House dust extracts elicit Toll-like receptor–dependent dendritic cell responses

Jared Boasen, BS,[a] Dugald Chisholm, BA,[a] Lev Lebet, BS,[a] Shizuo Akira, MD, PhD,[c] and Anthony A. Horner, MD[a,b] *La Jolla, Calif, and Osaka, Japan*

Background: Laboratory and epidemiological studies have provided indirect but compelling evidence that Toll-like receptors (TLRs) play an important role in innate host responsiveness to ambient immunostimulatory factors. However, little direct evidence exists.
Objective: To determine whether house dust extracts activate dendritic cells by TLR-dependent mechanisms.
Methods: In initial studies, bone marrow–derived dendritic cells were cultured with sterile house dust extracts, and cytokine production and costimulatory molecule expression were evaluated. In additional experiments, the TLR dependence of these responses was assessed.
Results: House dust extract–activated bone marrow–derived dendritic cells were found to produce IL-6 and IL-12 in a concentration-dependent manner and to increase their expression of CD40, CD80, CD86, and MHC class II molecules. Furthermore, correlations were seen between the relative bioactivities of house dust extracts and their endotoxin levels. Finally, bone marrow–derived dendritic cells from TLR (2, 4, and 9)–deficient mice all demonstrated attenuated responses, and MyD88 deficient bone marrow–derived dendritic cells were almost completely nonresponsive to house dust extracts.
Conclusion: These investigations provide direct evidence that TLR signaling pathways play a central role in at least a subset of dendritic cell responses to noninfectious factors ubiquitous in living environments. (J Allergy Clin Immunol 2005;116: 185-91.)

Key words: *Hygiene hypothesis, Toll-like receptor, house dust, endotoxin, dendritic cell, allergy, autoimmunity, autoinflammatory*

Abbreviations used
BMDDC: Bone marrow–derived dendritic cell
HDE: House dust extract
ISS: Immunostimulatory sequence phosphorothioate oligodeoxynucleotide
MAMP: Microbe-associated molecular pattern
MFIR: Mean fluorescence intensity ratio
P-3-C: Lipopeptide Pam-3-Cys
TLR: Toll-like receptor

Atopy, diabetes, colitis, and several other diseases of dysregulated immunity are far more prevalent in developed than underdeveloped countries.[1-5] The hygiene hypothesis proposes that modern public health practices have reduced the quantity and changed the quality of microbial exposures for people living in affluent countries, leading to aberrations in immune development during early childhood and an increased risk for allergic, autoinflammatory, and autoimmune diseases later in life.[1-8] Several epidemiological[2,6,7,9] and laboratory[10-13] studies provide indirect evidence that select infections and less invasive microbial exposures influence immune development, homeostasis, and host risk for developing these diseases. Nonetheless, our understanding of the mechanisms that underlie this apparent symbiosis between microbes and human immunity remains incomplete.

Mammalian cells express germline-encoded receptors that recognize a wide range of microbe-associated molecular patterns (MAMPs) not produced by higher eukaryotes.[14-16] Innate MAMP recognition provides for rapid, robust, and relatively microbe-specific immunity. Toll-like receptor (TLR)–mediated responses in particular have been found to play an integral role in host protection against infections by several pathogens.[15,17-19] With the possible exception of TLR11, all TLRs identified to date are expressed at varying levels by a wide range of mononuclear cells involved in innate and adaptive immunity and by the PMN cells that participate in end-organ inflammatory responses.[15,19] Moreover, heterogeneity in extracellular domains allows for TLR recognition of a wide range of biochemically distinct microbial elements, whereas variability in their intracellular signaling pathways suggests the potential for ligands of different TLRs to induce distinct immunological responses.[3,15] Such characteristics have prompted speculation that in addition to their role in innate defense against infection, TLRs

From [a]the Department of Medicine and [b]the Sam and Rose Stein Institute for Aging, University of California, San Diego; and [c]the Department of Host Defense, Research Institute for Microbial Diseases, Osaka University.
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Reprint requests: Anthony A. Horner, MD, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0663. E-mail: ahorner@ucsd.edu.
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might also mediate the modulatory influence of microbial exposures on diseases of immune dysregulation.[3,4,6-8] In line with this view, ambient levels of TLR ligands, including endotoxin (TLR4), muramic acid (a breakdown product of peptidoglycan; TLR2) and bacterial DNA (TLR9), have been found to be higher in homes with indoor pets and in homes on farms with livestock than in homes without these animal exposures.[6,7,20,21] Moreover, relative to infants raised in homes without animals, those raised in homes with regular animal exposures appear to be protected from atopy.

