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CD14, a Key Candidate Gene Associated with Specific Immune Response to Cockroach

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Summary

Background—Sensitization to cockroach allergen is one of the strongest predictors of asthma morbidity, especially among African Americans.

Objective—Our aims were to determine the genomic basis of cockroach sensitization and the specific response to cockroach antigen.

Methods—We investigated the Th1/Th2 cytokine profile of co-cultured plasmacytoid DCs (pDCs) and CD4⁺ T cells and the “transcript signature” of the immune response to cockroach antigen using high-throughput expression profiling of co-cultured cells.

Results—We observed significantly elevated levels of *IL-13*, *IL-10* and *TNF-α*, but undetectable levels of *IL-12p70* and *IFN-α*, when cultures were exposed to crude cockroach antigen. A significant difference was observed for *IL-13* between cockroach allergic and non-allergic individuals ($p = 0.039$). Microarray analyses demonstrated a greater response at 48 hours compared to 4 hours, with 50 genes being uniquely expressed in cockroach antigen-treated cells, including *CD14*, *S100A8*, *CCL8*, and *IFI44L*. The increased *CD14* expression was further observed in purified pDCs, human monocytic THP-1 cells, and supernatant of co-cultured pDCs and CD4⁺ T cells in exposure to cockroach extract. Furthermore, the most differential expression of *CD14* between cockroach allergy and non-cockroach allergy was only observed among individuals with the CC “high-risk” genotype of the *CD14* -260C/T. Ingenuity Pathways Analysis (IPA) analyses suggested the interferon-signaling as the most significant canonical pathway.

Conclusion—Our results suggest these differentially expressed genes, particularly *CD14*, and genes in the interferon-signaling pathway may be important candidates for further investigation of their role in the immune response to cockroach allergen.

Keywords

asthma; CD4⁺ T cells; CD14; cockroach sensitization; Dendritic cells (DCs); high-throughput expression profiling

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Introduction

Sensitization and exposure to indoor allergens is a well-established risk factor for asthma (1), but little is known about the precise causal relationship between cockroach exposure, cockroach sensitization, and asthma. Chronic exposure to low levels of indoor allergens (1–10 ug/year) is associated with production of specific IgE antibodies in susceptible individuals (2). Cockroach allergen exposure and sensitization, more than dust mite and cat allergen, is an especially strong risk factor for asthma morbidity among inner-city children with asthma(3),(4). Both *Blattella germanica* (German cockroach), and *Periplaneta americana* (American cockroach) are important producers of major allergens that elicit IgE-mediated responses (5). *B. germanica* is especially ubiquitous, particularly in large, crowded cities in the U.S.A. (6), and the prevalence of an IgE-mediated response to the major antigens, *Bla g 1* and *Bla g 2*, is high (30–50% and 60%, respectively). *Bla g 2* is an especially potent antigen, inducing IgE antibody responses at very low doses of exposure (0.33ug/g) (7). Although *Bla g 2* shares sequence homology with the aspartic proteinase family of proteolytic enzymes, it lacks proteolytic activity in a standard milk-clotting assay using casein as a substrate(8,9), suggesting factors other than enzymatic activity, such as genetic susceptibility, may play a role in sensitization. A previous study implicated a role for genetic susceptibility wherein cockroach sensitization was found to be more prevalent among African Americans compared to European Americans living in the Baltimore-Washington, D.C. metropolitan area, even after controlling for socioeconomic status(10), supporting the notion that African Americans are more susceptible to cockroach sensitization than other ethnic groups. Little is known, however, regarding the casual relationship between cockroach sensitization and allergic diseases, including asthma.

Dendritic cells (DCs) are the most powerful antigen-presenting cells (APCs) that process cockroach antigen and play a critical role in the initiation of the immune response and T cell polarization(11,12). Two major circulating DC subsets, plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), have been observed accumulating in the airway lumen of asthmatics following allergen challenge(13,14), suggesting that DCs might be especially relevant to asthma pathogenesis. A very recent study demonstrated that, in children with a family history of atopy, relative deficiency of circulating pDCs during infancy appears to be a risk factor for more frequent and more severe respiratory tract infection, wheezing, and asthma(15), suggesting that pDC is critical in protecting against these outcomes. Alternatively, CD4⁺ T cells, a key immune regulatory cell, produce large quantities of distinct cytokines upon stimulation of antigens (16,17). Therefore, our study focused on two of the major immune regulatory cells, peripheral blood DCs and CD4⁺ T cells.

