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Using house dust extracts to understand the immunostimulatory activities of living environments

Glenda Batzer[a], **Diane P. Lam**[a], **Petra Paulus**[a], **Jared Boasen**[a], **Nicholas Ng**[a], and **Anthony A. Horner**[a,b,c,*]

a*Department of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0663, USA*

b*Department of Pediatrics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0663, USA*

c*The Sam and Rose Stein Institute for Aging, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0663, USA*

Abstract

Laboratory and epidemiological studies have provided indirect but compelling evidence that toll like receptor (TLR) signaling pathways play an important role in host responsiveness to ambient immunostimulatory factors. Nonetheless, direct evidence is limited. This review will present our experience investigating the innate immunostimulatory activities of sterile house dust extracts (HDEs). In initial studies, bone marrow derived dendritic cells (BMDDCs) were cultured with HDEs and cytokine production and co-stimulatory molecule expression were evaluated. In additional experiments, the TLR dependence of these responses was determined. HDEs induced concentration dependent BMDDC activation. Moreover, the relative bioactivities of HDEs correlated with their endotoxin content. Finally, HDE mediated responses were found to be partially dependent on TLR2, TLR4, and TLR9 and almost completely dependent on MyD88. These investigations provide the first direct evidence that TLR signaling pathways play a key role in innate responsiveness to non-infectious factors ubiquitous in living environments.

Keywords

allergy; dendritic cell; endotoxin; house dust; hygiene hypothesis; toll like receptor

Introduction

Over the last century, prevalence rates for asthma and other allergic diseases have increased dramatically in the industrialized world but not in underdeveloped countries (Horner and Raz, 2003;Latvala et al., 2005;Wills-Karp et al., 2001). While a topic of intense speculation and investigation, it remains to be determined why. Nonetheless, there is consensus agreement that allergic disease prevalence rates in affected countries have increased too rapidly to be a consequence of genetic drift (Horner and Raz, 2003;Liu and Murphy, 2003;Martinez and Holt, 1999;von Mutius, 2002;Wills-Karp et al., 2001). From this perspective, there is a strong

*Address correspondence and reprint requests to Anthony A. Horner, M.D., University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0663, USA Phone: (858) 534-5435, Fax: (858) 534-0409, email: ahorner@ucsd.edu

imperative to understand how external factors impact on host immunity in general and allergic risk in particular.

Allergen exposure is a clear prerequisite for the development and persistence of antigen specific hypersensitivities (Huss et al., 2001;Platts-Mills et al., 1991;Platts-Mills et al., 2004). Nonetheless, while for some allergens (i.e. cockroach and mites), higher exposure levels have been associated with an increased risk of sensitization, for other allergens (dogs, cats, molds), this correlation has not been found (Frew, 2005;Huss et al., 2001). Furthermore, several studies have shown that for allergens associated with animals, increased levels of exposure are associated with decreased sensitization rates (Frew, 2005;Hesselmar et al., 2005;Platts-Mills et al., 2004). These and other observations have prompted speculation that aside from allergens, additional factors ubiquitous in living environments influence the balance between allergen tolerance and hypersensitivity. In line with this view, a number of epidemiological and laboratory studies provide indirect evidence that non-invasive contact with microbes influences immune development, homeostasis, and as a consequence, allergic risk. For example, intestinal flora have been shown to facilitate post-natal immune development (Mazmanian et al., 2005;Sudo et al., 1997) and prevent Th2 polarized responses to dietary allergens (Rask et al., 2005;Sudo et al., 2004) in mice. Moreover, human studies suggest that the intestines of atopic and non-atopic infants tend to be colonized with unique bacterial species(Bottcher et al., 2000). Finally, in several clinical trials, ingestion of probiotic bacteria has been found to be effective in treating eczema and in preventing development of additional allergic manifestations in high atopic risk infants (Bjorksten, 2005;Rautava et al., 2005). Nonetheless while such observations provide supportive evidence, our present understanding of the mechanisms that underlie this apparent symbiosis between physiologic microbial exposures and allergic risk remains incomplete.

