House dust bioactivities predict skin prick test reactivity for children with high risk of allergy

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Background: Although evidence suggests that ambient exposures to endotoxin and other immunostimulants during early life influence allergic risk, efforts to understand this host-environment relationship have been hampered by a paucity of relevant assays.

Objectives: These investigations determined whether parameters of house dust extract (HDE) bioactivity were predictive of allergen skin prick test (SPT) reactivity for infants at high risk of allergy participating in the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS).

Methods: We conducted a nested case-control study, selecting 99 CCAAPS children who had positive SPT results to at least 1 aeroallergen at age 3 years and 101 subjects with negative SPT results. HDEs were prepared from dust samples collected from the subjects' homes at age 1 year. Murine splenocytes and bone marrow–derived dendritic cells were incubated with HDEs, and supernatant cytokine concentrations were determined by means of ELISA. Alternatively, bone marrow–derived dendritic cells were preincubated with HDEs, and then LPS-induced IL-6 responses were assessed. HDE endotoxin levels were determined by using the limulus amebocyte lysate assay.

Results: HDEs derived from the homes of children with positive (cases) and negative (control subjects) SPT results had similar bioactivities. However, when cases were considered in isolation, HDEs with higher levels of bioactivity were significantly associated with children who had lower numbers of positive SPT results. Analogous statistical analyses did not identify any association between HDE endotoxin levels and the aeroallergen sensitization profiles of children included in this study.

Conclusion: HDE immunostimulatory activities predicted the aeroallergen sensitization status of CCAAPS subjects better than HDE endotoxin levels. These results provide the first published evidence that HDE bioassays have clinical relevance in predicting atopic risk. (J Allergy Clin Immunol 2012;129:1529-37.)

Key words: *House dust extract, aeroallergen sensitization, tolerance, allergy, skin prick test, human, innate immunity*

Allergic diseases have become far more common in industrialized countries in recent decades, whereas atopy rates remain low in underdeveloped countries.[1,2] Although reasons for these trends remain speculative, the rapidity with which allergic disease prevalence has increased in affected countries strongly suggests environmental factors are responsible. Adaptive responses associated with allergen tolerance and hypersensitivity appear to become imprinted early in life.[3,4] Therefore because infants/toddlers spend a majority of their time indoors, there is a great deal of interest in determining how home exposures affect allergic risk.

It is generally accepted that allergen exposure is a prerequisite for sensitization, and for some allergens (ie, cockroach and house dust mite), the risk of hypersensitivities increases significantly when levels in the home exceed a quantifiable threshold.[5-7] However, for other allergens (ie, dogs and cats), increased levels of home exposure have been linked to a decreased risk of sensitization, both to the allergen of interest and to unrelated allergens.[7-10] These and other findings suggest that aside from allergens themselves, living environments contain additional molecules that influence the immunologic balance between allergen tolerance and hypersensitivity.

Endotoxin (LPS), a molecule that activates immunocytes through Toll-like receptor (TLR) 4, has been reported to be present at higher concentrations in homes with regular animal exposures than in homes with none.[11-13] Moreover, in several but not all published investigations, infants raised in high-endotoxin homes were found to have a reduced incidence of atopic stigmata.[12,14] In consideration of these inconsistent results, it is important to recognize that in addition to endotoxin, living environments contain a variety of other immunostimulatory materials of microbial and nonmicrobial origin.[15-18]

Unfortunately, aside from endotoxin, there is little known about the potential for other house dust constituents to activate the innate immune system. Therefore, as an alternative approach to quantifying the immunomodulatory potential of living environments, our laboratory has begun to investigate the capacity of sterile house dust extracts (HDEs) to activate mononuclear cells. We previously published that HDEs contain molecules that induce murine bone marrow–derived dendritic cell (BMDDC) cytokine production by pathways that are partially dependent on signaling through TLR2, TLR4, and TLR9 and largely dependent on MyD88, a molecule required for signaling through all TLRs except TLR3.[16] The magnitude of the BMDDC cytokine response was highly dependent on the concentration of HDE used. Moreover, at a fixed concentration, HDEs derived from different homes elicited cytokine responses that varied in magnitude over a 2- to 3-log range, suggesting a high degree of variability in the

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Abbreviations used
ANCOVA: Analysis of covariance
aOR: Adjusted odds ratio
aRR: Adjusted rate ratio
BMDDC: Bone marrow–derived dendritic cell
CCAAPS: Cincinnati Childhood Allergy and Air Pollution Study
HDE: House dust extract
OVA: Ovalbumin
SPT: Skin prick test
TLR: Toll-like receptor
Treg: Regulatory T

immunostimulatory potential of different living environments. Recently, we discovered that along with TLR ligands, a majority of HDEs tested contained antigens that activated invariant natural killer T cells in a TLR-independent but CD1d-dependent manner.[15]

Like purified TLR ligands, HDEs were found to be potent mucosal adjuvants for antigens delivered through the intranasal route.[19,20] However, in a murine model more reflective of daily environmental immunostimulant exposures, mice exposed to HDEs through the airways on a daily basis had long-lived allergen tolerance.[19,20] In analogous experiments LPS, Pam-3-Cys (TLR2), and CpG motifs (TLR9) shared this tolerogenic potential with HDEs (unpublished data).[19,20] These observations might help explain why farm homes and other living environments that contain high concentrations of microbial products protect against atopy. Moreover, they suggest that the relative protective influence of living environments on the genesis of allergic diseases might be far more dependent on their total immunostimulatory load than on their specific content of individual molecules. Therefore the current studies determined whether the magnitude of splenocyte and BMDDC responses to HDEs derived from the homes of infants at high risk of allergy at age 1 year would predict their skin prick test reactivity to common aeroallergens at age 3 years.