Inspired by these investigations, the following experiments were designed to assess the role of TLRs in host immunoresponsiveness to factors ubiquitous in homes. We began with the simple assumption that bedroom dust samples processed by suspension in PBS and sterile filtration (house dust extracts; HDEs) would contain a majority of the immunostimulatory elements present in their environments of origin. Bone marrow–derived dendritic cells (BMDDCs) were cultured with HDEs, and cytokine production and costimulatory molecule expression were compared with responses induced by purified TLR ligands. Additional experiments assessed the TLR dependence of BMDDC activation by HDEs. The current series of investigations provides direct evidence that TLRs play a critical role in BMDDC responsiveness to sterile environmental samples collected from homes.

METHODS

Mice and purified TLR ligands

TLR2, TLR4, TLR9, and MyD88 KO mice were backcrossed for 10 generations onto the C157/B6 background. Only female mice were used for these studies. Age-matched female C157/B6 and female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Me). Animal protocols received previous approval from our institution's animal welfare committee. Lipopeptide Pam-3-Cys (P-3-C; EMC Microcollections, Tubingen, Germany), *Escherichia coli* 026-B6 LPS (Sigma, St Louis, Mo), and immunostimulatory sequence oligodeoxynucleotide (ISS; 5′-TGACTGTGAACGTTC-GAGATGA-3′; Trilink Biotechnologies, San Diego, Calif) were purchased from commercial vendors. To remove potential contaminants, LPS used for these studies was further purified with techniques described by Hirschfeld et al.[22]

Preparation of HDEs

With approval from our institution's human subjects committee, house dust samples were obtained by vacuuming a single carpeted bedroom in each of the homes selected for study. HDEs derived from 8 bedrooms without animal exposures and 7 bedrooms with daily cat and/or dog exposure were used in these experiments. Study bedrooms were left unvacuumed for 1 week before dust collection. On the day of sample collection, easily moveable items were taken out of the bedroom, and all exposed carpeting was vacuumed for 5 minutes with a Quick Broom (Hoover, Canton, Ohio) equipped with an acrylic trap for dust collection. House dust was initially run through a course sieve to remove large particulate matter. Residual fine dust was suspended in sterile PBS at 100 mg/mL; suspensions were placed in an ultrasonification bath for 15 minutes and then on a rotor at room temperature for 18 hours. Next, suspensions were filtered through glass wool, and finally through 0.22-μm Steriflip filters (Millipore, Bedford, Mass) to obtain sterile HDEs. In all studies presented here, HDE concentrations refer to the amount of house dust added per milliliter of PBS suspension before filtration.

After each house dust sample was collected, the removable vacuum dust trap was cleaned in a dishwasher. Internal surfaces of the vacuum were hand-washed with soapy water, followed by deionized water, and finally 70% ethyl alcohol. To establish that this cleaning procedure was adequate to prevent significant cross-contamination of house dust samples, internal surfaces of the cleaned vacuum were wiped with a sterile paper tissue that was then placed in 1 mL of sterile PBS. These samples were subsequently processed in the same manner as HDEs. However, unlike the HDEs under study (Figs 1-5), the control extracts (n = 3) demonstrated no immunological activities in BMDDC cultures (data not presented).

