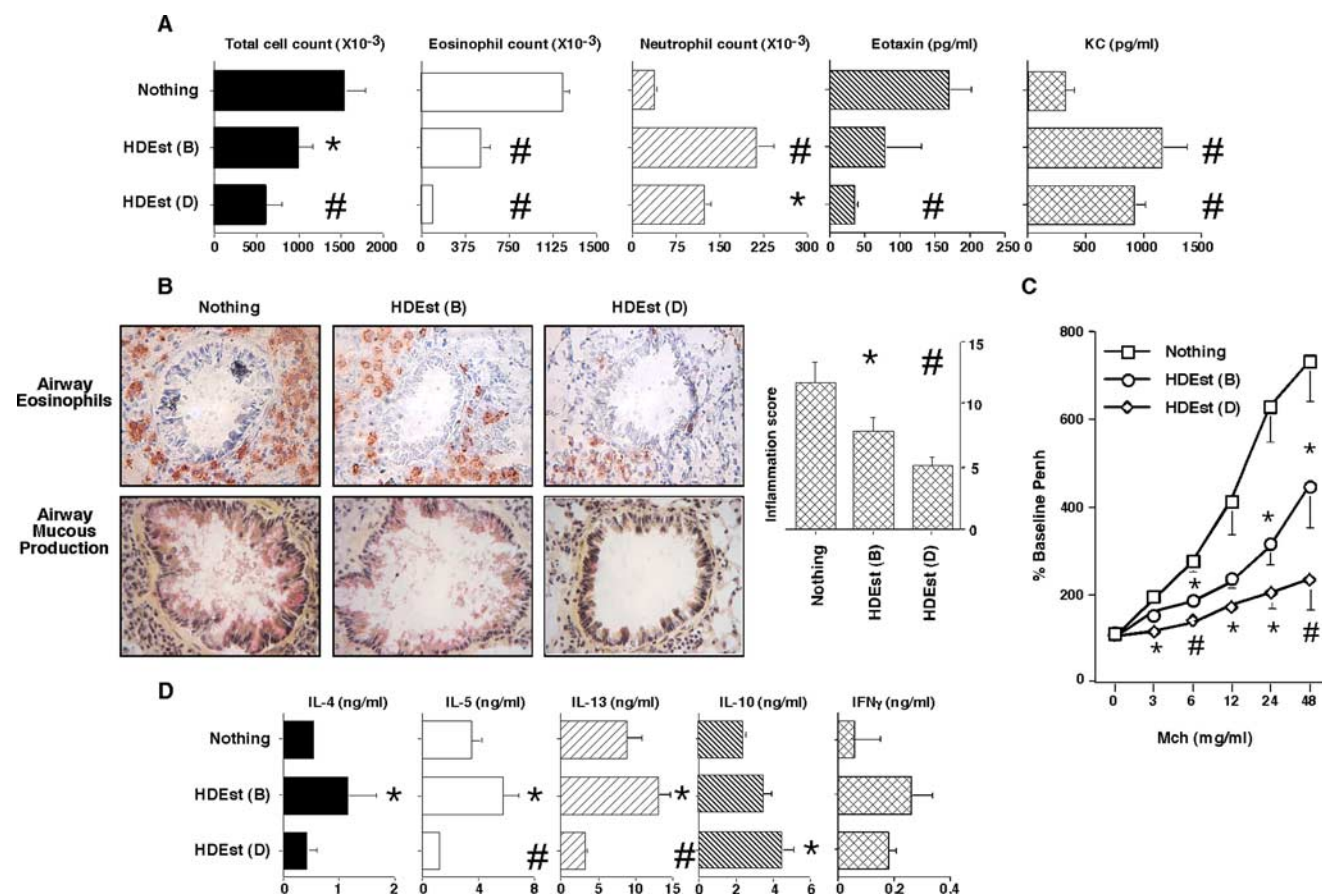


FIGURE 2. Concurrent airway HDEst exposures modify allergen-induced AHRs. OVA/alum-sensitized BALB/c mice ($n = 4$ per group) were i.n. allergen challenged. Select groups of mice received i.n. HDEst during the OVA challenge period in accordance with the bolus (B) and daily (D) delivery schedules described in Fig. 1A. AHRs and OVA-induced BLN cytokine responses were assessed 24 h later. Reported results were reproduced in three independent experiments, and bar and line graph data points present mean values with SEs. *, $p \leq 0.05$; #, $p \leq 0.01$, for HDEst-treated vs HDEst-untreated mice. A, BALF total cell, eosinophil, neutrophil, counts, and eotaxin and KC levels. B, Lung histology and inflammation scores. C, Airway Mch sensitivity. D, OVA-induced BLN cell cytokine production.

dust suspensions were then placed on a rotor at room temperature for 18 h, filtered through glass wool, and finally through 0.22- μ m Steriflip filters (Millipore) to obtain sterile HDEs. In previously published studies, we compared the relative bioactivities of these HDEs (28). For the current studies, eight of the HDEs found to be highly bioactive were combined to prepare a large volume of high bioactivity HDEst that could be used for all experiments presented in this study. The endotoxin concentration of the HDEst discussed in this work was determined with the QCL-1000 kit (Bio-Whittaker), according to the manufacturer's instructions.