Atopy and Toll-Like Receptors

In order to ensure survival, the major task of mammalian immunity is the rapid detection and neutralization of infectious organisms (Gazzinelli et al., 2004;Picard et al., 2003;Takeda et al., 2003;Zhang et al., 2004). In part, surveillance is achieved by germline-encoded receptors that recognize a wide range of microbe associated molecular patterns (MAMPs) not produced by higher eukaryotes (Medzhitov and Janeway, 2002;Philpott and Girardin, 2004;Takeda et al., 2003). Innate MAMP recognition provides for rapid, robust, and relatively microbe specific immunity. 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 polymorphonuclear cells that participate in end organ inflammatory responses (Takeda et al., 2003;Zhang et al., 2004). Moreover, heterogeneity in extra-cellular domains, allows for TLR recognition of a wide range of biochemically distinct microbial elements, while variability in their intra-cellular signaling pathways suggests the potential for ligands of different TLRs to induce distinct immunological responses (Horner and Raz, 2003;Takeda et al., 2003). Finally, purified ligands for several TLRs have been found to prevent or promote the development of Th2 biased hypersensitivities, in animal models of asthma and other atopic diseases (Chisholm et al., 2004;Eisenbarth et al., 2002;Horner and Raz, 2002;Horner and Raz, 2003;Racila and Kline, 2005;Tsalik, 2005). Such characteristics have prompted speculation that in addition to their role in innate defense against infection, TLRs might also mediate the modulatory influence of microbial exposures on allergic diseases and other diseases of immune dysregulation (Braun-Fahrlander et al., 2002;Gereda et al., 2000;Horner and Raz, 2003;Martinez and Holt, 1999;Wills-Karp et al., 2001).

In support of this view, endotoxin (TLR4), has been found to be ubiquitous in living environments, with higher concentrations reported in homes that have regular exposures to animals than in homes without animal exposures (Braun-Fahrlander et al., 2002;Gereda et al.,

2001). Moreover, infants raised in homes with high ambient endotoxin levels have been suggested to be at low relative risk for developing allergic hypersensitivities in many, although not all published reports (Braun-Fahrlander et al., 2002;Gereda et al., 2000). However, it is important to note that despite this apparent association between ambient endotoxin exposure levels and allergic risk, endotoxin rich environments also generally contain elevated levels of other immunostimulatory microbial products. These include muramic acid, a breakdown product of peptidoglycan (TLR2), and bacterial DNA (TLR9) (Roy et al., 2003;van Strien et al., 2004). Furthermore, several man made pollutants have been found to promote the development of allergic hypersensitivities (Saxon and Diaz-Sanchez, 2005). While much has been learned in recent years, such complexity in the content of daily exposures has hampered efforts to develop a comprehensive understanding of their impact on the development of allergic diseases.

Rationale for studying the immunological activities of house dust extracts

Given the difficulties inherent in determining which environmental exposures have the greatest impact on host immunity, we reasoned that direct study of the immunological activities of unpurified but clinically relevant environmental samples might prove enlightening. Sterile house dust extracts (HDEs) were chosen for investigations, as we believed gravity would concentrate most, if not all, ambient immunomodulatory particulates present within living environments into settled dust. Furthermore, house dust allergen and endotoxin levels have already proven useful surrogate markers in epidemiological studies of allergic risk (Braun-Fahrlander et al., 2002;Gereda et al., 2000;Huss et al., 2001). In the following sections, we will review our brief experience, investigating the immunological activities of HDEs (Boasen et al., 2005).

Dendritic cell activation by HDEs

In order to conduct experiments, dust samples were collected from bedrooms in fifteen suburban homes in San Diego, California (Boasen et al., 2005). All bedrooms were carpeted; seven were in homes with indoor pets (dog and/or cats); the rest had no identified animal exposures. House dust samples were processed by standardized techniques that included suspension in PBS, physical agitation, and sterile filtration. The sterility of each house dust extract (HDE) was determined by culturing an aliquot in bacterial growth medium. HDE toxicity was assessed by co-culturing TLR ligand hyporesponsive (MyD88 deficient) bone marrow derived dendritic cells (BMDDCs) with aliquots of these HDEs. Only HDEs deemed to be sterile and non-toxic were used in investigations.