Determining the sterility and toxicity of HDEs

A 20-μL aliquot of each HDE stock solution (100 mg/mL) was placed in 5 mL tryptic soy broth on a shaker at 37°C for 48 hours. HDE cultures were then spun down, and 4.9 mL tryptic soy broth was removed. Any precipitates were then resuspended, and aliquots were gram-stained and viewed under a microscope for evidence of bacterial growth. Additional aliquots of each HDE were placed on blood agar plates and cultured in a 37°C incubator for 72 hours before plates were viewed for evidence of bacterial growth. To assess for TLR independent toxicity, MyD88 KO BMDDCs (2×10^6 cells/mL) were incubated in triplicate in media without or with HDEs (1 mg/mL) for 48 hours. Cells were then stained with trypan blue, and total, live, and dead cell counts were determined with a microscope and hemocytometer.

Endotoxin analysis

House dust extract endotoxin levels were determined with the QCL-1000 kit (Bio-Whittaker, Walkersville, Md) according to the manufacturer's instructions. HDE stock solutions were assayed in duplicate at 25,000-fold, 125,000-fold, and 625,000-fold dilutions.

Preparation of myeloid BMDDCs

Mouse femurs were harvested with sterile techniques. Bones were flushed with complete media (RPMI, 1 mmol/L Na pyruvate, penicillin/streptomycin, 10% heat-inactivated, low LPS FCS). Cells were spun down, washed, counted, and resuspended at 2×10^5 cells/mL in media with GM-CSF (R&D Systems, Minneapolis, Minn) at 5 ng/mL, and cultured in large petri dishes. Fresh media and GM-CSF were added to cultures on day 3 and 6, and BMDDCs were harvested for experiments on day 7.

Cytokine responses

Bone marrow–derived dendritic cells were cultured in triplicate at 1×10^6 cells/mL in complete media alone or supplemented with P-3-C (5 μg/mL), LPS (100 ng/mL), or ISS (10 μg/mL), at optimized concentrations, or with HDEs (0.001-10 mg/mL). In additional experiments, MyD88 KO and WT BMDDCs were cultured on CD40 mAb (2 μg/mL; Pharmingen, San Diego, Calif)–coated plates without other stimuli. Supernatants from all cultures were harvested at 24 hours to assess IL-6 and IL-12p40 production with standard ELISA techniques and commercial reagents (Pharmingen).

Flow-cytometric analyses

Bone marrow–derived dendritic cells were cultured in media alone or with P-3-C, LPS, ISS, or HDEs (1 mg/mL) for 24 hours before harvesting cells. BMDDCs were stained with APC-linked CD11c and PE-linked CD40, CD80, CD86, MHC class II, or isotype control mAbs. All mAbs were purchased from Pharmingen. A FACSCalibur flow-cytometry unit (Becton Dickinson, San Jose, Calif) was used for