OVA sensitization and airway challenge

Mice ($n = 4$ /group) were initially Th2 sensitized by three weekly i.p. injections of OVA (100 μ g) and alum (1 mg) in a volume of 500 μ l of PBS, as outlined in Fig. 1. One month after the final injection, mice received two i.n. OVA (10 μ g) challenges, delivered 7 days apart, in a volume of 30 μ l of PBS, divided equally and delivered bilaterally to each nare. In each experiment, one group of mice additionally received 21 μ l of HDEst or 70 ng of LPS (700 EU) with each OVA challenge. Another group of mice began receiving i.n. HDEst (3 μ l) or LPS (10 ng or 70 EU) on a daily basis, beginning 7 days before the first and ending with the last OVA challenge. A third control group of OVA-challenged HDEst/LPS naive mice was included in each experiment. Mice were lightly anesthetized (isoflurane; Abbott Laboratories) before i.n. delivery of all reagents. For experiments described in Figs. 2, 4, and 5, outcome parameters were assessed 24 h after the second airway OVA challenge (Fig. 1A). For experiments presented in Fig. 3, mice were observed for an additional month before receiving a second round of OVA challenges without HDEst coadministration (Fig. 1B).

Assessment of AHRs

Airway responsiveness to methacholine (Mch) was assessed with a single-chamber whole-body plethysmograph from Buxco Electronics. Mice were

exposed to increasing concentrations of nebulized Mch (Sigma-Aldrich; 3–48 mg/ml) by Aerosonic ultrasonic nebulizer (DeVilbiss), and the percentage of increase in enhanced pause from baseline for each Mch challenge dose was determined. After Mch challenge, mice were sacrificed, lungs were lavaged with 800 μ l of PBS, and bronchoalveolar lavage fluid (BALF) was collected and centrifuged. Supernatants were saved for chemokine ELISA. Cell pellets were resuspended in 1 ml of PBS, and total BALF cell counts were determined with a hemocytometer. In addition, BALF cytopins were prepared, slides were fixed in acetone, and then Wright-Giemsa was stained. A blinded observer determined the percentage of eosinophils, neutrophils, and mononuclear cells on each slide by counting a minimum of 200 cells in random high-power fields with a light microscope. Lung tissue was flash frozen, cryosectioned, acetone fixed onto poly(L-lysine)-coated slides, and stained with H&E, peroxidase/diaminobenzidine, and periodic acid-Schiff stain, by standardized techniques. To quantitate peribronchial inflammation, eosinophil infiltration, and airway mucous production, a scoring system (0–5) was devised in which a blinded observer scored four to eight airways per mouse for each of these parameters. Mean inflammation scores were determined by averaging the total cellular infiltration, eosinophil infiltration, and airway mucous production scores for each mouse group and combining them to generate a total score (0–15). Experimental techniques used for these analyses are further described in our previous publications (10, 29).

BALF chemokine and OVA-specific cytokine responses

BALF KC and eotaxin levels were determined with R&D Systems reagents, according to the manufacturer's instructions. OVA-specific bronchial lymph node (BLN) cytokine responses were assessed by previously published methods (10, 29). Briefly, BLNs harvested from each group of experimental mice were pooled, and single-cell suspensions were prepared by enzymatic digestion with collagenase VIII (300 U/ml; Sigma-Aldrich) and DNase-I (1.5 μ g/ml; Sigma-Aldrich). BLN cells were cultured in