In initial experiments, we determined whether HDEs could activate BMDDCs (Boasen et al., 2005), as these cells have previously been shown to be highly responsive to purified TLR ligands (Akira and Takeda, 2004). BMDDCs were cultured in serial dilutions of HDEs for 24 hours before supernatant cytokine levels were assessed. BMDDCs cultured with Pam-3-Cys (P-3-C; TLR2), LPS(TLR4), or immunostimulatory sequence oligodeoxynucleotide (ISS, TLR9) were used as benchmarks for comparative analyses. While relative bioactivities varied widely, all HDEs studied, induced concentration dependent IL-6 and IL-12p40 responses by BMDDCs (Fig. 1A). Moreover, higher concentrations of most HDEs and optimized concentrations of TLR ligands elicited similar levels of IL-6 production. In contrast, LPS (TLR4) and ISS (TLR9) induced stronger IL-12p40 responses than any of the HDEs studied. In a subsequent study, we determined whether a sampling of HDEs induced the production of bioactive IL-12 (IL-12p70). HDE induced BMDDC IL-12p70 responses were relatively weak compared to those induced by LPS and ISS but similar to responses elicited by P-3-C (Fig. 1B). Moreover, R848, a TLR7 ligand used as an additional control for this study, elicited BMDDC IL-12p70 responses that were 10 fold greater than those induced by HDEs. Purified

TLR ligands and HDEs also induced low levels of BMDDC IL-10 production, while IL-4, IL-13 and TNF-α were not detected in any culture supernatants.

In additional studies, HDE regulation of BMDDC co-stimulatory molecule expression was assessed. BMDDCs stimulated with HDEs displayed up-regulation of CD40, CD80, CD86 and MHC Class II expression compared to unstimulated BMDDCs (Boasen et al., 2005). Moreover, co-stimulatory molecule expression levels were similar on BMDDCs activated with HDEs or purified TLR ligands.

Correlations between HDE endotoxin levels and bioactivities

Epidemiological investigations have established that ambient endotoxin levels are generally high, and higher still in homes with regular animal exposures (Braun-Fahrlander et al., 2002;Gereda et al., 2001). Consistent with these studies, we found that the mean endotoxin content of house dust samples obtained from homes with pets (n = 7; 45 ± 9.4 ng/mg) was more than twice that for house dust samples obtained from homes without pets (n = 8; 17.1 ± 6.4 ng /mg)(Boasen et al., 2005). In additional experiments, the immunostimulatory activities of HDEs derived from homes with and without animal exposures were compared. Although mean IL-6 responses were similar, HDEs from homes with pets elicited IL-12p40 responses that were 60% stronger (Boasen et al., 2005). Nonetheless, the number of HDEs tested was small, differences in IL-12 production were not statistically significant, and substantial overlap was found in the cytokine inducing abilities of HDEs derived from homes with and without pets (Fig. 2).

In further analyses, correlations between HDE endotoxin levels and BMDDC cytokine inducing capacities were assessed (Fig. 2)(Boasen et al., 2005). 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, while r values were not strengthened, correlations between endotoxin levels and IL-6 and IL-12p40 inducing activities did reach statistical significance when all HDEs were considered together.

The role of TLRs in HDE responsiveness

To further evaluate the contribution of TLR4 to the HDE induced responses described in Fig. 1, experiments were repeated in parallel with wild type (WT) and TLR4 knockout (ko) BMDDCs (Boasen et al., 2005). Ten HDEs found to have the greatest bioactivity were selected for these studies. Compared to WT BMDDCs, TLR4 ko BMDDCs demonstrated a marked reduction in HDE induced cytokine production (Fig. 3A) and a decrease in co-stimulatory molecule expression (Boasen et al., 2005). As predicted, while TLR4 ko BMDDCs responses to LPS were lost, responses to P-3-C and ISS stimulation were similar to those of WT BMDDCs. In additional experiments we found that even HDEs with the lowest endotoxin levels induced attenuated responses by TLR4 ko compared to WT BMDDCs (data not presented). Taken together, these observations provided strong evidence that TLR4 had a major role in mediating BMDDC responses to HDEs.