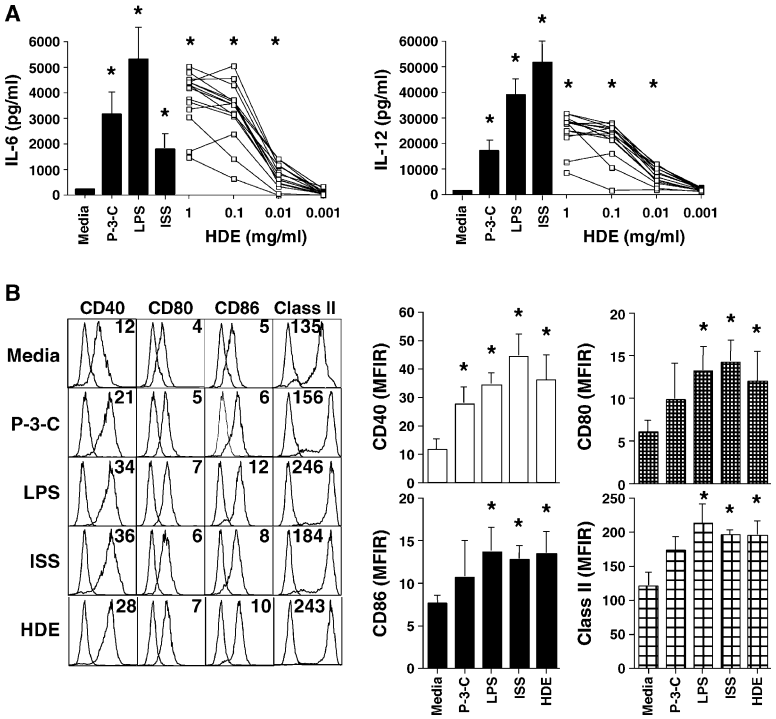

FIG 1. HDEs activate BMDDCs. BALB/c BMDDCs were cultured with P-3-C, LPS, ISS, or HDEs (n = 15). Presented results are reflective of 3 or more experiments (*$P \leq .05$ vs unstimulated BMDDCs. Results for individual HDEs combined). **A,** IL-6 and IL-12 production. **B,** BMDDC costimulatory molecule expression. *Left,* Representative histograms of isotype and costimulatory molecule staining with individual MFIRs. *Right,* Mean MFIRs ± SEs (n ≥ 4 per data point).

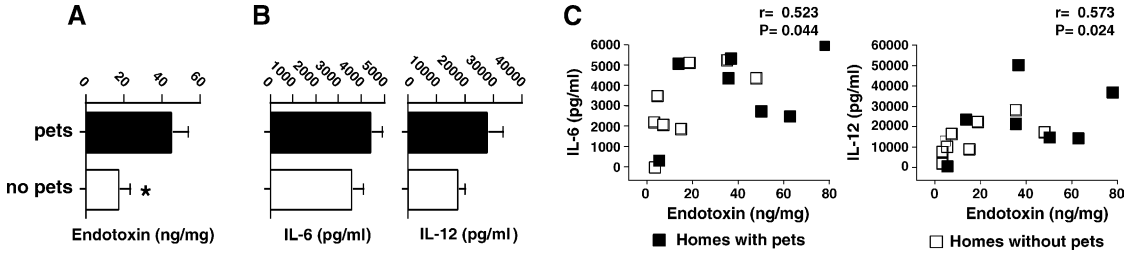

FIG 2. HDE immunostimulatory activities correlate with their endotoxin content. HDEs from homes with (n = 7) and without (n = 8) indoor pets were studied. **A,** Mean endotoxin levels ± SEs (*$P \leq .05$). **B,** Mean IL-6 and IL-12 production for BALB/c BMDDCs cultured with HDEs (0.1 mg/mL) ± SEs. Differences between groups were not statistically significant. **C,** Individual HDE endotoxin levels plotted against cytokine responses induced by those HDEs (0.1 mg/mL).

these studies, recording 50,000 $CD11c^+$ events per sample. In Fig 1, costimulatory molecule expression on $CD11c^+$ cells is quantified by mean fluorescence intensity ratio (MFIR; mean fluorescence intensity for specific staining divided by the mean fluorescence intensity for isotype control staining). In Figs 3 to 5, results are presented as percentages of baseline MFIRs for unstimulated BMDDCs (=100%) to accommodate differences in costimulatory molecule expression by unstimulated BMDDCs from the different mouse strains under study.

Statistics

Statistical analyses were conducted by using Statview software (Abacus Concepts, Berkley, Calif). Two-tailed unpaired Student *t* tests were conducted for analyses of all data except for correlations, for which the correlation *z* test was used.

RESULTS

Sterility and nonspecific toxicity of HDEs

In initial studies, the sterility of HDEs was assessed. Neither bacterial nor fungal growth was observed in tryptic soy broth cultures supplemented with any of the HDEs under study. In addition, no microbial growth was seen when HDE aliquots were cultured on blood agar plates. Recognizing that HDEs contain TLR ligands,[6,7,20,21] MyD88 KO BMDDCs were incubated with HDEs for 48 hours to determine whether they might reduce cell viability by TLR independent mechanisms (ie, contamination with intracellular pathogens or nonspecific toxicity). In these experiments, cell yields and viability from MyD88 KO