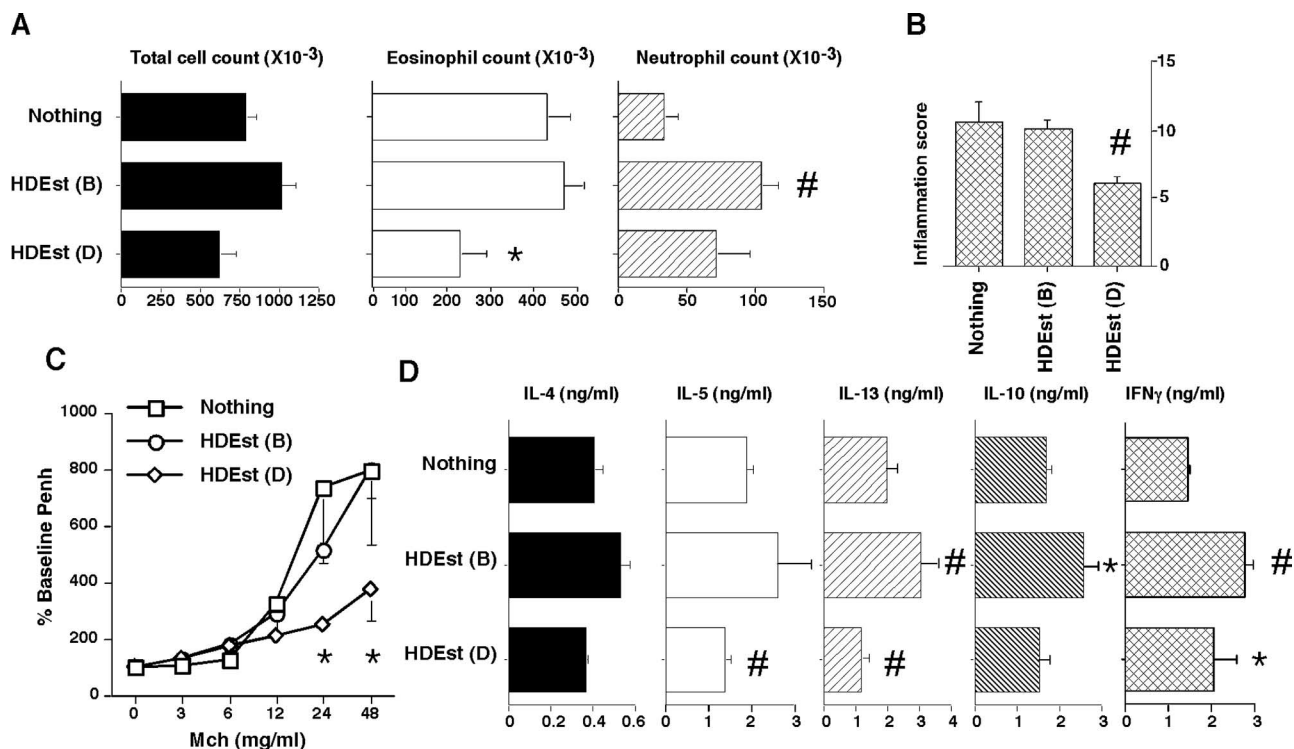


FIGURE 3. Intranasal HDEst exposures during primary AHRs lead to long-lived changes in allergen responsiveness. OVA/alum-sensitized BALB/c mice ($n = 4$ per group) received primary i.n. allergen challenges, and select mouse groups received i.n. HDEst in accordance with the bolus (B) and daily (D) delivery schedules described in Fig. 1B. Mice had secondary i.n. OVA challenges delivered 30 days later, and responses were assessed 24 h after the last. Reported results were reproduced in a second experiment. Bar and line graph data points present mean values with SEs. *, $p \leq 0.05$; #, $p \leq 0.01$, for HDEst-treated vs HDEst-untreated mice. A, BALF total cell, eosinophil, and neutrophil counts. B, Lung inflammation scores. C, Airway Mch sensitivity. D, OVA-induced BLN cell cytokine production.

triplicate at 1×10^6 cells/ml in medium with or without OVA (50 μ g/ml) for 72 h before harvesting supernatants. IL-4, IL-5, IL-10, IL-13, and IFN- γ levels in culture supernatants were determined by ELISA using BD Pharmingen reagents, according to the manufacturer's recommendations. BLN cytokine responses were calculated by subtracting background cytokine production from responses of BLN cells cultured with OVA.

Statistical considerations

Statistical analyses were conducted using Statview software. Two-tailed unpaired Student's t tests were used to analyze all data. Outcome measures for mice receiving HDEst/LPS by the daily or intermittent delivery schedules were compared with those of mice that remained HDEst/LPS naive. The Bonferroni correction factor was included in the calculation of p values, to account for the increased probability of type-I errors when multiple groups are statistically compared. Results were considered statistically significant if p values were ≤ 0.05 (*) or ≤ 0.01 (#).

Results

i.n. HDEst exposures during allergen challenge modify the AHR

To assess the immunomodulatory influence of intermittent and daily airway HDEst exposures on allergen-induced AHRs, experiments were conducted in accordance with a schedule outlined in Fig. 1A. Mice were first Th2 sensitized to OVA. Airway OVA challenges were initiated 1 mo after the final sensitization. One group of mice received a high-dose HDEst bolus concurrently with each i.n. OVA challenge. Another group of mice received low-dose i.n. HDEst (one-seventh bolus dose) on a daily basis beginning 7 days before the first and ending with the final OVA challenge. Bolus and daily delivery schedules were standardized to provide the same total amount of HDEst to mice over the course of the experiment.