Results presented in Fig. 3A suggested that while playing a role, TLR4 was not the only receptor involved in HDE responsiveness, as 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 and TLR9 (Roy et al., 2003;van Strien et al., 2004). Therefore, in additional experiments, WT, TLR2 ko and TLR9 ko BMDDCs were cultured with HDEs (n = 10) and cytokine production and co-stimulatory molecule expression profiles were compared. While HDE-stimulated TLR2 ko BMDDCs produced less IL-6 than WT BMDDCs, IL-12p40 production and co-stimulatory molecule expression were preserved (Fig. 3B)(Boasen et al., 2005). In contrast, HDE-stimulated TLR9 ko BMDDCs were found

to produce less IL-6 and IL-12p40 than WT BMDDCs (Fig. 3C). Furthermore, while TLR4 ko BMDDCs displayed a greater deficit, HDE activated TLR9 ko BMDDCs also expressed lower levels of co-stimulatory molecules than WT BMDDCs (Boasen et al., 2005). Importantly, except for the relevant ligand, TLR2 and TLR9 ko BMDDC responses to purified TLR ligands remained intact. These findings support the view that in addition to TLR4, both TLR2 and TLR9 contributed to the HDE mediated BMDDC responses.

Experimental findings presented thus far suggested that TLR signaling pathways played an important role in mediating HDE induced BMDDC responses. Nonetheless, these results 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 (Oshiumi et al., 2003;Takeda et al., 2003), a final series of experiments was conducted to compare cytokine production and co-stimulatory molecule up-regulation by HDE-activated WT and MyD88 ko BMDDCs. Compared to WT BMDDCs, MyD88 ko BMDDCs incubated with HDEs (n = 10) or purified TLR ligands produced negligible amounts of IL-6 and IL-12 (Fig. 3D).

Moreover, while MyD88 ko BMDDCs consistently demonstrated a slight increase in co-stimulatory molecule expression after culture with LPS or HDEs, expression levels were markedly attenuated compared to WT BMDDCs (Boasen et al., 2005). However, 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. 3D). As 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

Experts generally agree that living environments have a significant impact on allergic risk (Horner and Raz, 2003;Liu and Murphy, 2003;Martinez and Holt, 1999;von Mutius, 2002;Wills-Karp et al., 2001). In support of this view, a number of immunostimulatory materials, including allergens, microbial products, and manmade pollutants are fairly ubiquitous in inspired air (Becker et al., 2002;Rabinovitch et al., 2005;Saxon and Diaz-Sanchez, 2005) and house dust samples (Braun-Fahrlander et al., 2002;Gereda et al., 2001;Platts-Mills et al., 2004;Rabinovitch et al., 2005;Roy et al., 2003;van Strien et al., 2004). Moreover, in animal models, purified preparations of these ambient factors have a significant influence on allergic disease outcome measures (Akbari et al., 2001;Chisholm et al., 2004;Eisenbarth et al., 2002;Saxon and Diaz-Sanchez, 2005). Nonetheless, high atopic risk children live in a world in which they are continually exposed to a wide range of immunostimulatory molecules. Therefore, traditional reductionist investigations with purified materials may not adequately model the immunomodulatory potential of living environments.

Considerations just discussed prompted us to directly investigate whether HDEs derived from environments thought to have an impact on allergic risk could induce innate immune activation (Boasen et al., 2005). Experiments conducted to date establish that sterile HDEs have dose dependent immunostimulatory activities (Fig. 1). In addition, the relative bioactivities of HDEs correlated loosely with their endotoxin content (Fig. 2). Finally, the HDE-mediated responses under investigation were shown to be partially dependent on TLR2, TLR4, and TLR9 and almost completely dependent on MyD88 (Fig. 3). Taken together, these experimental observations 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.

It should be emphasized that while experiments described herein established the TLR dependence of BMDDC responses to HDEs, they did 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 (Gantner et al., 2003). Several zymosan-induced responses were found to be absolutely dependent on TLR2 but required a lectin like receptor (Dectin-1) to facilitate ligand-TLR2 interactions. In contrast, a small molecular weight synthetic TLR2 ligand (P-3-C) elicited analogous responses independent of Dectin-1 (Gantner et al., 2003). As HDEs are likely to contain zymosan and other complex materials of microbial origin, it remains to be determined whether TLRs function alone or in conjunction with additional MAMP receptors, in mediating innate responses to HDEs.