In this study, we investigated whether exposure of DCs (in co-culture with CD4⁺ T cells) to crude cockroach allergen extract instructs DCs to preferentially skew CD4⁺ T cells toward a Th2 response. Because pDCs responded poorly to LPS (no TLR4 expression) (14,18), our study focused on pDCs as an initial study DC subtype, which could avoid endotoxin contamination in cockroach extract induced immune response. We speculated that, upon exposure to cockroach extract, pDCs affect CD4⁺ T cell activation to promote allergic inflammation. Because of the limited numbers of circulating human pDCs, we used co-cultured pDCs and CD4⁺ T cells for microarray analysis with the aim of identifying genes for the specific immune response to cockroach allergen among cockroach-sensitized individuals.

Materials and Methods

Study population

Atopic African American subjects aged 18–50 years with and without cockroach sensitization were recruited at the Johns Hopkins Asthma & Allergy Center for the microarray studies. Atopic status and cockroach sensitization were determined by a positive skin prick test (ST; wheal ≥ 3 mm greater than the saline control) and/or positive specific IgE (≥ 0.35 kU/L) to common aeroallergens (Greer Laboratories, Lenoir, NC, USA) as described in the Methods Section of the Online Data Supplement. The study protocol was approved by the Institutional Review Board of Johns Hopkins School of Medicine and written informed consent was obtained from each of the study subjects.

Cell isolation and cockroach allergen stimulation

Target cells were freshly isolated from PBMCs using a standardized method(19) as described in the Methods Section of the Online Data Supplement. The purity of pDCs was determined according to the manufacturer's instructions ($>97\%$). The isolated pDCs and CD4⁺ T cells were plated in a ratio of 1:20 ($\sim 5.0 \times 10^4$ pDCs and 1.0×10^6 CD4⁺ T cells in a total volume of 1 ml) and co-cultured in the presence or absence of German cockroach extracts (100 ng/ml, B46, GREER Laboratories) or Bermuda grass (100 ng/ml, D8, GREER Laboratories). The decision for a 1:20 ratio for isolated pDCs and CD4⁺ T cells ($\sim 5.0 \times 10^4$ pDCs and 1.0×10^6 CD4⁺ T cells in a total volume of 1 ml) was based on previous reports (20,21), and a series of pilot studies using 1:10, 1:20 and 1:50 detecting the CD69 expression by Flow-cytometric analysis (data not shown). The concentration of allergen extract to be used in these studies was determined by the T cell activation (data not shown) and TNF- α production (Figure E1) when cells were exposed to various concentrations (*i.e.*, 1–200 μ g/ml) of antigen extract. Endotoxin in both extracts was detected by Limulus Amebocyte Lysate (LAL) QCL-1000R (Lonza, Walkersville). The cultured cells were harvested at 4 and 48 hours and stored for RNA extraction and microarray analysis.

Cytokine assay

The concentrations of cytokines secreted by both pDCs and CD4⁺ T cells from atopic subjects in the culture supernatant were determined by ELISA (BD Biosciences), according to the manufacturer's instructions. Selected cytokines included *IL-12p70*, *IL-13*, *TNF- α* , *IL-10*, and *CD14*.

RNA extraction and transcript profiling with Illumina Arrays

RNA extraction and Illumina GeneChip profiling was performed at the Johns Hopkins Bayview Lowe Family Genomics Core(22) (23). In brief, total RNA (7–10 μ g) was isolated from each sample using TriZol (Gibco-BRL) and RNeasy columns (QIAGEN, Inc.), and RNA quality was confirmed on the Agilent 2100 bio-analyzer (Agilent Technologies, Palo Alto, CA, USA). Biotin-labeled cRNAs were prepared as described in the Illumina expression analysis technical manual (Illumina, Inc., San Diego, CA) using 0.5 μ g of purified total RNA as template for the reaction. The biotin-labeled cRNA samples were hybridized (16 hours) to Illumina's Sentrix HumanRef-8 Expression BeadChips comprised of 24,000 transcripts and variants. The Array images were acquired using Illumina's BeadStation 500GX Genetic Analysis System scanner and analyzed with Illumina BeadStudio software. Additionally, resulting digitized matrix was modified for Illumina platform approach described previously(22) (23). Power prediction analysis for minimum array requirements and microarray data analysis are described in the Methods section of the Online Data Supplement. In particular, microarray data analyses were structured to address three central questions: (1) what is the overall pattern of gene expression in pDCs and CD4⁺

T cells in three categories (medium only, cockroach antigen stimulation and Bermuda grass antigen stimulation) at early (4hrs) and late (48 hrs) time points?; (2) is the cockroach allergen response distinct from the response to an unrelated (i.e., non-cockroach) antigen?; and (3) does the pattern of gene expression differ in subjects with and without cockroach allergy?