In one representative experiment, mice receiving daily i.n. HDEst during the airway allergen challenge period had mean reductions

of ~58 and 92% in their BALF total cell and eosinophil counts and a 233% average increase in BALF neutrophil counts, compared with sensitized mice challenged with OVA alone (Fig. 2A). Consistent with these findings, levels of eotaxin, an eosinophil-specific chemokine, were reduced 79%, and levels of KC, a neutrophil-specific chemokine, were increased 180% in BALF recovered from daily HDEst-treated vs HDE naive mice. Evaluation of lung histology confirmed that daily i.n. HDEst delivery during the OVA challenge period reduced total cellular accumulation, eosinophilic inflammation, mucous secretion, and goblet cell hyperplasia in and around the airways, with a 57% mean reduction in inflammation scores, compared with those of HDEst-nontreated mice (Fig. 2B). Although less sensitive and specific than invasive measures of bronchial hyperresponsiveness, which were unavailable at the time of these investigations, enhanced pause measurements further demonstrated that daily HDEst-treated mice were less responsive to inhaled Mch than HDEst-unexposed mice (Fig. 2C).

Compared with HDEst delivery by the daily low-dose schedule, intermittent high-dose HDEst delivery was less effective, but also attenuated features of the Th2-biased AHR. BALF analyses demonstrated 34 and 59% average reductions in total cell and eosinophil counts compared with BALF from HDEst-unexposed mice, whereas neutrophil counts rose 476%. In line with these findings, mean BALF eotaxin and KC levels for mice treated with intermittent high-dose HDEst were 53% lower and 225% higher, respectively, than for control mice. Lung sections from mice receiving HDEst only on airway OVA challenge days also displayed reductions in airway inflammation compared with those of HDEst-unexposed mice (average inflammation score reduced 34%), and their airways were less sensitive to Mch inhalation.