In addition to TLR and lectin-like receptor ligands, a number of microbes are known to produce B and/or T cell superantigens (Davison et al., 2000;Silverman and Goodyear, 2002). Therefore, as HDEs are laden with microbial products, a rationale exists for suggesting that HDEs might also contain biologically significant amounts of these oligoclonal lymphocyte mitogens. Additionally, NK T cells can influence the allergic phenotype but have an extremely limited TCR repertoire (Crowe et al., 2003;Meyer et al., 2006). Given that natural ligands for NK T cells have not been clearly identified and the heterogeneous composition of HDEs, it is also reasonable to consider the possibility that HDEs might contain ligands for NK T cells.

Clearly, far more study will be needed to fully characterize the molecular pathways by which HDEs and the environments they represent activate innate immunity. Nonetheless, the interpretable results generated from this series of experiments serve as a proof of principal that studies of the allergen independent immunostimulatory activities of clinically relevant environmental samples are feasible. We suggest that as a complement to studies with purified materials, investigations with HDEs have the potential to provide important insights about how ambient exposures influence not only innate immunity but also adaptive immunity and allergic risk.

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Abbreviations

BMDDC, bone marrow derived dendritic cell; HDE, house dust extract; ISS, immunostimulatory sequence phosphorothioate oligodeoxynucleotide; MAMP, microbe associated molecular pattern; P-3-C, lipopeptide Pam-3-Cys; TLR, toll-like receptor.

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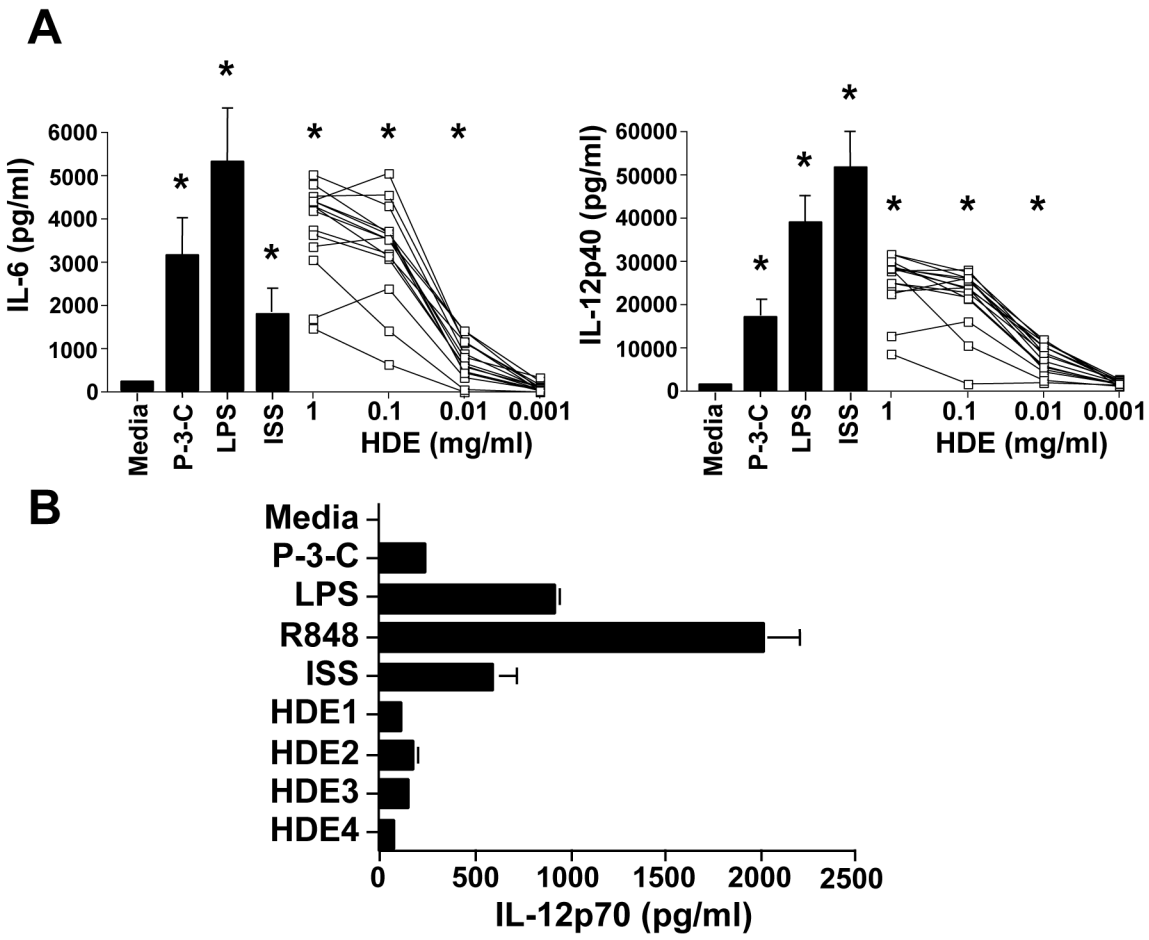