Quantitative RT-PCR

Quantitative PCR was used to validate the differential expression of selected top ranking genes. Isolated RNA from purified pDCs and CD4⁺ T cells was used to synthesize cDNA using the Superscript First-Strand cDNA Synthesis kit (Invitrogen), according to the manufacturer's protocol. The cDNA was used as a template for quantitative RT-PCR using SYBR Green Master Mix (BioRad). Fold changes were calculated using the $\delta\delta Ct$ method. The significance of the obtained differences was assessed by using *t* tests.

Flow-cytometric analysis of cell surface-expressed molecules

The allergen-treated and untreated cells were harvested at 48 hours, washed in phosphate-buffered saline (PBS) once, and then incubated for 30 minutes at 4°C with blocking antibody FcR (1:5, Miltenyi Biotec, Bergisch Gladbach, Germany) and followed by adding either FITC-conjugated-BDCA-2, PE-conjugated *CD14* (R&D System, Minneapolis, MN), or IgG1 isotype control antibodies (2.5 µg/ml). After washing, cells were analyzed by fluorescence-activated cell sorting (FACS, Calibur, Becton-Dickinson, San Jose, CA) (24).

SNP genotyping

Genotyping for the *CD14*-260 variant (rs2569190) was performed in a total of 807 African American subjects with the TaqMan assay as previously described(25). Detailed information regarding the study population is present in the Online Data Supplement. Data primers and probes were synthesized by Applied Biosystems (Foster City, CA).

Statistical analysis

Statistical analysis was performed by using SPSS (version 11; SPSS Inc., Chicago, Ill). Total cytokine mean levels between different groups were compared by means of 1-Way ANOVA. A *p*-value<0.05 was considered significant. The Cochran-Armitage trend test was used to test for association between individual SNP and disease status using PLINK software (26) (27).

Results

Study population

A final group of 18 atopic African American subjects were selected for this study, including eight who were cockroach sensitized and 10 who were not cockroach sensitized. Clinical characteristics and demographics of these subjects are summarized in Table E1. All individuals were atopic and all were sensitized to Bermuda grass. Asthma was confirmed in 75% of the cockroach sensitized group and 50% of the cockroach non-sensitized group. No difference in total serum levels of IgE was observed between cockroach sensitized and non-cockroach sensitized group (2.73 ± 0.45 vs 2.76 ± 0.42 , *P*=0.859).

Cockroach antigen induced Th2 cytokine production

To test the Th1/Th2 cytokine profile of co-cultured pDCs and CD4⁺ T cells, Th1/Th2 cytokines were measured in supernatants of co-cultured pDCs and CD4⁺ T cells in the presence or absence of cockroach and Bermuda grass antigen. Spontaneous production of *TNF-α* at 4 hours and *IL-10* at 48 hours was observed in resting DCs and CD4⁺ T cells

(Figure 1A and B, respectively). Significantly increased production was observed for *TNF- α* at 4 hours, and *IL-10* and *IL-13* at 48 hours when co-cultures were exposed to cockroach antigen, compared to resting cells isolated from all subjects without any differences between those with and without cockroach allergy. *IL-13* production at 48 hours was significantly higher from subjects with compared to without cockroach allergy ($p = 0.039$, Figure 1C). A similar pattern was observed for Bermuda grass allergen. However, in contrast to cockroach antigen, *IL-10* was only significantly increased in subjects without cockroach allergy and not in those with cockroach allergy. Also, no statistically significant difference in the levels of *IL-13*, *IL10*, and *TNF- α* was observed between those with and without cockroach allergy and between those in response to cockroach and Bermuda grass allergen. There were no detectable levels of *IL-12p70* and *IFN- α* .