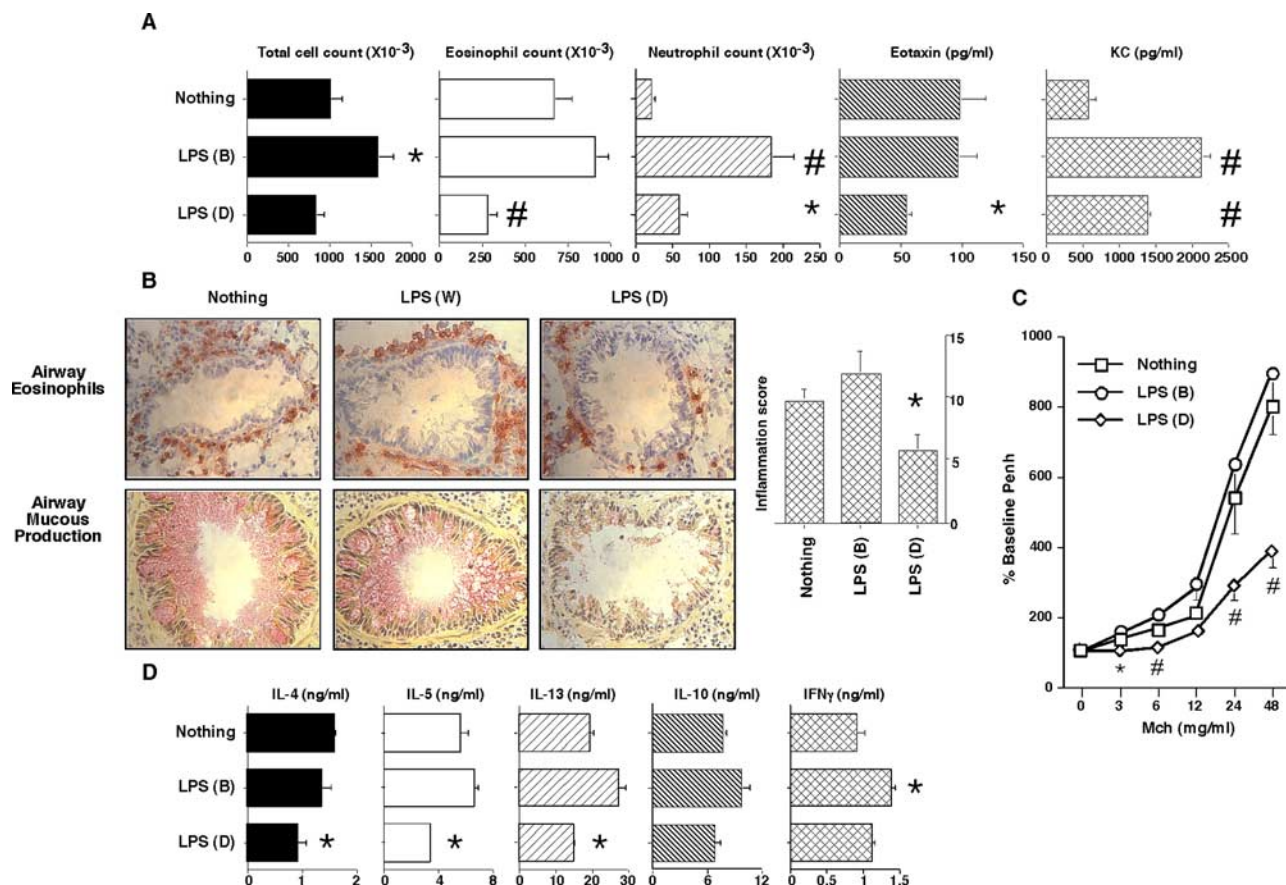


FIGURE 4. Airway LPS exposures modify allergen-induced AHRs. OVA/alum-sensitized BALB/c mice ($n = 4$ per group) were i.n. allergen challenged. Select groups of mice received i.n. LPS during the OVA challenge period in accordance with the bolus (B) and daily (D) delivery schedules described in Fig. 1A. AHRs and OVA-induced BLN cytokine responses were assessed 24 h later. Reported results were reproduced in two independent experiments, and bar and line graph data points present mean values with SEs. *, $p \leq 0.05$; #, $p \leq 0.01$, for LPS-treated vs LPS-untreated mice. A, BALF total cell, eosinophil, neutrophil, counts, and eotaxin and KC levels. B, Lung histology and inflammation scores. C, Airway Mch sensitivity. D, OVA-induced BLN cell cytokine production.

Consistent with changes noted in their AHRs, BLN cells from mice receiving daily i.n. HDEst during the airway allergen challenge period produced lower levels of the proasthmatic cytokines, IL-4 (19%), IL-5 (65%), and IL-13 (68%), and higher levels of cytokines antagonistic to the Th2-biased AHR, i.e., IL-10 (77%) and IFN- γ (220%), than BLN cells from control mice (Fig. 2D). In contrast, BLN cells from mice receiving intermittent high-dose HDEst with each challenge dose of OVA produced higher levels of IL-4 (104%), IL-5 (62%), IL-13 (49%), IL-10 (38%), and IFN- γ (370%) than BLN cells from HDEst-unexposed mice. Interestingly, despite reproducible and significant differences in cytokine production, at sacrifice, the OVA-specific serum IgE, IgG1, and IgG2a levels of mice from experimental and control groups were similar.

i.n. HDEst delivery during primary allergen challenges leads to persistent changes in airway allergen responsiveness

To determine whether the modulatory effects of daily and bolus i.n. HDEst exposures on AHRs were long-lived, mice described in Fig. 2 were not sacrificed after the primary airway allergen challenge period, but instead, were observed for an additional month before receiving a second round of OVA challenges without HDEst, as depicted in Fig. 1B. Although effects on primary challenge responses were more dramatic, after secondary allergen challenge, BALF from mice receiving daily i.n. HDEst continued to display reductions in total cell (22%) and eosinophil (50%) counts and

increases in neutrophil (115%) counts compared with HDEst-untreated mice (Fig. 3A). Likewise, lung sections from daily HDEst-treated mice had quantifiable reductions in airway inflammation (Fig. 3B; mean inflammation score reduced 43%) and their airway responses to Mch inhalation were attenuated (Fig. 3C) compared with HDEst-untreated mice. In contrast, mice receiving intermittent i.n. HDEst during the primary OVA challenge period had increased BALF total cell (22%) and eosinophil (10%) counts, and substantially increased neutrophil (215%) counts compared with control mice. However, little difference was appreciated in the lung section inflammation scores and Mch sensitivity of bolus HDEst-treated and HDEst-untreated mice.

In addition to long-lived modifications in their AHRs, OVA-stimulated BLN cells from Th2-sensitized mice receiving daily low-dose HDEst during the primary allergen challenge period produced 10–40% less IL-4, IL-5, IL-13, and IL-10 than BLN cells from HDEst-unexposed mice, whereas IFN- γ production was 42% higher. In contrast, BLN cells from Th2-sensitized mice exposed to high-dose HDEst intermittently during the primary OVA challenge period produced 33–92% higher levels of all cytokines measured than BLN cells from HDEst naive mice.

Bolus and daily i.n. LPS exposures attenuate and augment AHRs, respectively

Additional investigations determined whether the modulatory influence of HDEst on experimental asthma could be replicated with