Fig 1.

HDEs induce BMDDC cytokine production. BALB/c BMDDCs were cultured at 1×10^6 cells/ml with P-3-C (5μg/ml), LPS (100 ng/ml), ISS (10μg/ml), R848 (1μg/ml), or HDEs (n = 15) for 24 h before supernatants were harvested for cytokine ELISA. HDE preparation and other methodological details for these experiments have been described previously. Presented results are reflective of 3 or more experiments (*$P \leq 0.05$ versus unstimulated BMDDCs). For statistical analyses, results with individual HDEs were combined. (A) IL-6 and IL-12p40 production. (B) IL-12p70 production. HDEs were used at 1 mg/ml in these experiments.

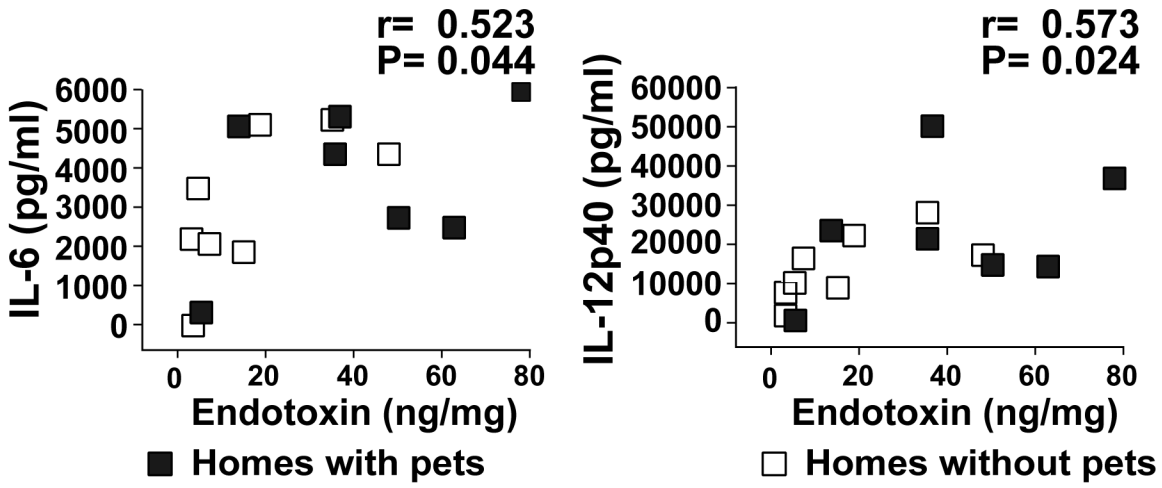

Fig 2.

Associations between HDE endotoxin levels and bioactivities. BMDDCs were cultured with HDEs (0.1 mg/ml), as in Fig. 1 and HDE endotoxin levels were measured. Individual HDE endotoxin levels were then plotted against the cytokine responses induced by those HDEs (0.1 mg/ml). R = correlation coefficient.