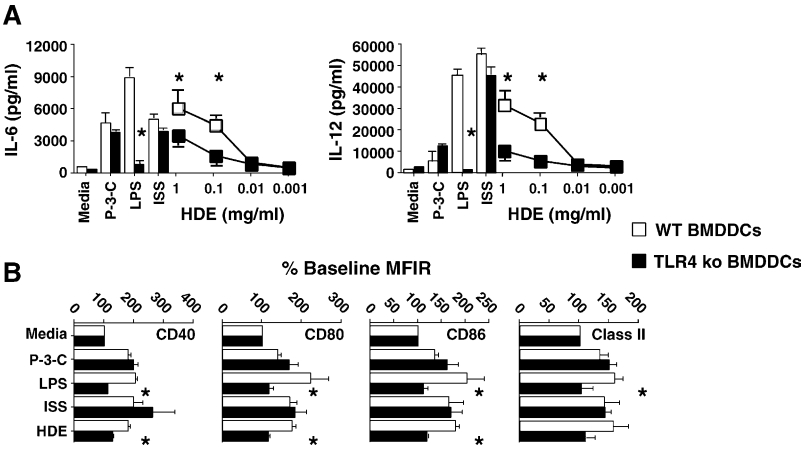

FIG 3. HDE-induced BMDDC responses are partially TLR4-dependent. TLR4 KO and WT BMDDCs (C157/B6 background) were cultured with purified TLR ligands or HDEs (n = 10) with high bioactivities. Results are presented as means ± SEs (*$P \leq .05$ for WT vs TLR4 KO BMDDCs). **A,** IL-6 and IL-12 production. **B,** Costimulatory molecule expression. Results are presented as percentages of baseline MFIRs for unstimulated BMDDCs (see Methods).

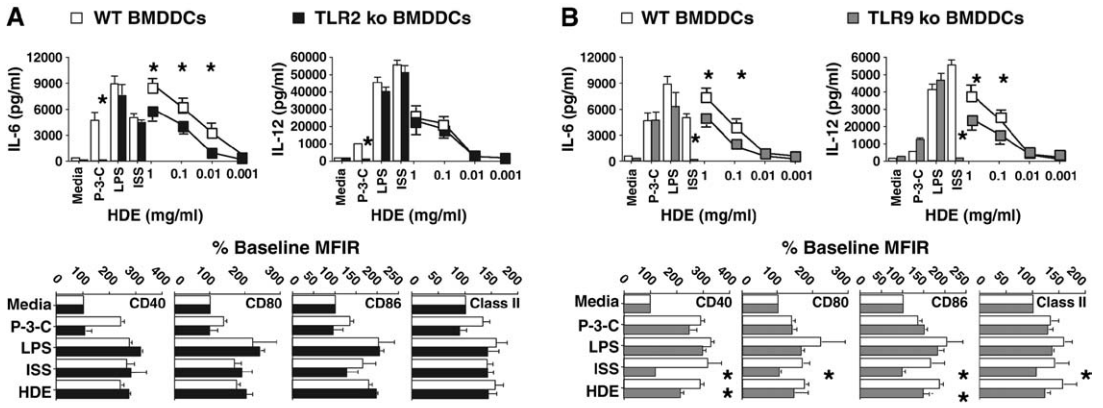

FIG 4. HDE-induced BMDDC responses are partially TLR2-dependent and TLR9-dependent. TLR2 KO, TLR9 KO, and WT BMDDCs (C157/B6 background) were cultured with purified TLR ligands or HDEs (n = 10). Cytokine production and costimulatory molecule expression are presented as in Fig 3 (*$P \leq .05$ for WT and KO BMDDCs). **A,** TLR2 KO and WT BMDDCs. **B,** TLR9 KO vs WT BMDDCs.

BMDDC cultures with and without HDE addition were similar (data not shown).

Immunostimulatory activities of HDEs

Myeloid BMDDCs were cultured with serial dilutions of HDEs, and proinflammatory cytokine responses were evaluated. All HDEs studied (n = 15) induced dose-dependent IL-6 and IL-12 production, but they had a relatively wide range of biological potencies (Fig 1, *A*). Furthermore, higher concentrations of most HDEs and optimized concentrations of P-3-C (TLR2), LPS (TLR4), and ISS (TLR9) elicited similar levels of BMDDC IL-6 production. Nonetheless, purified LPS and ISS consistently induced stronger IL-12 responses than all of the HDEs studied. In addition to IL-6 and IL-12, HDEs and purified TLR ligands stimulated low-level IL-10 production, whereas TNF-α, IL-4, and IL-13 levels were below the limits of ELISA detection in all culture supernatants (data not shown).