Differentially expressed genes in response to cockroach allergen

To identify genes uniquely associated with response to cockroach allergen, we characterized gene expression profiles in allergen-stimulated and un-stimulated pDCs and CD4⁺ T cells at 4 and 48 hours using the 24,000 oligonucleotide human BeadChip (Illumina, Inc.). Because we observed a very low response at 4 hours, we limited our analysis on the immune responses at 48 hours. A total of 13,512 genes were identified as being present in at least 75% of samples. Using the SAM approach, with a FDR of 5% and 1.5-fold cutoff, we identified a total of 89 genes that were significantly differentially expressed among cockroach-allergic individuals and 87 genes that were differentially expressed in non-cockroach allergic individuals (Figure 2). Of these, 36 genes overlapped. Twelve genes were differentially expressed when co-cultures were exposed to the unrelated allergen (Bermuda grass) among cockroach allergic subjects. Importantly, a total of 50 genes were found to be uniquely expressed specifically to cockroach allergen among all cockroach-allergic individuals (48 genes up-regulated, two genes down-regulated). The genes most significantly up-regulated were *CD14* (5.43 fold), *S100A8* (5.26-fold), *CCL8* (5.18-fold), *IFI44L* (4.82-fold) and *GIP2* (3.99-fold) (Table 1).

We imported the 50 genes into the Ingenuity Pathways Analysis (IPA) software to identify potential biological pathways. Six major canonical pathways were found to be most significant, including interferon signaling ($P < 0.000001$), nicotinate and nicotinamide metabolism ($P < 0.0001$) and Toll-like receptor (TLR) signaling ($P < 0.01$), followed by arginine and proline metabolism, lysine biosynthesis and actin cytoskeleton signaling ($P < 0.05$; Figure 3).

Microarray results were validated by RT-PCR

Eight of the top-ranking known genes (*CD14*, *S100A8*, *IFI44L*, *GIP2*, *IL-24*, *S100A9*, *IFI44* and *IRF7*) were selected from among the 50 specifically expressed genes and subjected to RT-PCR in cockroach allergic and non-allergic individuals ($n=5$ for each group). All selected genes were confirmed by RT-PCR in the same samples that were used for the microarray study (Figure 4). These eight genes were further validated in the human THP-1 cells, a widely used monocytic cell line, by RT-PCR (Figure E2). Results showed mRNA levels of seven of the eight genes evaluated were increased after exposure to cockroach allergen extract (100 $\mu\text{g/ml}$) for 48 hours, and the increased expression was higher compared with that exposure to Bermuda grass (100 $\mu\text{g/ml}$) and LPS (100 ng/ml, an equal amount of LPS in the cockroach extract).

CD14 expression at the protein level was validated

The increased expression of *CD14* at the protein level was also detected on purified human pDCs using flow cytometry (Figure 5A). The percentage of CD14⁺ cells among BDCA2⁺ cells was 3.2% in the presence of cockroach allergen (100 $\mu\text{g/ml}$), which was much higher

compared to that in the presence of media (0.4%) and Bermuda grass (1.6%). To validate the findings, we used the human THP-1 cells and observed a similar increase in *CD14* expression in the presence of cockroach extract (21.2%), but a limited (2.08%) and no increase (0.59%) in the presence of LPS and Bermuda grass, respectively (Figure 5B). Additionally, soluble *CD14* (sCD14) was significantly higher in supernatants of co-cultured pDCs and CD4⁺ T cells in exposure to cockroach antigen for 48 hrs, compared to resting cells isolated from all subjects ($P = 0.034$) or just from cockroach allergic subjects ($P = 0.012$) (Figure 5C).

Functional -260C/T variant in CD14 contributes to the differential gene expression

We examined whether the *CD14* functional polymorphism, *CD14* -260 C/T (28), was associated with asthma in this African American population. Genotype frequencies were in Hardy-Weinberg equilibrium in our genotyped 807 African Americans and were consistent with previous reports (29). Detailed information on the study population is described in the Methods Section of the Online Data Supplement, and clinical characteristics are presented in Table E2. We observed a significant association for *CD14* -260 genotype with asthma (TT vs CC: odds ratio (OR), 0.62, 95%CI, 0.38–0.99, $P = 0.037$; T allele vs C allele: OR, 0.79, 95%CI, 0.64–0.97, $P = 0.023$, Table 2). Moreover, we examined whether the same asthma associated genetic variant contributes to differential *CD14* gene expression. Among subjects with *CD14* -260 CC (wildtype) genotype ($n = 13$), *CD14* gene expression was significantly increased in cells from cockroach allergic subjects ($n=6$) compared to those from non-allergic individuals ($n=7$) at baseline ($P = 0.011$) (Figure 6). Increased expression was even stronger when cultures were exposed to cockroach allergen for 48 hours ($P = 0.002$). No difference in *CD14* gene expression was observed among subjects with the CT genotype ($n = 5$) between those with and without cockroach allergy. This finding suggests an interrelationship between cockroach allergen exposure and cockroach sensitization may depend partly upon the *CD14* -260C/T functional variant.