METHODS

The Cincinnati Childhood Allergy and Air Pollution Study cohort

The Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) is a prospective epidemiologic study of children at risk for atopy that has been described in detail previously.[13,21,22] Briefly, infants were identified from birth records from the greater Cincinnati metropolitan area between 2001 and 2003. Parents were screened for allergy and asthma symptoms with a 12-question interview and underwent skin prick tests (SPTs) with a panel of aeroallergens. If 1 or both parents had symptoms of eczema, rhinitis, or asthma and had at least 1 positive SPT result, their infant was invited into the study. At a mean age of 9 ± 2.8 months of age, 779 infants had home visits by trained members of the CCAAPS team, and dust samples were collected from the child's area of primary activity. At a mean age of 3.1 years, 646 children returned to the clinic and had SPTs placed for the same 15 aeroallergens used to screen parents. All parents whose infants participated in the CCAAPS provided informed consent before enrollment, and the study was approved by the University of Cincinnati Institutional Review Board. Children had to have no change of address between the year 1 home visit and their year 3 SPT and had to have at least 400 mg of their original house dust sample available for analyses to be included in this study. Children were assigned case (positive SPT result or results) or control (negative SPT results) status according to the results of their 3-year SPTs. Ninety-nine cases and 101 control subjects were randomly chosen from 268 children eligible for this study to approximate equal numbers in each group.

Allergen SPTs and clinical assessments

During screening, parents of potential CCAAPS participants underwent SPTs with 15 aeroallergens endemic in Cincinnati, including white oak, elm, maple mix, eastern red cedar, fescue and timothy grasses, short ragweed, 4 mold allergens (*Alternaria alternata*, *Aspergillus fumigatus*, *Penicillium* species mix, and *Cladosporium* species), cat, dog, house dust mite mix (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), and German cockroach (ALK-Abelló, Hørsholm, Denmark). The same allergen SPTs were performed on CCAAPS children at age 3 years. All SPTs were placed with a bifurcated device (Accusets; ALK-Abelló). A positive SPT result was defined as a wheal at least 3 mm larger than that elicited by the negative saline control. In addition to allergen SPTs, CCAAPS participants underwent a clinical evaluation by a study physician who categorized subjects as unlikely, possibly, probably, or definitely having eczema, asthma, or both.

HDE preparation

Methods used for the collection and processing of house dust samples have been described previously.[13] Briefly, during year 1 CCAAPS home visits, house dust samples were collected with a Filter Queen Majestic vacuum cleaner (Health-Mor; HMI Industries, Inc, Seven Hills, Ohio) from the floor of the infant's primary activity room by vacuuming a 2-m^2 area at a rate of 2 min/m^2. After collection, samples were sieved with a pyrogen-free 355-μm mesh screen. Filtered dust was stored desiccated at −20°C until further analysis. Collected house dust was run through a course sieve to remove large particulate matter and suspended in sterile PBS at 100 mg/mL to prepare HDEs. House dust suspensions were placed on a rotor at room temperature for 24 hours and then filtered through 0.22-μm Steriflip filters (Millipore, Bedford, Mass) to obtain sterile HDEs. In all studies presented herein, HDE concentrations refer to the amount of house dust added per milliliter of PBS suspension before filtration.

Murine bioassays

Splenocytes and BMDDCs (GM-CSF derived) were prepared from BALB/c mice by using standard methods. For splenocyte assays, mononuclear cells were cultured in triplicate at 2.5×10^6 cells/mL in media alone or with 1 of the 200 CCAAPS HDEs included in these analyses at a final concentration of 0.01 to 1.0 mg/mL. Supernatants were harvested at 48 hours, and IFN-γ levels were determined with standard ELISA techniques and commercial reagents (PharMingen, San Jose, Calif). In analogous assays BMDDCs were cultured in triplicate at 1×10^6 cells/mL in media supplemented with GM-CSF (5 ng/mL; R&D Systems, Minneapolis, Minn) with or without CCAAPS HDEs (0.01-1 mg/mL). Supernatants from BMDDC cultures were harvested at 24 hours, and IL-6 and IL-12p40 levels were determined by using ELISA with PharMingen reagents. For LPS tolerance assays, BMDDCs were cultured in triplicate at 1×10^6 cells/mL in GM-CSF–supplemented media with or without CCAAPS HDEs (0.01-1 mg/mL). After 24 hours, cells were washed and resuspended in fresh media with LPS (100 ng/mL). After an additional 24 hours, supernatant IL-6 levels were determined by means of ELISA.

House dust endotoxin levels

House dust endotoxin concentrations were determined by using the limulus amebocyte lysate assay (Associates of Cape Cod Inc, Falmouth, Mass), according to methods described by Milton et al.[23] All glassware and materials used were endotoxin and pyrogen free.

Data analysis

Exploratory analyses demonstrated that cytokine and endotoxin levels were approximately $\log_e$ normally distributed. Therefore $\log_e$-transformed data were used for all statistical analyses. A 2-part hurdle regression model determined whether parameters of HDE bioactivity and HDE endotoxin content

predicted the case-control status of study subjects (adjusted odds ratios [aORs]), the number of allergens to which children with positive SPT results were allergic (adjusted rate ratios [aRRs]), or both. For these analyses, parameters of HDE bioactivity and HDE endotoxin levels were considered the independent variable. The first part of the hurdle model was used to conduct logistic regression comparisons of HDE parameters associated with children with positive and negative SPT results adjusted for the following patient characteristics: age at the time of SPTs, race (African American vs white or "other"), and sex. The second part of the hurdle model used Poisson regression analyses of association between measures of HDE bioactivity or HDE endotoxin levels and the number of positive SPT results for cases adjusted for race, the only variable found to significantly affect SPT reactivity in the logistic regression model. Analyses of covariance (ANCOVAs) were used to compare geometric mean HDE bioactivity measures and HDE endotoxin levels of case and control subjects adjusted for age, race, and sex. ANCOVAs were also conducted for HDEs associated with subjects with positive SPT results, with SPT reactivity as a 4-category variable (1, 2, 3, and ≥4 positive SPT results). Again, age, race, and sex were included as covariates. We set up a contingency table and conducted a χ^2 analysis to assess eczema and asthma prevalence as a function of SPT reactivity. These analyses were performed with SAS 9.2 software (SAS Institute, Inc, Cary, NC). For analyses of correlation, Pearson coefficients of correlation (r values) were calculated, and the significance of these values was determined with 2-tailed t tests by using Statview software. Only P values of .05 or less were considered significant.