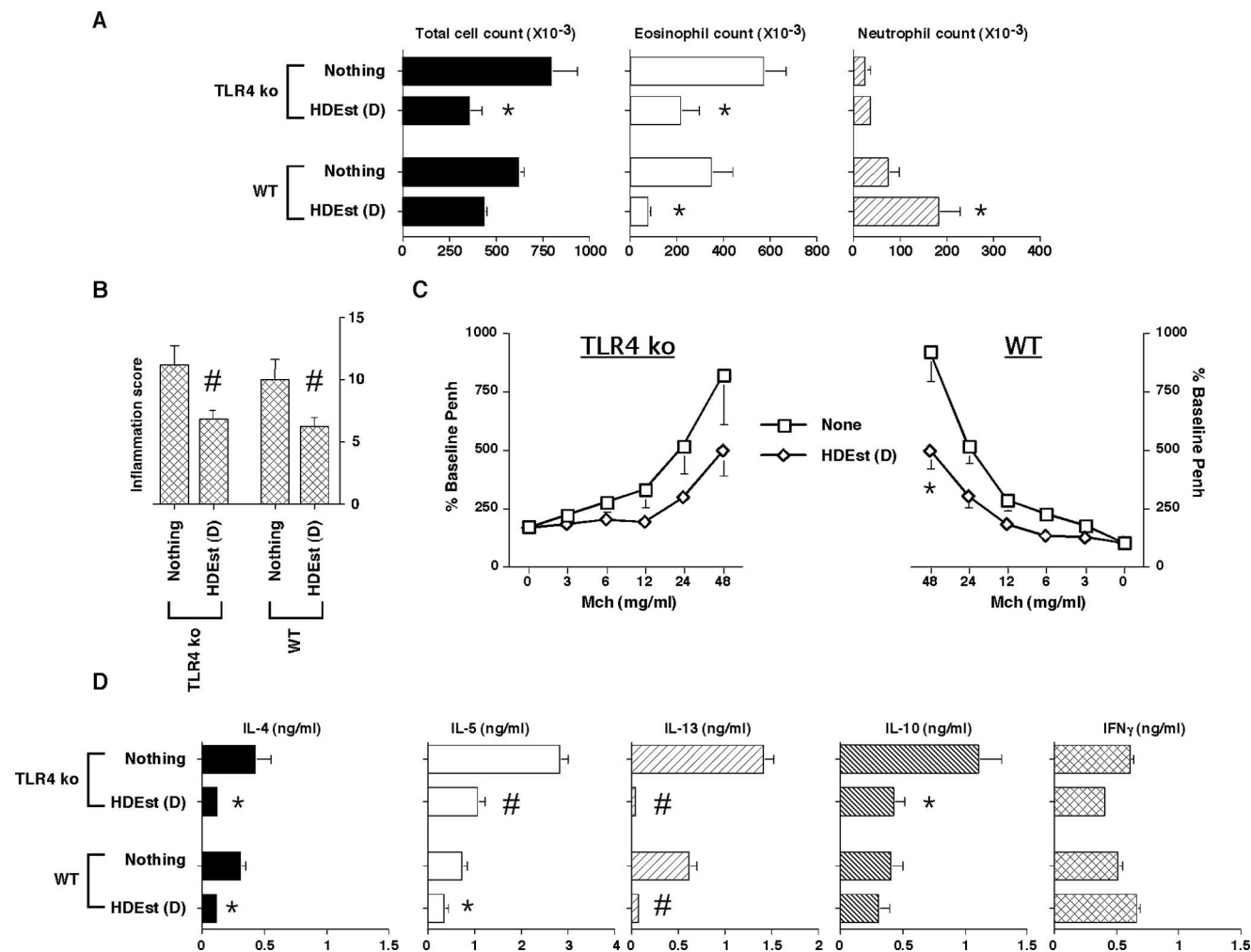


FIGURE 5. Daily airway HDEst exposures modify allergen-induced AHRs in TLR4-deficient mice. OVA/alum-sensitized WT (C57BL/6) and TLR4-deficient (C57BL/6 background) mice ($n = 4$ per group) were i.n. allergen challenged. Select mouse groups received i.n. HDEst during the OVA challenge period in accordance with the daily (D) delivery schedules described in Fig. 1A. AHRs and OVA-induced BLN cytokine responses were assessed 24 h later. Reported results were reproduced in a second experiment. Bar and line graph data points present mean values with SEs. *, $p \leq 0.05$; #, $p \leq 0.01$, for HDEst-treated vs HDEst-untreated mice. A, BALF total cell, eosinophil, and neutrophil counts. B, Lung inflammation scores. C, Airway Mch sensitivity. D, OVA-induced BLN cell cytokine production.

purified LPS. The endotoxin content of the HDEst was first determined by *limulus* lysate assay. Bolus (21 μ l) and daily (3 μ l) HDEst delivery doses used in Fig. 2 experiments were found to contain the equivalent of ~ 700 and 100 EU (70 and 10 ng) of LPS, respectively. Therefore, these LPS doses were used to conduct studies analogous to those executed with HDEst (Figs. 1A and 2). In one representative experiment, BALF samples from mice receiving daily i.n. LPS during the airway allergen challenge period had 17, 58, and 44% mean reductions in total cell and eosinophil counts and eotaxin levels, whereas neutrophil counts and KC levels increased by 163 and 141% compared with LPS-untreated mice, respectively (Fig. 4A). Histological evaluation of lung sections confirmed that daily LPS-treated mice had fewer inflammatory changes than LPS naive mice (Fig. 4B; average inflammation score reduced 43%), and pulmonary function testing demonstrated reductions in Mch sensitivity (Fig. 4C). Unlike daily LPS delivery, intermittent LPS delivery only on OVA challenge days consistently provoked increased BALF total cell (60%) and eosinophil counts (36%), and lung inflammation scores (23%), whereas BALF eotaxin levels and airway responses to inhaled Mch were similar to those of LPS-unexposed mice. Likewise, intermittent LPS delivery led to dramatic increases in allergen challenge-in-