Discussion

Previous studies on allergen-induced, immune responses have suggested a functional interaction between innate immunity and development of allergic diseases, wherein DCs are critical APCs that respond immediately in innate immune responses and then subsequently initiate and regulate adaptive immune responses (30–32). Two major DC subtypes, pDCs and mDCs, induce different types of immune responses in the presence of different stimuli(33). It has been suggested both DC subsets recognize different stimuli by expression of distinct repertoires of TLRs, and induce different types of innate immune responses depending on environmental factors(34). A recent study has shown both pDCs and mDCs accumulate in the airway lumen after allergen challenge in patients with asthma(13), and pDCs are critical in protecting against severe respiratory tract infections, wheezing, and risk of asthma(15). Given that DCs have abilities to initiate immune response to common allergens and virus, we postulate they might be especially relevant to asthma pathogenesis. We therefore selected pDCs as an initial study DC subtype to investigate cockroach allergen induced immune responses, especially examining the Th1/Th2 cytokine profile of co-cultured pDCs and CD4⁺ T cells upon cockroach exposure. We observed an increased production of *IL-13*, *IL-10*, and *TNF- α* when co-cultures were exposed to cockroach allergen compared to resting cells, but detectable levels of *IL-12p70* and *IFN- α* were not observed. It is notable that *IL-13* was higher in individuals with cockroach allergy compared to atopic individuals without cockroach allergy, and Bermuda grass exposure did not produce a significant increase in *IL-13* expression in these same individuals (who were also allergic to Bermuda grass). Given that *IL-13* is the foremost cytokine and a key player in mediating Th2-dependent diseases (35,36), the increased expression in cockroach allergic individuals

may suggest that cockroach allergen, similar to other allergens such as dust mite (37,38), may be capable of activating DCs and favoring a Th2 response. Indeed, we observed an increased expression of CD69 (an early activation marker in T cells) in pDCs and co-cultured pDCs and CD4⁺ T cells in exposure to cockroach allergen (data not shown). Interestingly, the increased expression was proportionate to the ratio of co-cultured pDCs and CD4⁺ T cells, suggesting cockroach allergen-induced pDCs are able to activate CD4⁺ T cells.

Parallel to analyses of cytokine secretion, we performed high-throughput genomics studies to characterize the transcriptional signature for cockroach sensitization and specific immune response to cockroach allergen. To avoid heterogeneity among leukocyte subpopulations and the potential diversity of the cell-type specific response(39), we limited our focus to the two major cell types, pDCs and CD4⁺ T cells. Because of the limited number of pDCs in blood (*i.e.*, only $\sim 5 \times 10^5$ pDCs can be isolated from 60 ml of blood for one individual), we had insufficient pDCs for microarray analysis (*i.e.*, at least 1 million cells are needed per experimental condition). We therefore co-cultured both pDCs and CD4⁺ T cells and speculated genes differentially expressed in these co-cultured cells are those expressed in either pDCs or CD4⁺ T cells or both, and are likely associated with initiation and maintenance of T-cell immunity to cockroach allergens as well as Th2 effector function. We focused on the mRNA profile at 4 and 48 hours, which allowed us to detect genes transiently up-regulated after initial exposure to antigen and genes induced by both continuous exposure to antigen stimulation and previously secreted mediators. As expected, very few genes were identified at 4 hours and this likely reflects the very few pDCs in these cultures. Among these genes, the *GABARAPL1* (GABA(A) receptor-associated protein like 1) was the most differentially expressed gene, but its function remains unknown(40). In contrast, we identified a total of 89 genes differentially expressed in cockroach allergic individuals at 48 hours, demonstrating a greater response in the cockroach antigen treated cells at 48 hours compared to an earlier timeframe. More importantly, we identified 50 genes uniquely expressed in cockroach sensitized individuals upon exposure to cockroach allergen by excluding genes differentially expressed in non-cockroach allergic individuals and genes differentially expressed in individuals upon exposure to control allergen (*Bermuda* grass). These differentially expressed genes include several well-known genes such as *CD14*, *S100A8*, *CCL8*, *IFI44L* and *GIP2*. Those genes were further validated by RT-PCR in the same samples used for the microarray analysis. Although the fold changes in co-cultures between pre- and post-cockroach antigen stimulation were systematically higher using TaqMan, the relationship was highly concordant, with *CD14* being the most up-regulated gene with a 21.3 fold increase according to RT-PCR among all validated genes (5.43 in microarray). *CD14* is a surface protein preferentially expressed on monocytes/macrophages, and subset of blood mDCs. *CD14* serves as a receptor for lipopolysaccharide (LPS) (41) and binding of LPS to *CD14* induces macrophage activation with release of cytokines such as interleukin (*IL*)-6, *IL*-8 and tumor necrosis factor (*TNF*- α) (42). *CD14*-mediated cell activation following segmental allergen provocation (standardized doses of rye pollen, birch pollen or house-dust mite allergen) has been suggested to play a role in asthmatic inflammation(43). In this study, we found an increased *CD14* expression in co-cultured pDCs and CD4⁺ T cells following cockroach allergen stimulation; in particular, in the purified pDCs (>97% purity) as determined by the percentages of CD14⁺ cells among BDCA2⁺ cells. However, it remains unknown if the percentage of CD14⁺ cells among the purified pDCs increases concomitantly with the increased levels of *CD14* mRNA in pDCs and CD4⁺ T cells observed in microarray analysis. In addition, to further examine whether *CD14* was expressed in different cell types, we selected THP-1 cells, a human monocytic cell line, and detected *CD14* expression at the RNA and protein levels. As expected, we observed an increase in *CD14* expression, and, interestingly, the most up-regulation was noted in cockroach allergen extract-treated cells compared to cells treated with the amount