RESULTS

Characteristics of the CCAAPS study cohort

The demographics of the study cohort are presented in Table I. A total of 99 CCAAPS children with 1 or more positive SPT results (cases) and 101 control subjects with negative SPT results and their associated HDEs were included in these investigations. The mean ages of subjects with positive and negative SPT results were 3.05 years (range, 2.29-3.64 years) and 3.03 years (range 2.00-3.50 years), respectively. Of the subjects with positive SPT results, 54.5% were male, whereas 51.5% of the subjects with negative SPT results were male. African Americans represented 22.2% of the children with positive SPT results and 12.9% of the children with negative SPT results. On average, children with positive SPT results were reactive to 2.05 of the 15 aeroallergens in the SPT panel. Only 5 of the 47 children with 2 or more positive SPT results reacted to a single family of allergens. The number of positive SPT results was similar for male and female subjects, but on average, African Americans reacted to 33.5% more allergens than non–African Americans.

TABLE I. Demographics of the study groups with positive and negative SPT results

	Cases (≥1 positive SPT results)	Control subjects (negative SPT results)
No. of children	99	101
Age (y)	3.05 ± 0.13 (2.29-3.64)	3.03 ± 0.14 (2.00-3.50)
Sex (male/female)	54/45	52/49
Race (African American/white or other)	22/77	13/88
Mean no. of positive SPT results	2.05 ± 1.4 (1.0-7.0)	NA
Male	2.06 ± 1.48	
Female	2.04 ± 1.31	
African American	2.55 ± 1.57	
White or other	1.91 ± 1.33	

NA, Not applicable.

Levels of HDE-induced splenocyte IFN-γ production predict trends in SPT reactivity for CCAAPS children with positive SPT results but not those with negative SPT results

In pilot studies splenocytes were cultured with HDEs (0.01-1 mg/mL) to determine which cytokines were produced. None of the HDEs elicited IL-4, IL-5, or IL-13 responses, whereas about half induced detectable levels of IL-6, IL-10, and/or IL-12p40 production at higher HDE concentrations, and the vast majority elicited the production of IFN-γ. Although the magnitude of IFN-γ responses was HDE concentration dependent, the range of responses detected was greatest when splenocytes were stimulated with HDEs at the lowest concentration tested (0.01 mg/mL). Therefore this concentration was the focus of analyses in subsequent experiments with all 200 HDEs. However, in conflict with our working hypothesis, HDEs derived from the homes of CCAAPS children with positive SPT results and CCAAPS children with negative SPT results were found to induce similar mean splenocyte IFN-γ responses (0.54 and 0.41 ng/mL, respectively), and differences were not significant by means of ANCOVA (Fig 1, *A*). Likewise, levels of HDE-induced IFN-γ production by splenocytes did not predict case or control status (aOR, 1.05; 95% CI, 0.93-1.20; not significant).

One potential confounding variable in these analyses stems from the rigorous criteria used to select infants at high risk of allergy for CCAAPS participation coupled with the fact that allergic risk is largely inherited. Therefore it stands to reason that subjects identified as having positive and negative SPT results at 3 years of age would be more and less likely to have inherited their parent's genetic tendencies toward atopy, respectively. HDE-induced splenic IFN-γ responses of CCAAPS subjects with positive SPT results were also assessed in isolation to partially control for this. In this case significant inverse trends were found between HDE-induced IFN-γ responses and the number of allergens to which these children reacted (Fig 1, *B*). Mean IFN-γ responses for HDEs associated with children with 1 and 4 or more positive SPT results were 0.83 and 0.18 ng/mL, respectively ($P = .02$, ANCOVA), and the overall aRR for SPT reactivity in CCAAPS subjects as a function of HDE-induced IFN-γ production was 0.90 (95% CI, 0.83-0.97; $P = .005$). These findings provide direct evidence to support the view that higher levels of ambient immunostimulant exposure limits the number of allergens to which infants at high risk of allergy become sensitized.

Levels of HDE-induced BMDDC cytokine production predict trends in SPT reactivity for CCAAPS children with positive SPT results but not those with negative SPT results

In pilot studies about half of the HDEs tested (n = 20) induced detectable IL-10, IL-12p70, and/or IFN-γ production by BMDDCs, but almost all induced the production of IL-12p40 and IL-6. Therefore we next determined whether case and control HDEs induced IL-12p40 and IL-6 responses that were significantly different. As shown in Fig 2, *A*, mean IL-12p40 production levels induced by HDEs from the homes of subjects with positive SPT results were higher than those induced by HDEs associated with subjects with negative SPT results (6.26 vs 4.63 ng/mL, respectively), as were IL-6 responses (6.35 vs 3.0 ng/mL, respectively), but differences were not significant by means of