In additional experiments, HDE activated BMDDCs underwent flow-cytometric analyses of costimulatory molecule expression. HDE-stimulated BMDDCs expressed CD40, CD80, CD86, and MHC class II at substantially higher levels than unstimulated BMDDCs (Fig 1, *B*). Moreover, BMDDCs activated with HDEs or purified TLR ligands expressed costimulatory molecules at comparable levels.

Influence of pets on HDE endotoxin content and immunological activity

Epidemiological investigations have established that ambient endotoxin levels are generally high, and higher

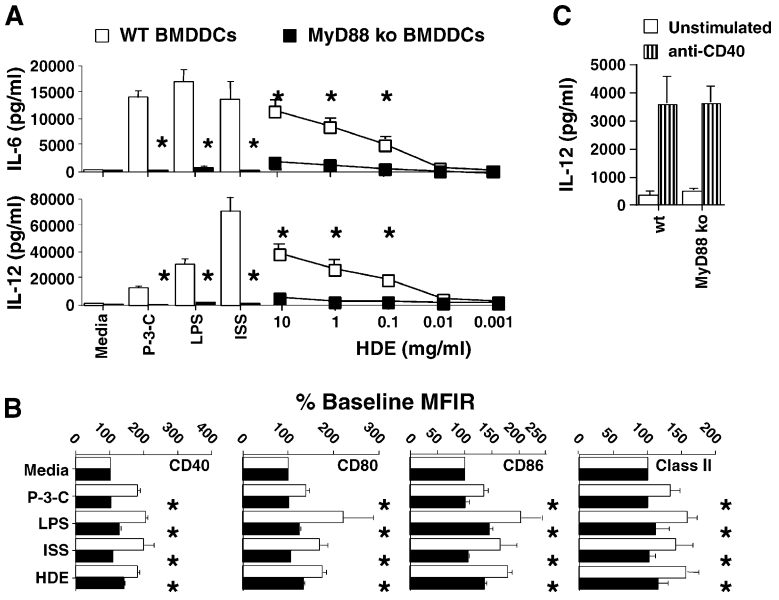

FIG 5. HDE-induced BMDDC responses are MyD88-dependent. MyD88 KO and WT BMDDCs (C157/B6 background) were cultured with purified TLR ligands or HDEs (n = 10). Cytokine production and costimulatory molecule expression are presented as in Fig 3 (*$P \leq .05$ for WT vs KO BMDDCs). **A,** HDE-induced cytokine production. **B,** HDE-induced costimulatory molecule expression. **C,** IL-12 production by BMDDCs stimulated with plate-bound CD40.

still in homes with regular animal exposures.[6,7] Consistent with these reports, we found that on average, HDEs derived from homes with pets (n = 7) contained significantly more endotoxin (range, 3.8-77.5 ng/mL) than HDEs derived from the homes without pets (n = 8; range, 2.4-46.3 ng/mL; Fig 2, *A*). HDEs derived from homes with and without pet exposures were also compared for their ability to induce BMDDC cytokine production. Although HDEs from these homes induced similar mean IL-6 responses, HDEs from homes with pets elicited IL-12 responses that averaged about 60% higher than those induced by HDEs from pet-free homes (Fig 2, *B*). Nonetheless, differences in IL-12 production were not statistically significant, and substantial overlap was found in the cytokine-inducing abilities of HDEs derived from pet and no-pet homes (Fig 2, *C*).

In further analyses, correlations between HDE endotoxin levels and BMDDC cytokine-inducing capacities were assessed (Fig 2, *C*). Considered separately, HDEs from homes with and without pet exposures had correlation coefficients (r values) above 0.5, but these were not statistically significant by z testing (data not shown). However, whereas r values were not strengthened, correlations between endotoxin levels and IL-6 and IL-12–inducing activities did reach statistical significance when all HDEs were considered together (Fig 2, *C*).