of LPS typically present in the cockroach extract (1.14 ng/μl LPS) and Bermuda grass extract. These observations suggest cockroach allergen – not the LPS present in cockroach extract - may induce *CD14* expression on pDCs and subsequently lead to an allergic inflammation.

A functional single nucleotide polymorphism (SNP), *CD14* (-260C/T), is one of the most replicated genetic associations with asthma and associated traits (44,45). Recent studies suggest this functional polymorphism in the promoter of *CD14* may modulate specific responses to environmental aeroallergens, at least among individuals predisposed to atopy (46),(47). Our results in this study supported the previous evidence of association between the *CD14* -260 C allele or CC genotype and increased asthma, and further demonstrated cells from subjects with the CC ‘high-risk’ genotype exposure to cockroach allergen had the greatest differential expression of *CD14* between cockroach allergy and non-cockroach allergy. This result supports the notion that different genotypes may result in significant variations in response to allergen, suggesting some interaction between cockroach allergen exposure and *CD14* genotype may contribute to cockroach allergy susceptibility. However, no association was observed for the C variant, which has been associated with asthma by multiple groups, and cockroach allergy (data not shown). There are several possibilities for failure to observe this association, including the very small sample size, heterogeneity of the phenotype, and importantly, differences in environmental exposure (*e.g.*, different exposure levels of cockroach allergen). Unfortunately, data on current exposure to cockroach allergen among the volunteers in this study were not available. Also, it is possible that *CD14* SNPs other than the C-260T allele are conferring risk to cockroach allergy.

Ingenuity Pathways Analysis analyses identified six major canonical pathways mediating cockroach allergen induced immune responses, including two of the most common pathways: interferon signaling and TLR signaling pathways. pDCs are specialized producers of type I IFN production, and triggering through TLR activates pDCs to produce type I IFNs (48),(49). A receptor, PDC-TREM, was recently identified to be preferentially expressed on TLR-stimulated pDCs and responsible for augmented production of type I IFN (50). Surface expression of PDC-TREM requires both TLR and IFN signaling. It would be of interest to investigate if cockroach allergen can bind to either PDC-TREM or TLRs on pDCs. We speculate cockroach allergen may activate TLR and IFN signaling pathways and lead to an augmented production of IFNs. Indeed, among these differentially expressed genes identified here for cockroach sensitization, several of the top genes are interferon-related genes, including *IFI44* (Interferon-induced protein 44), its ligand *IFI44L*, *GIP2* (Interferon, alpha-inducible protein), *GIP3*, and *IFIT1*. This finding was unexpected, because it is known that type-I interferon is mainly produced by pDCs, and plays an important role in DC activation, maturation, and T cell differentiation toward Th1 type immune response (51). Thus, the results in this study appear contradictory, wherein a strong Th2 response and a dominant type-I interferon response was observed when co-cultured pDCs and CD4⁺ T cells were exposed to cockroach allergen. We speculate that there may be cross-regulation by type I interferon between pDCs and CD4⁺ T cells after 48 hours culture. In this instance, a feedback mechanism might exist whereby CD4⁺ T cells eventually communicate back with pDCs to up-regulate IFN-alpha genes-perhaps in an attempt to shut down Th2 activity. Validation of these findings in single cell types rather than in co-cultured conditions is forthcoming.