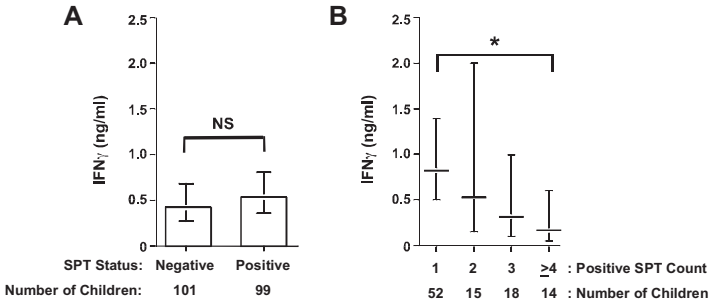


FIG 1. Associations between SPT status and HDE-induced IFN-γ production by splenocytes. IFN-γ levels are presented as geometric means ± 95% CIs and compared by using ANOVA. IFN-γ responses of subjects with positive SPT results (case) and subjects with negative SPT results (control subjects) were further analyzed with a 2-part hurdle regression model. **A,** Differences in IFN-γ responses of case and control HDEs and the aOR of being a case based on HDE-induced IFN-γ production were not significant (*NS*; aOR, 1.05; 95% CI, 0.93-1.20). **B,** *HDE-induced IFN-γ responses associated with subjects having 1 and 4 or more positive SPT results were significantly different ($P = .02$). There was also a significant inverse trend between a subject's positive SPT result count and the magnitude of the IFN-γ response induced by their associated HDEs (aRR, 0.90; 95% CI, 0.83-0.97; $P = .005$).

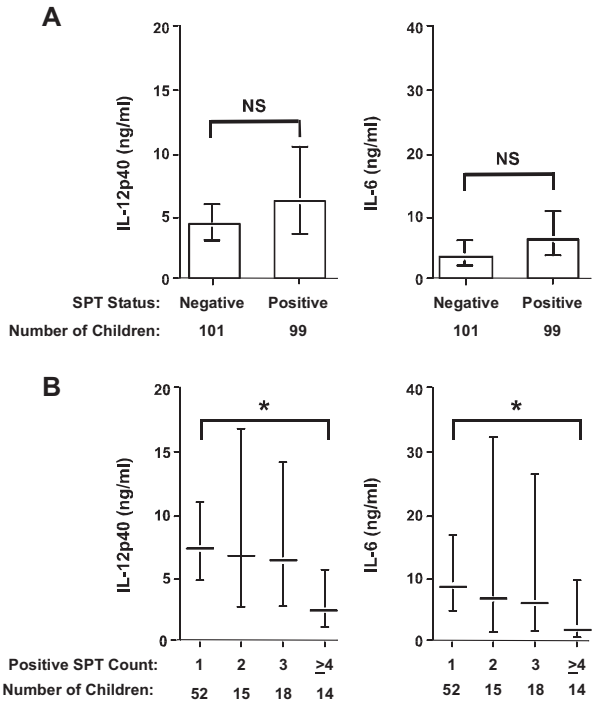


FIG 2. Associations between SPT status and HDE-induced IL-12p40 and IL-6 production by BMDDCs. Cytokine levels are presented as geometric means ± 95% CIs and were compared by using ANOVA. Cytokine responses of subjects with positive SPT results (cases) and subjects with negative SPT results (control subjects) were further analyzed with a 2-part hurdle regression model. **A,** Differences in cytokine responses of case and control HDEs and the aOR of being a case based on HDE-induced IL-12p40 and IL-6 production were not significant (*NS*; aOR for IL-12p40, 1.13 [95% CI, 0.94-1.37]; aOR for IL-6, 1.10 [95% CI, 0.99-1.22]). **B,** *HDE-induced IL-12p40 and IL-6 responses associated with subjects having 1 and 4 or more positive SPT results were significantly different (IL-12p40, $P = .02$; IL-6, $P = .07$). There were also significant inverse trends between a subject's positive SPT result count and the magnitude of the IL-12p40 and IL-6 responses induced by their associated HDEs (aRR for IL-12p40, 0.88 [95% CI, 0.79-0.99; $P = .034$]; aOR for IL-6, 0.94 [95% CI, 0.89-0.99; $P = .03$]).

ANCOVA. Likewise, the aOR was not significant for being a case versus control based on BMDDC IL-12p40 (aOR, 1.13; 95% CI, 0.94-1.37) or IL-6 (aOR, 1.10; 95% CI, 0.99-1.22; not significant) production. In contrast, when subjects with positive SPT results were considered in isolation, significant inverse trends were seen between levels of HDE-induced BMDDC cytokine production and the number of positive SPT results (Fig 2, *B*). For example, HDEs linked to children with only 1 positive SPT result induced geometric mean IL-12p40 and IL-6 responses of 6.83 and 8.07 ng/mL, respectively, whereas HDEs associated with subjects with 4 or more positive SPT results induced geometric mean IL-12p40 responses of 2.61 ng/mL ($P = .02$, ANCOVA) and IL-6 responses of 1.69 ng/mL ($P = .07$, ANCOVA). Moreover, the overall aRRs for SPT reactivity in CCAAPS subjects as a function of HDE-induced IL-12p40 and IL-6 production were 0.88 (95% CI, 0.79-0.99; $P = .034$) and 0.94 (95% CI, 0.89-0.99; $P = .03$), respectively. These findings provide additional evidence that for infants at high risk of allergy who have aeroallergen hypersensitivities, higher levels of ambient immunostimulant exposure decreases the number of aeroallergens to which they ultimately become sensitized.