duced BALF neutrophil (700%) counts and KC (269%) levels, compared with those of control mice.

Along with attenuating characteristic features of the Th2-biased AHR, daily LPS delivery was found to inhibit proinflammatory Th2 cytokine production by OVA-stimulated BLN cells harvested from experimental mice (IL-4, IL-5, and IL-13 responses reduced 45, 50, and 25%, respectively), whereas IL-10 production was relatively preserved and IFN- γ production increased 18% (Fig. 4D). In juxtaposition, with the exception of IL-4 (production reduced 17%), BLN cells from mice treated with LPS only on OVA challenge days produced modestly increased amounts of IL-5, IL-13, IL-10, and IFN- γ (15, 42, 28, and 52%, respectively) compared with BLN cells from LPS naive mice.

Daily i.n. HDEst exposures modify allergen-induced AHRs by TLR4-deficient mice

Despite determining the endotoxin content of HDEst and using LPS at equivalent doses, HDEst proved more effective than LPS at protecting against allergen-induced Th2 hypersensitivity responses, by both the daily and intermittent delivery schedules (Fig. 2 vs 4). These observations led us to speculate that aside from LPS, HDEst might contain additional immunostimulatory molecules

that contributed to its protective influence on Th2-biased airway hypersensitivities. To test this hypothesis, wild-type (WT; C57BL/6) and TLR4 ko (C57BL/6 background) mice were Th2 sensitized and challenged with OVA, as outlined in Fig. 1A, with experimental groups receiving daily low-dose or no HDEst during the airway challenge period, as in Fig. 2 experiments.

As seen with BALB/c mice, daily i.n. HDEst delivery attenuated the AHR of OVA-sensitized C57BL/6 mice undergoing airway OVA challenge (Fig. 5). Moreover, TLR4-deficient and competent mice treated with daily i.n. HDEst delivery had similar reductions in BALF total cell (56 vs 30%) and eosinophil (62 vs 80%) counts compared with their corresponding controls (Fig. 5A). However, daily HDEst exposures during the allergen challenge period elicited far smaller increases in BALF neutrophil counts in TLR4 ko than in WT mice (54 vs 148%). As in experiments presented earlier, histological analyses (Fig. 5B) and pulmonary function testing (Fig. 5C) confirmed that daily i.n. HDEst delivery reduced airway inflammation and bronchial hyperresponsiveness to Mch in both TLR4 ko and WT mice. Moreover, BLN cells from TLR4 ko and WT mice treated by daily i.n. HDEst delivery demonstrated similar changes in OVA-induced BLN cell cytokine production compared with their respective controls (Fig. 5D).

Discussion

These investigations considered how airway exposures to ambient allergen-nonspecific immunostimulants modify allergen-induced AHRs of previously Th2-sensitized mice. Study results established that local delivery of the immunostimulatory contents of living environments, in the form of HDEst, had a long-lived effect on airway responses to allergen challenge and allergen-specific immunity. Delivery schedule and dose proved important variables in determining how HDEst exposures impacted on relevant outcome variables. Additional experiments demonstrated that in addition to LPS, HDEst contained immunostimulatory molecules that modified allergen-induced AHRs by TLR4-independent mechanisms.

Experiments presented in Fig. 2 established that concurrent airway HDEst exposures during allergen challenge attenuated outcome measures of the Th2-biased AHR, while increasing airway neutrophilia. Recognizing that human airways are regularly exposed to the contents of HDEst, these findings may help explain why airway neutrophilia is far more prominent in asthma patients than in mouse models that depend on allergen/alum sensitization and subsequent airway challenges with allergen alone (30, 31). Furthermore, these experimental results highlight the impact that allergen-nonspecific immunostimulants ubiquitous in living environments can have on the functionality of cells that contribute to allergic asthma.