We recognize co-cultured pDCs and CD4⁺ T cells may lead to cell heterogeneity (pDCs and CD4⁺ T cells) for microarray analysis; however, results from this study can be further validated on purified pDCs at both mRNA and protein levels. We also acknowledge commercially-prepared, crude cockroach extract may not be the optimum antigen in terms of potency. However, this extract is clinically relevant and is routinely used for diagnostic

purposes (*i.e.*, skin testing) to identify cockroach allergic subjects. In fact, the cockroach whole body extract has been shown to contain clinically relevant allergens and also possesses allergenicity similar to fecal material and egg casings, but is more potent than cockroach detritus. Although recombinant and purified native cockroach allergens are commercially available (*e.g.*, Bla g1, and Bla g2), they represent only a few components of the whole extract, and therefore, relevant sensitizations are missed in a portion of the human subjects tested. For example, only 30–50% of patients with cockroach allergy have serum specific-IgE to Bla g1, and 60–68% have serum IgE to Bla g2 (52). By using crude cockroach extract, we are able to determine the ‘generic’ responses to cockroach allergen (*e.g.*, cockroach allergy, yes/no). It is also noteworthy that, although potential contamination in cockroach extract may be a confounding stimulus, the human pDCs selected in this study reportedly lack CD14 receptor expression (TLR4). For follow-up studies, we plan to quantify the amounts of endotoxin and set additional controls with an equivalent dose of endotoxin to that found in cockroach extract.

In summary, we have demonstrated a strong Th2 response when co-cultured pDCs and CD4⁺ T cells are exposed to cockroach antigen. Using an array platform representing most human genes, we also identified a group of genes that may uniquely contribute to a specific immune response to cockroach allergen. Functional genomics analyses of these differentially expressed genes suggest both interferon signaling pathway and TLR-signaling pathways are two of the most significant canonical pathways. As part of our continuing efforts, these differentially expressed genes and genes within the identified signaling pathways will be targeted for further investigation into their biological relevance to specific immune response to cockroach allergens and cockroach sensitization. More extensive research with a panel of different cockroach allergens (recombinant and purified natural allergens, *e.g.*, rBla g2 and nBla g2) and two major DC subtypes (pDCs, mDCs) may provide a better understanding of how these genes are involved in the specific allergic response compared to those governing overall inflammatory response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Bla g	<i>Blattella germanica</i>
DC	Dendritic cell
pDC	Plasmacytoid dendritic cell
SAM	Significant analysis of microarray
IPA	Ingenuity Pathways Analysis

FDR	False discovery rate
SNP	Single nucleotide polymorphism

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Figure 1.

Cockroach allergen induces the production of TNF- α , IL-10, and IL-13 in supernatants of co-cultured pDCs and CD4⁺ T cells at 4 or 48 hours. (A) The levels of TNF- α at 4 hours. (B) The levels of IL-10 at 48 hours. (C) The levels of IL-13 at 48 hours. The black bars represent the cockroach allergic subjects (N= 8) and white bars represent the cockroach non-allergic subjects (N=10). * P <0.05, ** P <0.01.

Figure 2. Genes significant differentially expressed in subjects with (n=8) and without cockroach allergen (n=10). Gene expression profiles of cultures were analyzed using GCOS 1.4 and SAM. Fifty genes (n=50, within dark red circle) were selected from differentially expressed genes in cockroach allergic individuals by filtering genes differentially expressed in cockroach non-allergic individuals (n=36, within brown circle) and cultures in exposure to Bermuda grass (n=12, within green circle).

Inguenity Pathways Analysis of canonical pathways for target genes. The significance represents the degrees of our candidate genes involved in the represented function. The significance is expressed as a log-transformed p-value. The threshold line represents significant p-value 0.05.

Figure 4. Validation of the microarray results by using RT-PCR. Each mRNA value in RT-PCR is expressed relative to *GAPDH* mRNA in five subjects (N=5) and is expressed as fold change. Filled bar represents fold change by microarray, and open bar represents fold change by RT-PCR. Results are expressed as the mean $\pm$ SD.

C.

Figure 5.