Levels of LPS-induced IL-6 production after preincubation with HDEs predict trends in SPT reactivity for CCAAPS children with positive SPT results but not those with negative SPT results

Bioassays presented in the first 2 figures of this article demonstrate the capacity of HDEs to act as immunostimulants. However, in addition to inducing cytokine production on primary exposure, many immunostimulant exposures render cells hyporesponsive to subsequent provocation by the same or a different stimulus.[24,25] We hypothesized that the inhibitory influence of HDE preincubation on BMDDC responsiveness to subsequent LPS stimulation might be another parameter of HDE bioactivity that would predict the SPT reactivity profiles of CCAAPS subjects. To test this theory, BMDDCs were first incubated with HDEs for 24 hours, as in the Fig 1 studies. BMDDCs were then washed and restimulated with LPS for 24 hours before supernatants were harvested to determine their cytokine content. As with previous analyses, preincubation of BMDDCs with case and control HDEs had similar effects on subsequent LPS-induced IL-6 production (11.16 and 11.64 ng/mL, respectively; not significant by means of ANCOVA; Fig 3, *A*), and the aOR

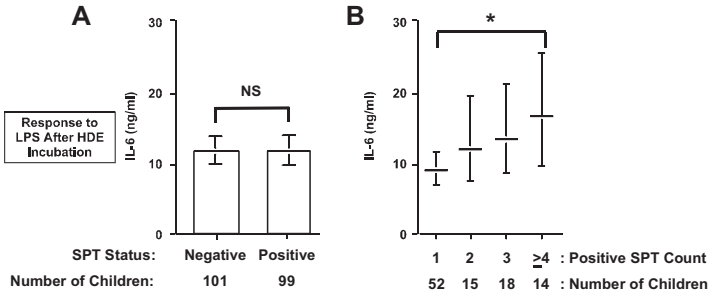


FIG 3. Associations between SPT status and LPS-induced IL-6 production after preincubation with HDEs. IL-6 levels are presented as geometric means ± 95% CIs and were compared by using ANOVA. IL-6 responses in subjects with positive SPT results (cases) and subjects with negative SPT results (control subjects) were further analyzed with a 2-part hurdle regression model. **A,** Differences in IL-6 responses of case and control HDEs and the aOR of being a case based on LPS-induced BMDDC IL-6 production after preincubation with HDE were not significant (*NS*; aOR, 0.96; 95% CI, 0.69-1.33). **B,** *LPS-induced IL-6 responses after preincubation with HDEs associated with subjects having 1 and 4 or more positive SPT results were significantly different ($P = .05$). There was also a significant inverse trend between a subject's positive SPT result count and the magnitude of the LPS-induced IL-6 response induced after preincubation with their associated HDEs (aRR, 1.24; 95% CI, 1.02-1.50; $P = .03$).

of being a case versus control based on this outcome measure of HDE bioactivity was 0.96 (95% CI, 0.69-1.33; not significant). In contrast, for case HDEs (Fig 3, *B*), geometric mean LPS-induced IL-6 responses for BMDDCs preincubated with HDEs associated with subjects having 1 and 4 or more positive SPT results were 9.71 and 16.4 ng/mL, respectively ($P = .05$, ANCOVA). The overall aRR for CCAAPS subject SPT reactivity as a function HDE-mediated inhibition of LPS-induced IL-6 production was 1.24 (95% CI. 1.02-1.50; $P = .03$). In a previous study children living in homes with high endotoxin loads were found to be at reduced risk for atopic asthma, and their PBMCs were hyporesponsive to LPS stimulation.[12] The current analyses offer additional evidence that living environments that better protect against the development of allergen SPT reactivity also better attenuate innate responsiveness to LPS stimulation.

Eczema and asthma prevalence is highest for CCAAPS subjects with 4 or more positive SPT results

Although case and control subjects were selected based on their SPT status, they were also assessed by a study physician at their year 3 CCAAPS clinic visit. By blinded assessment, 22.3% of cases and 21.8% of control subjects were thought to have possible, probable, or definite eczema (see Table E1 in this article's Online Repository at www.jacionline.org). Additionally, although none of the children were given a diagnosis of definite asthma, 14.6% and 13.5% of cases and control subjects, respectively, were considered to possibly or probably have asthma (see Table E2 in this article's Online Repository at www.jacionline.org). In contrast, on the basis of these same diagnostic criteria, 38.5% of the children with 4 or more positive SPT results had eczema, and 38.5% had asthma. Although not statistically significant, these trends suggest that in addition to protecting against aeroallergen hypersensitivity, living environments with high levels of immunostimulatory activity protect against other clinically relevant manifestations of atopy. Moreover, these findings are consistent with previous reports that allergen-polysensitized children are more likely to manifest additional atopic manifestations than children sensitized to 1 or just a few aeroallergens.[26,27]

HDE endotoxin levels do not predict trends in SPT reactivity for CCAAPS children

Many, but not all, infant observational studies have shown that house dust endotoxin levels are predictive of allergic risk.[11-13,28-32] Therefore the geometric mean endotoxin levels of HDEs associated with CCAAPS children with positive SPT results and CCAAPS children with negative SPT results were compared (Fig 4, *A*). As with previously discussed bioassays, HDEs associated with children with positive SPT results were found to have higher endotoxin levels than those associated with children with negative SPT results (78.09 and 66.48 EU/mg dust, respectively), but differences were not significant by means of ANOVA. HDE endotoxin levels also did not predict case or control status (aOR, 1.12; 95% CI, 0.87-1.45). However, unlike the previously described bioassays, when CCAAPS subjects with positive SPT results were analyzed in isolation, no trend was found between HDE endotoxin levels and the number of positive SPT results in these subjects (aRR, 1.05; 95% CI, 0.90-1.23; not significant; Fig 4, *B*). Considered with results presented in Figs 1 to 3, this finding suggests that HDE bioactivity assays might be superior to HDE endotoxin assays in their ability to predict the allergic status of infants raised in their homes of origin.