Determining the contribution of TLR4 to HDE responsiveness

To evaluate directly the contribution of TLR4 to the HDE-induced responses described in this report, experiments analogous to those presented in Fig 1 were conducted in parallel with WT and TLR4 KO BMDDCs. Ten HDEs found to have the greatest bioactivity in experiments outlined in Figs 1 and 2 were selected for these studies. Compared with WT BMDDCs, TLR4 KO BMDDCs demonstrated a marked reduction in HDE-induced cytokine production (Fig 3, *A*) and costimulatory molecule expression (Fig 3, *B*). In additional experiments, even HDEs with the lowest endotoxin levels were found to induce attenuated responses by TLR4 KO compared with WT BMDDCs (data not presented). Of note, although LPS responsiveness was lost, TLR4 KO BMDDCs responses to P-3-C and ISS stimulation were similar to those of WT BMDDCs. Taken together, these observations provided strong and direct evidence that endotoxin was bioactive in all HDEs, and that TLR4 played an important role in the HDE-induced BMDDC responses under investigation.

Determining the contribution of TLR2 and TLR9 to HDE responsiveness

Results presented in Fig 3 suggested that TLR4 was not the only receptor involved in HDE responsiveness, because TLR4 KO BMDDCs cultured with HDEs displayed an attenuated but nonetheless activated phenotype. Consistent with this finding, previous reports have demonstrated that HDEs contain ligands for TLR2 (muramic acid, a breakdown product of peptidoglycan) and TLR9 (bacterial DNA).[20,21] Therefore, in additional experiments, WT, TLR2 KO, and TLR9 KO BMDDCs were cultured with HDEs (n = 10, as in Fig 3), and cytokine production and costimulatory molecule expression profiles were compared. Although HDE-stimulated TLR2

KO BMDDCs produced less IL-6 than WT BMDDCs, IL-12 production and costimulatory molecule upregulation were preserved (Fig 4, *A*). In contrast, HDE-stimulated TLR9 KO BMDDCs were found to produce less IL-6 and IL-12 than WT BMDDCs (Fig 4, *B*). Furthermore, although TLR4 KO BMDDCs displayed a greater deficit, HDE-activated TLR9 KO BMDDCs also expressed lower levels of costimulatory molecules than WT BMDDCs. Importantly, except for the relevant ligand, TLR2 and TLR9 KO BMDDC responses to purified TLR ligands remained intact. These findings support the position that in addition to TLR4, both TLR2 and TLR9 contributed to the HDE-mediated BMDDC responses described in this article.

Determining the global contribution of TLRs to HDE responsiveness

Results presented thus far suggested that TLR signaling pathways played a central role in the HDE-induced BMDDC responses under investigation. Nonetheless, our findings did not exclude the possibility that HDEs might also activate BMDDCs by completely TLR-independent pathways. Therefore, given that MyD88 plays a critical role in signaling through all TLRs except TLR3,[15,23] a final series of experiments was conducted to compare cytokine production and costimulatory molecule upregulation by HDE-activated WT and MyD88 KO BMDDCs. Compared with WT BMDDCs, MyD88 KO BMDDCs incubated with HDEs (n = 10, as in Figs 3 and 4) or purified TLR ligands produced negligible amounts of IL-6 and IL-12 (Fig 5, *A*). Moreover, although MyD88 KO BMDDCs consistently demonstrated a slight increase in costimulatory molecule expression after culture with LPS or HDEs, expression levels were markedly attenuated compared with WT BMDDCs (Fig 4, *B*). Despite a severe deficit in responsiveness to HDEs and purified TLR ligands, MyD88 KO and WT BMDDCs produced IL-12 at similar levels when activated with plate-bound CD40 mAb (Fig 4, *C*). Because MyD88 KO BMDDCs had a specific deficit in HDE but not CD40 responsiveness, these results confirmed that TLR signaling pathways were critical for BMDDC activation by HDEs.