CD14 expression was detected by FACS. (A) Detection of *CD14* expression on purified pDCs. pDCs were exposed to media, cockroach allergen extract (100 ug/ml), and Bermuda grass (100 ug/ml) for 48 hours and then stained with BDCA2, CD14, and isotype antibodies. (B) Detection of CD14 expression on human THP-1 cells. THP-1 cells were exposed to media, cockroach allergen extract (200 ug/ml), LPS (200 ng/ml), and Bermuda grass (200 ug/ml) for 48 hours and then stained with CD14. Numbers in (A) and (B) indicate percentage of CD14⁺ cells, and data in both (A) and (B) represent one of three independent experiments. (C) The levels of sCD14 were detected by ELISA in supernatants of co-cultured pDCs and CD4⁺ T cells from cockroach sensitized [black bar, n=8] and cockroach non-sensitized [open bar, n=8] individuals. *P<0.05.

Figure 6.

CD14 gene expression was compared between individuals with (N=8) and without cockroach allergy (N = 10) stratified by cockroach exposure and *CD14* -260 genotype (CC, N = 13, and CT, N = 5). The mRNA expression was determined by signal densities (mean $\pm$ SD). Results represent normalized expression values (relative units). * P <0.05, ** P <0.01.

Table 1

Cockroach allergen induced genes uniquely expressed in cells from subjects with cockroach allergy

Symbol	Description	Fold change*	P-value
Up-regulation			
CD14	CD14 antigen	5.43	0.017
S100A8	S100 calcium binding protein A8 (calgranulin A)	5.26	0.015
CCL8	Chemokine (C-C motif) ligand 8	5.18	0.005
IFI44L	Interferon-induced protein 44-like	4.82	0.031
G1P2	Interferon, alpha-inducible protein (clone IFI-15K)	3.99	0.036
IL24	Interleukin 24	3.94	0.050
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78	3.61	0.028
S100A9	S100 calcium binding protein A9 (calgranulin B)	3.20	0.033
G1P3	Interferon, alpha-inducible protein (clone IFI-6-16) (G1P3)	3.06	0.032
IFI44	Interferon-induced protein 44	3.02	0.037
RSAD2	Radical S-adenosyl methionine domain containing 2	2.95	0.019
LYN	V-src-1 Yamaguchi sarcoma viral related oncogene homolog	2.86	0.021
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	2.83	0.019
LY6E	Lymphocyte antigen 6 complex, locus E	2.55	0.024
HERC5	Hect domain and RLD 5	2.51	0.034
PRIC285	Peroxisomal proliferator-activated receptor A interacting complex 285	2.44	0.032
BASP1	Brain abundant, membrane attached signal protein 1	2.43	0.033
EPSTI1	Epithelial stromal interaction 1 (breast)	2.41	0.033
MARCKS	Myristoylated alanine-rich protein kinase C substrate	2.29	0.048
IRF7	Interferon regulatory factor 7	2.26	0.029
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	2.24	0.026
HERC6	Hect domain and RLD 6	2.21	0.026
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	2.21	0.027
INDO	Indoleamine-pyrrole 2,3 dioxygenase	2.16	0.019
OAS1	2',5'-oligoadenylate synthetase 1	2.09	0.015
OAS3	2'-5'-oligoadenylate synthetase 3	2.08	0.027
IFITM3	Interferon induced transmembrane protein 3 (1-8U)	2.02	0.015
VNN2	Vanin 2	2.01	0.037
PLSCR1	Phospholipid scramblase 1	2.00	0.001
Down-regulation			
SGK	Serum/glucocorticoid regulated kinase	-1.62	0.022
CIR	CBF1 interacting corepressor	-1.96	0.025

* The fold change was determined by circulating the ratio of global normalized signals from the co-cultured pDCs and CD4⁺ T cells of cockroach allergic group.

Among the 50 cockroach allergen induced genes, 29 genes with 2-fold up-regulation and 2 genes with >1.5 – fold down-regulation were listed in this table.

Table 2

Association between the *CD14* -260C/T variant and asthma in an African American case-control population

Characteristic	No. of cases	Asthmatic N (%)	Non-asthmatic N (%)	OR	95%CI	P value [*]
CC	345	182 (46.3)	163 (39.4)			
CT	364	171 (43.5)	193 (46.6)	0.79	(0.58–1.08)	0.124
TT	98	40 (10.2)	58 (14.0)	0.62	(0.38–0.99)	0.037
C	1054	535 (68.1)	519 (62.7)			
T	560	251 (31.9)	309 (37.3)	0.79	(0.64–0.97)	0.023

^{*}This SNP was not significantly associated with cockroach sensitization