Significant correlations exist between CCAAPS HDE-induced cytokine responses

Because all HDE bioactivity measures displayed similar association characteristics with the SPT status of CCAAPS children, we next determined whether these HDE bioactivity measures correlated with one another. As shown in Fig 5, significant correlations were found between BMDDC IL-12p40 versus splenocyte IFN-γ responses ($r = 0.151$, $P = .032$), BMDDC IL-6 versus splenocyte IFN-γ responses ($r = 0.188$, $P = .007$), BMDDC IL-12p40 versus IL-6 responses ($r = 0.751$, $P < .0001$), and BMDDC IL-6 responses to HDEs versus LPS-induced IL-6 responses after preincubation with HDEs ($r = -0.585$, $P < .0001$). These correlations support the view that the bioassays under investigation were responding to the same immunostimulatory molecules within HDEs or that a majority of the

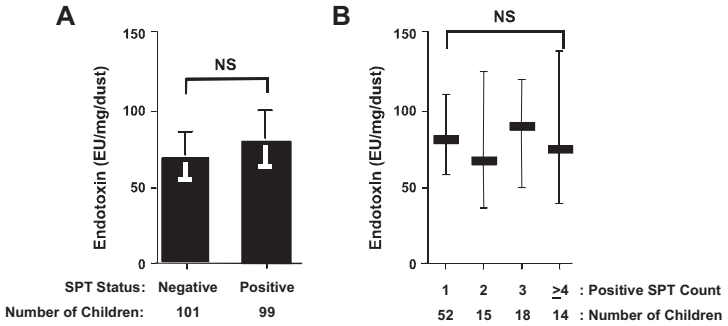


FIG 4. Associations between SPT status and HDE endotoxin content. HDE endotoxin levels are presented as geometric means ± 95% CIs and were compared by using ANOVA. HDE endotoxin levels in subjects with positive SPT results (cases) and subjects with negative SPT results (control subjects) were further analyzed with a 2-part hurdle regression model. **A,** Differences in geometric mean endotoxin levels of case and control HDEs and the aOR of being a case based on HDE endotoxin levels were not significant (*NS*; aOR, 1.12; 95% CI, 0.87-1.45). **B,** Differences in the endotoxin levels of HDEs associated with subjects having 1 and 4 or more positive SPT results were not significant *(NS)*. The correlation between the number of SPTs to which subjects with positive SPT results reacted and the endotoxin content of their associated HDEs was also not significant (aRR, 1.05; 95% CI, 0.90-1.23).

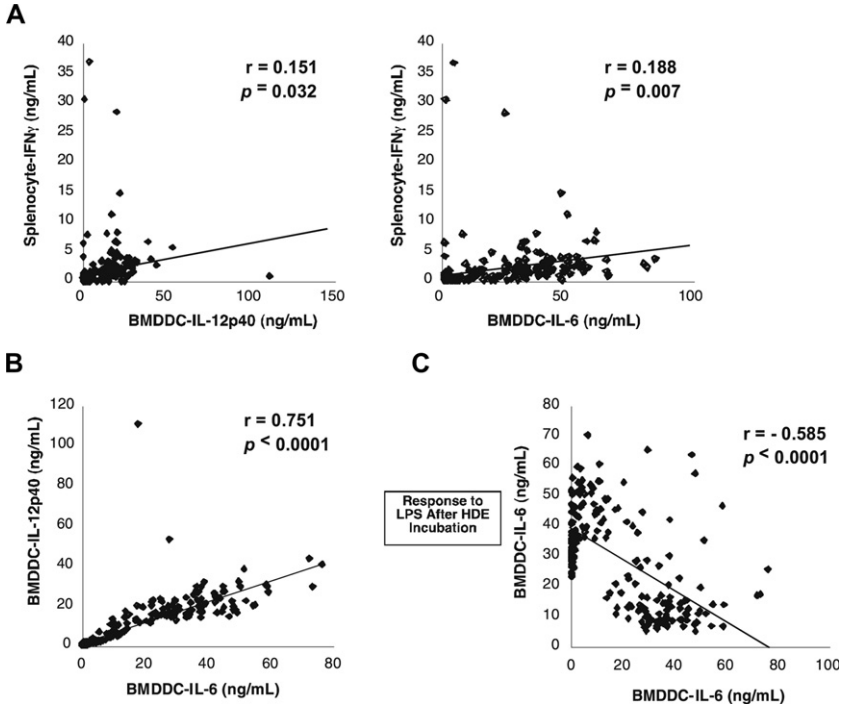


FIG 5. Correlations between measures of HDE bioactivity. **A,** HDE-induced BMDDC IL-6 and IL-12p40 responses were compared with HDE-induced splenocyte IFN-γ responses. **B,** HDE-induced BMDDC IL-6 and IL-12p40 responses were compared with each other. **C,** HDE-induced IL-6 production by BMDDCs was compared with LPS-induced IL-6 production after HDE desensitization. Pearson correlation coefficients *(r)* and *P* values were calculated by using standard methods.

immunostimulants ubiquitous in homes induce similar patterns of immune activation and LPS tolerance.

CCAAPS subjects' HDE-induced cytokine responses do not correlate strongly or significantly with their endotoxin content

Endotoxin is ubiquitously distributed and a very potent immunostimulant.[1,12] Moreover, investigators interested in studying the immunostimulatory potential of living environments have often focused on endotoxin content because assays are commercially available and associations between home endotoxin levels and allergic status have been established.[11-13,28-32] Therefore it might be assumed that the bioassays described in Figs 1 to 3 were responding primarily to endotoxin contained within the HDEs. Given these considerations, additional statistical analyses were performed to determine the strength of associations between HDE-induced