Another finding presented in Fig. 2 is that the same total dose of HDEst divided into 2 (bolus) or 14 (daily) treatments had qualitatively different modulatory effects on outcome measures in this murine experimental asthma model. For example, i.n. HDEst delivery by the daily schedule was consistently more effective than by the bolus schedule in attenuating all outcome measures associated with the Th2-biased AHR, whereas bolus delivery was more effective at inducing airway neutrophilia. Likewise, only daily HDEst delivery led to decreases in BLN Th2 cytokine production, whereas bolus HDEst delivery led to increased BLN cell production of all cytokines measured. These experimental results are of potential clinical relevance, because air-sampling studies demonstrate that although the content of endotoxin and other allergen-nonspecific immunostimulants in ambient air can vary by 5 logs or more, human airways are generally exposed to relatively low levels of ambient immunostimulants on a continuous basis (23, 32).

The cellular and molecular mechanisms by which HDEst exposures modify responses of Th2-sensitized mice undergoing concurrent airway allergen challenges require additional characterization. Nonetheless, we have observed that within hours of i.n. HDEst delivery, a “cytokine storm” develops in the airways of allergen naive mice. Cytokines released include IL-12 (13), IL-10, IL-17, and IL-23 (our unpublished observations), all of which have the potential to inhibit features of the Th2-biased AHR and/or promote neutrophil recruitment (33–35). Therefore, the local cytokine milieu created by airway HDEst delivery may temporarily inhibit airway responses to allergen challenge. In support of this view, a previous report found that ISS-ODN exposures inhibited allergen-induced conjunctivitis by an IL-12-dependent mechanism (16). Airway ISS-ODN delivery during allergen challenge has also been shown to compromise the ability of resident dendritic cells to present Ag and support Th2 effector cell responses (36). Given that dendritic cell activation by HDEs is largely TLR dependent (28), it is reasonable to suggest that airway HDE exposures may also inhibit Th2-biased AHRs by modifying the functional characteristics of dendritic cells and other APCs within the lungs and their draining lymph nodes. Alternatively, a growing body of literature suggests that CD4 cells and, in particular, T regulatory cells and activated effector CD4 cells, express TLRs and can respond directly to TLR ligands (37–39). Therefore, the capacity of HDEs to modify the allergen-induced AHR could in part be due to direct effects on TLR-expressing CD4 cells. These considerations are the focus of ongoing and planned investigations.

Additional experiments demonstrated that compared with HDEst-unexposed mice, Th2-sensitized mice receiving daily i.n. HDEst during a primary series of airway allergen challenges continued to display modest reductions in all outcome measures of the Th2 AHR and elevated BALF neutrophil counts when challenged with allergen alone, 1 mo later. In contrast, secondary airway allergen challenge outcome measures of mice receiving bolus HDEst during the initial challenge period were equivalent to or greater than those of control mice. Consistent with these findings, BLN cells from daily and bolus HDEst-exposed mice continued to have attenuated and augmented Th2 cytokine responses after secondary airway allergen challenge, respectively. Design considerations limited the number of OVA/HDEst coexposures given to mice in these experiments. Nonetheless, we speculate that immunological themes identified in these studies could have a far more profound influence on clinical manifestations of allergic asthma for patients exposed to ubiquitous aeroallergens and allergen-nonspecific immunostimulants on a semicontinuous basis.

Despite mimicking the antiasthmatic activities of HDEst, when delivered at endotoxin dose equivalence, LPS was found to be generally less effective at attenuating outcome measures of the Th2-biased AHR (Fig. 4 vs Fig. 2). This suggested HDEst might contain molecules capable of modifying the AHR by TLR4-independent mechanisms. The impression was confirmed in a final series of experiments in which daily HDEst delivery was observed to be highly effective in attenuating outcome measures of experimental asthma and the BLN cell Th2 cytokine responses of TLR4 ko mice. However, BALF neutrophil increases associated with daily HDEst delivery in WT mice were greatly attenuated in TLR4 ko mice. These results established that LPS is not the only immunostimulatory molecule within HDEst responsible for its protective influence on the Th2-biased AHR, but that it had a major role in recruiting neutrophils to the airways of mice treated with HDEst.

Experimental results presented in this paper demonstrate that airway exposures to allergen-nonspecific immunostimulants contained in HDEst and ubiquitous in living environments modify the allergen-induced AHR of Th2-sensitized mice for 1 mo or more.

We previously published that dendritic cells respond to HDEs by mechanisms that are partially TLR2, TLR4, and TLR9, and largely MyD88 dependent (28). Additional studies demonstrated that weekly airway HDE delivery provided MyD88-dependent Th2 adjuvant activity in naive mice receiving concurrent i.n. OVA vaccinations, whereas daily HDE delivery promoted both short-term (13) and long-term (our unpublished observations) OVA-specific tolerance. These observations are consistent with results described in this study, and lead us to suggest that the immunomodulatory potential of living environments is a sword that cuts both ways in the natural history of allergic respiratory diseases. Our ongoing investigations suggest that the absolute level and frequency of airway exposures to ambient TLR ligands and potentially other allergen-nonspecific immunostimulants will prove critically important variables in determining their net influence on the genesis and duration of aeroallergen-driven diseases.

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Disclosures

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