DISCUSSION

Although theories abound, the factors responsible for the increased health burden of many allergic, autoinflammatory, and autoimmune diseases in developed compared with underdeveloped countries remain a subject of ongoing debate. Results from epidemiological investigations suggest that infants raised in homes that collect large amounts of endotoxin and/or other TLR ligands are relatively protected from developing allergic diseases, whereas infants raised in homes with low ambient levels of these microbial products have an increased atopic risk.[6,7,20,21] Laboratory studies have further established that TLR ligands can profoundly influence susceptibility to a variety of immune-mediated diseases, including asthma, arthritis, and colitis.[10-13,24-26] Such findings have prompted speculation that early life exposures to endotoxin and other TLR ligands may modulate immunity in a manner that protects infants from diseases fueled by immune dysregulation.[1-5,8] However, despite an extensive literature on TLRs and their ligands, the relative contribution of TLR signaling in host responsiveness to ambient immunomodulatory factors has not been adequately assessed with samples reflective of these exposures.

Building on the assumption that gravity leads all particulates present in bedrooms to accumulate on floors, we hypothesized that HDEs would contain a relatively complete sampling of immunomodulatory factors present in their environments of origin. In Fig 1, HDEs derived from 15 bedrooms were shown to induce IL-6 and IL-12 production by BMDDCs in a concentration-dependent manner and to induce their increased surface expression of costimulatory molecules. Further investigations demonstrated that on average, endotoxin levels of HDEs derived from homes with pets were significantly higher than those of HDEs derived from homes without animal exposures (Fig 2, *A*), consistent with previous reports.[6,7] However, independent of the pet exposure status of their homes of origin, HDE endotoxin levels were found to correlate with their relative biopotencies (Fig 2, *C*). In additional experiments, BMDDCs from TLR2, TLR4, and TLR9 KO mice all displayed attenuated responses, and MyD88 KO BMDDCs were almost nonresponsive to HDE stimulation (Figs 3-5). Residual MyD88 KO BMDDC responsiveness to HDEs (Fig 5) may be explained by endotoxin induced BMDDC activation via MyD88-independent mechanisms, as described by others.[27,28] Consistent with this view, in studies analogous to those presented in Fig 5, LPS neutralization by addition of polymyxin to cultures completely extinguished MyD88 KO BMDDC responsiveness to HDEs (Chisholm D, Horner AA, unpublished data, May 2003). Although TLRs have previously been found to play a key role in innate protection against infectious agents,[15,17-19] this report demonstrates for the first time that a subset of BMDDC responses to sterile environmental samples also requires intact TLR signaling pathways.

It should be emphasized that although the current results establish the TLR dependence of BMDDC activation by HDEs, they do not exclude a synergistic role for additional MAMP receptors. This scenario has recently been described for zymosan, a complex macromolecular constituent of fungal cell walls.[29] In these studies, several zymosan induced responses were found to be absolutely dependent on TLR2 but required an additional MAMP receptor (Dectin-1) to facilitate ligand-TLR2 interactions. In contrast, a small-molecular-weight synthetic TLR2 ligand (P-3-C) elicited analogous responses independently of Dectin-1.[29] Because HDEs are likely to contain zymosan and other complex materials of microbial origin, future investigations will need to consider whether TLRs function alone or in conjunction with additional MAMP receptors in mediating the HDE responses described.

Clearly, far more study will be required to understand fully the symphony of immunomodulatory interactions

that continually take place between host MAMP receptors and immunostimulatory elements present in environments of daily living. Nonetheless, the interpretable results generated from this series of investigations serve as a proof of principle that immunological studies of clinically relevant environmental samples are feasible. More importantly, our findings suggest that even in the absence of infection, TLRs play a critical role in at least some aspects of host responsiveness to immunomodulatory elements ubiquitous in the world in which we live. Because living environments appear to have a profound influence on immune homeostasis, we suggest that a comprehensive understanding of the mechanisms by which ambient factors influence the genesis of atopy and other diseases of immune dysregulation could lead to environmental interventions for their prevention and treatment.

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