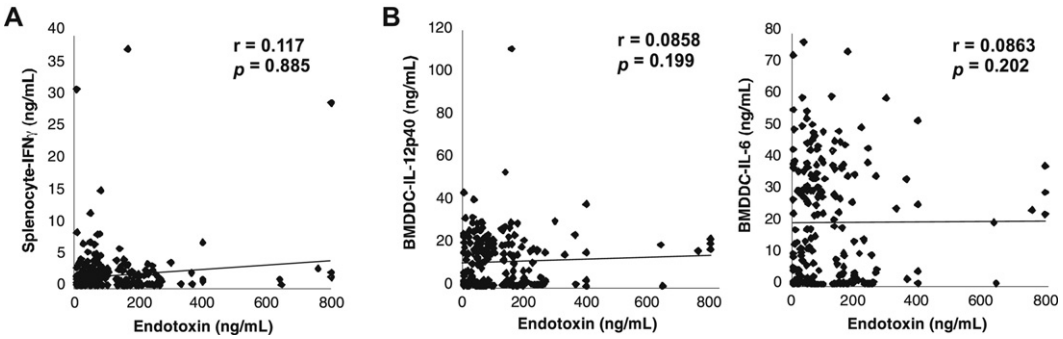


FIG 6. Correlations between HDE endotoxin content and HDE-induced cytokine production. HDE-induced splenocyte IFN-γ responses **(A)** and BMDDC IL-6 and IL-12p40 responses **(B)** were plotted against HDE endotoxin levels on scatter graphs. Pearson correlation coefficients *(r)* and *P* values were calculated by using standard methods.

responses and endotoxin content. However, as shown in Fig 6, correlations were weak ($r < 0.12$) and not significant, suggesting that endotoxin was not the major immunostimulatory molecule contained within these HDEs.

DISCUSSION

It has long been recognized that atopic risk is influenced by both genetic and environmental factors.[33,34] The current investigations determined whether assays of HDE immunostimulatory potential could predict the effect of home environments on SPT reactivity for a pediatric cohort at high allergic risk. Unexpectedly, HDEs associated with children with positive SPT results and children with negative SPT results did not display significant differences in their bioactivities. However, when subjects with positive SPT results were considered in isolation, significant correlations were found between parameters of HDE bioactivity and the number of allergens to which these subjects reacted. In this regard these assays of immunostimulatory activity proved superior to assays of HDE endotoxin content. Moreover, although significant correlations were found between HDE bioactivity measures, little correlation was found between the immunostimulatory activities of HDEs and their endotoxin content. Taken together, these findings provide the first published evidence that quantitative measures of HDE immunostimulatory activity have clinical relevance and that HDE bioactivities are not simply a consequence of their endotoxin content.

High levels of home endotoxin exposure are thought to protect against the allergic march, and endotoxin induces cytokine production in cellular assays.[12,16,29] We therefore hypothesized that HDEs derived from the homes of children with negative SPT results would induce more cytokine production than HDEs associated with children with positive SPT results. Surprisingly, this did not prove to be the case (Figs 1 and 2, *A*). There are several potential explanations for this negative finding. The most obvious is that the immunostimulatory potential of living environments at 1 year of age did not influence allergen SPT reactivity at age 3 years. Alternatively, selected measures of HDE bioactivity might not have adequately quantified the immunostimulatory potential of the homes from which they were derived. However, neither of these interpretations account for the strong association found between the number of positive SPT results in children and the splenocyte and BMDDC responses induced by their associated HDEs (Figs 1-3).

In retrospect, we recognize a potentially important confounding variable inherent to this study's design that might have contributed to its failure to detect differences in the immunostimulatory activities of HDEs associated with cases with positive SPT results and control subjects with negative SPT results. Rigorous criteria were used in CCAAPS for the initial selection of infants at high genetic risk for allergic hypersensitivities. However, on the basis of genetics alone, infants inheriting and not inheriting their parent's allergic tendencies would be more and less likely to have positive SPT results at 3 years of age, respectively. Although speculative, this logic suggests that differences in the genetic makeup of children retrospectively identified as cases and control subjects might have confounded the effect of environment on SPT reactivity. Alternatively, living environments with high levels of immunostimulatory activity might prevent the development of polysensitization to aeroallergens rather than preventing conversion from SPT-negative to SPT-positive status. Data presented in Tables E1 and E2 further suggest that living environments that protect against allergen polysensitization also protect against the development of atopic eczema and asthma. These findings provide support for the view that innate immune stimulation during early life offers protection against the allergic march.

There are many genetic and environmental variables that have the potential to influence the SPT reactivity of 3-year-old children and many environmental factors that might also influence the bioactivity of HDEs prepared from their bedrooms. Presented results were adjusted for age, sex, and ethnicity because these genetic variables were found to significantly affect SPT status. In early analyses we also considered whether socioeconomic status, breast-feeding, the smoking habits of family members, outdoor air pollution, or pet exposures might be associated with trends in the SPT status of children included in this study, the bioactivities of their associated HDEs, or both. In univariate analyses HDE dog allergen levels, exposure to air pollution (measured as elemental carbon attributable to traffic), and the smoking history of subject family members did not predict SPT reactivity or HDE bioactivity. In univariate analyses socioeconomic status was initially found to be a marginally significant predictor of HDE bioactivity and subjects' breast-feeding histories and HDE cat allergen levels were marginally significant predictors of SPT status. However, after inclusion in the multivariate model, these associations were also lost. Although this study was not designed for this purpose, these observations suggest that HDE bioactivities predicted the

allergen SPT status of CCAAPS participants in a manner that was not strongly linked to other nongenetic variables previously found to affect the atopic status of young children.[35-38]

Many investigators believe that ambient exposures to TLR ligands and other molecules with immunostimulatory activities during early life have a long-lasting influence on allergic status.[1,12] Nonetheless, it has proved difficult to develop quantifiable and reproducible assays to measure the immunostimulatory potential of homes. Endotoxin levels can be readily measured, and homes with high endotoxin levels have been associated with protection against the development of allergic stigmata in some but not all studies.[12,32] However, consistent with previously published data for the entire CCAAPS cohort,[13] results presented in Fig 4 did not detect any association between HDE endotoxin levels and the allergen SPT status of subjects associated with the HDEs. One potential explanation is that other environmental immunostimulants played a more important role in attenuating the development of allergic stigmata in these children. Consistent with this view we previously found that HDEs contain immunostimulatory molecules that activate cells through TLR2, TLR4, and TLR9,[16] as well as natural killer T-cell antigens.[15] Furthermore, analyses presented in Fig 6 demonstrated little association between HDE endotoxin levels and parameters of HDE immunostimulatory activity. These observations suggest that endotoxin was not the dominant immunostimulant contained within the HDEs under study. In this regard it is of interest that in a previous study of house dust samples collected in San Diego, we found a significant correlation between HDE-induced cytokine responses and their endotoxin levels. One intriguing explanation for this discordance between HDEs collected in San Diego and Cincinnati is that climatic differences had a significant effect on the microbial and molecular makeup of house dust samples collected in these cities.