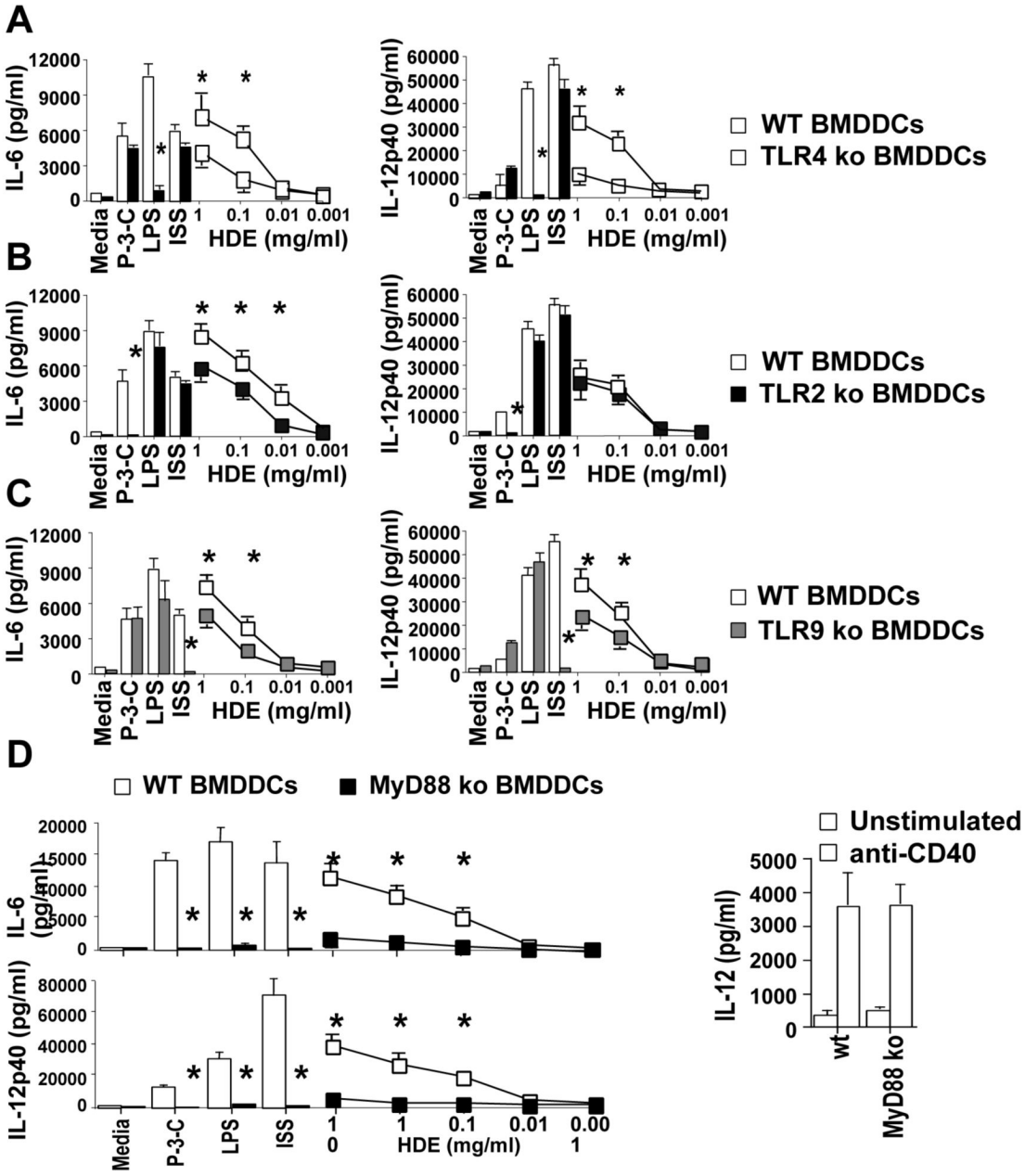

Fig 3.

HDE induced BMDDC responses are TLR dependent. TLR2, TLR4, TLR9, MyD88 ko and WT (C157/B6) BMDDCs were cultured with purified TLR ligands or HDEs (n = 10) with high bioactivities, as in Fig. 1. HDE results are presented as means ± standard errors (* $P \leq 0.05$ for WT versus KO BMDDCs). (A) WT versus TLR4 ko mice. (B) WT versus TLR2 ko mice. (C) WT versus TLR9 ko mice. (D) WT versus MyD88 ko mice.