Recognizing that HDEs and the living environments they represent contain a variety of different immunostimulatory molecules, it was anticipated that the relative concentrations of each would vary from HDE to HDE and that this heterogeneity in molecular content would lead to qualitative differences in the immune responses induced by individual HDEs. However, as shown in Fig 5, there was a great deal of concordance in the magnitude of responses induced by individual HDEs. These findings lead to 2 potential conclusions. The first is that all of the HDE-induced responses reported in this article (Figs 1-3) were dependent on the same molecules. Alternatively, these responses might have been less dependent on the relative concentrations of specific molecules and more dependent on the net immunostimulatory content of the HDEs, with a wide range of molecules engaging different cellular receptors and acting in a synergistic manner to induce the measured responses.

The current investigations do not explain why homes with high levels of innate immunostimulatory potential protect against allergen-specific SPT reactivity. It might be tempting to speculate that because HDEs induced an innate IFN-γ and IL-12 response in bioassays that the immunostimulatory contents of homes would promote the development of protective T_H1 responses against potential aeroallergens. However, this explanation is at odds with results from our previous murine vaccination studies. When mice received intranasal vaccinations with ovalbumin (OVA) and HDE at weekly intervals, all doses and HDEs tested ($n = 10$ homes) were found to induce OVA-specific T_H2-biased airway hypersensitivities.[19] In contrast, if mice received weekly intranasal OVA immunizations, as in previously described studies, while HDE was intranasally delivered daily at a lower dose beginning 1 week before the first and ending with the last OVA immunization,[19,20] mice had long-lived OVA tolerance by mechanisms that were regulatory T (Treg) cell dependent.[19,20] Moreover, daily airway HDE exposures were found to selectively induce IL-10 production and the recruitment, expansion, or both of natural Treg cells in the lungs and bronchial lymph nodes of mice.[19,20] Considered in the context of the findings presented in this article, we speculate that regular skin and airway contact with ambient immunostimulants promotes aeroallergen tolerance through mechanisms that involve IL-10, natural Treg cells, and probably other factors that remain to be elucidated.

This article provides evidence that bioassays of house dust collected from the homes of infants at high risk of allergy have the potential to predict their SPT reactivity at age 3 years. It will be important to confirm these observations with other birth cohorts and, in particular, to determine whether HDE bioactivity measures predict the allergen SPT status of children who do not come from families at high allergic risk. Moreover, recognizing that murine cells were used to measure immunostimulatory potential in these investigations, it stands to reason that the use of human cells might improve the ability of HDE bioassays to predict clinical outcomes.

Nonetheless, the current findings are compelling for several reasons. First, they provide a rationale for using bioassays, including those discussed in this article, to better characterize the immunostimulatory content of living environments. In this regard functional assays of HDE bioactivity complement endotoxin assays and other assays of molecular content. Second, these investigations highlight the limits of using endotoxin assays alone to predict and understand how living environments affect allergic risk. Finally, HDEs were prepared without purification, and strong correlations were found in their ability to induce IFN-γ, IL-12p40, and IL-6 production and inhibit LPS-induced IL-6 production. This suggests that many immunostimulatory molecules contained within homes act in a complementary and synergistic fashion rather than each having a unique and discordant effect on allergic risk. If assays of HDE immunostimulatory activity prove highly predictive of allergic risk in future investigations, they have the potential to teach us a great deal about how living environments and their immunostimulatory potential influence the genesis of allergic diseases and, potentially, other diseases of immune dysregulation.

Clinical implications: This report suggests that exposures to stimulants of innate immunity protect against aeroallergen sensitization, and it provides evidence that the immunostimulatory potential of house dust is not explained by endotoxin content alone.

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TABLE E1. Prevalence of physician-diagnosed eczema among CCAAPS children at age 3 years

	Total no. of children	Unlikely	Possible	Probable	Definite	Probable or definite	Possible, probable, or definite
Control subjects (negative SPT results)	96	74	9	12	1	13 (13.5%)	22 (22.3%)
Cases (positive SPT results)	96	75	10	9	2	11 (11.4%)	21 (21.8%)
No. of positive SPT results							
1	51	42	4	3	2	5 (9.8%)	9 (17.6%)
2	14	10	3	1	0	1 (7.1%)	4 (28.6%)
3	18	15	1	2	0	2(11.1%)	3 (16.7%)
≥4	13	8	2	3	0	3 (23.1%)	5 (38.5%)

Data are missing for 3 cases and 5 control subjects. Differences in eczema prevalence were not statistically significant for children with greater than 4 positive SPT results by means of χ^2 analyses.

TABLE E2. Prevalence of physician-diagnosed asthma among CCAAPS children at age 3 years

	Total no. of children	Unlikely	Possible	Probable	Definite	Possible or probable
Control subjects (negative SPT results)	96	82	6	8	0	14 (14.6%)
Cases (positive SPT results)	96	83	8	5	0	13 (13.5%)
No. of positive SPT results						
1	51	44	4	3	0	7 (15.9%)
2	14	14	0	0	0	0 (0%)
3	18	17	1	0	0	1 (5.6%)
≥4	13	8	3	2	0	5 (38.5%)

Data are missing for 3 cases and 5 control subjects. Differences in asthma prevalence were not statistically significant for children with greater than 4 positive SPT results by means of χ^2 analyses.