

## ORIGINAL ARTICLE

# CD14, a key candidate gene associated with a specific immune response to cockroach

P. Gao<sup>1</sup>, D. N. Grigoryev<sup>1</sup>, N. M. Rafaels<sup>1</sup>, D. Mu<sup>1</sup>, J. M. Wright<sup>2</sup>, C. Cheadle<sup>1</sup>, A. Togias<sup>1</sup>, T. H. Beaty<sup>3</sup>, R. A. Mathias<sup>1,4</sup>, J. T. Schroeder<sup>1</sup> and K. C. Barnes<sup>1</sup>

Departments of <sup>1</sup>Medicine, Division of Allergy and Clinical Immunology, <sup>2</sup>Physiology, <sup>3</sup>Epidemiology, Bloomberg School of Public Health and <sup>4</sup>Medicine, Division of General Internal Medicine, The Johns Hopkins University, Baltimore, MD, USA

## Clinical & Experimental Allergy

### 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 dendritic cells (pDCs) and CD4<sup>+</sup> 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- $\alpha$ , but undetectable levels of IL-12p70 and IFN- $\alpha$ , 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 h compared with 4 h, 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 the supernatant of co-cultured pDCs and CD4<sup>+</sup> T cells on 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 analyses suggested the IFN signalling as the most significant canonical pathway.

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

**Keywords** asthma, CD4<sup>+</sup> T cells, *CD14*, cockroach sensitization, dendritic cells (DCs), high-throughput expression profiling

*Submitted 1 March 2010; revised 27 April 2010; accepted 11 May 2010*

### Correspondence:

Kathleen C. Barnes, The Johns Hopkins Asthma & Allergy Center, 5501 Hopkins Bayview Circle, Room 3A.62, Baltimore, MD 21224, USA.

E-mail: kbarnes@jhmi.edu

### 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  $\mu\text{g}/\text{year}$ ) is associated with the 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 United States [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 (0.33  $\mu\text{g}/\text{g}$ ) [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 that 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 with European Americans living in the Baltimore–Washington, DC 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 dendritic cells (pDCs) and myeloid dendritic cells (mDCs), have been observed accumulating in the airway lumen of asthmatics following an 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, the 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<sup>+</sup> 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<sup>+</sup> T cells.

In this study, we investigated whether exposure of DCs (in co-culture with CD4<sup>+</sup> T cells) to crude cockroach allergen extract instructs DCs to preferentially skew CD4<sup>+</sup> T cells towards a T-helper type 2 (Th2) response. Because pDCs responded poorly to lipopolysaccharide (LPS) [no Toll-like receptor (TLR)-4 expression] [14, 18], our study focused on pDCs as an initial study DC subtype, which could avoid endotoxin contamination in a cockroach extract-induced immune response. We speculated that, upon exposure to cockroach extract, pDCs affect CD4<sup>+</sup> T-cell activation to promote allergic inflammation. Because of the limited numbers of circulating human pDCs, we used co-cultured pDCs and CD4<sup>+</sup> 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 (weal  $\geq 3$  mm greater than the saline control) and/or positive specific IgE ( $\geq 0.35$  kU/L) to common aeroallergens (Greer Laboratories, Lenoir, NC, USA) as described in 'Methods' of the supporting information. 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 peripheral blood mononuclear cells using a standardized method [19] as described in 'Methods' of the supporting information. The purity of pDCs was determined according to the manufacturer's instructions ( $>97\%$ ). The isolated pDCs and CD4<sup>+</sup> T cells were plated in a ratio of 1 : 20 ( $\sim 5.0 \times 10^4$  pDCs and  $1.0 \times 10^6$  CD4<sup>+</sup> 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<sup>+</sup> T cells ( $\sim 5.0 \times 10^4$  pDCs and  $1.0 \times 10^6$  CD4<sup>+</sup> 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- $\alpha$  production (Fig. S1) when cells were exposed to various concentrations (i.e. 1–200  $\mu\text{g/mL}$ ) of antigen extract. Endotoxin in both extracts was detected by Limulus amebocyte lysate QCL-1000<sup>®</sup> (Lonza, Walkersville, MD, USA). The cultured cells were harvested at 4 and 48 h and stored for RNA extraction and microarray analysis.

### Cytokine assay

The concentrations of cytokines secreted by both pDCs and CD4<sup>+</sup> T cells from atopic subjects in the culture supernatant were determined by ELISA (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Selected cytokines included IL-12p70, IL-13, TNF- $\alpha$ , 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  $\mu\text{g}$ ) was isolated from each sample using TriZol (Gibco-BRL, Carlsbad, CA, USA) and RNeasy columns (Qiagen Inc.,

Hilden, Germany), and RNA quality was confirmed on the Agilent 2100 bio-analyzer (Agilent technologies, Palo Alto, CA, USA). Biotin-labelled cRNAs were prepared as described in the Illumina expression analysis technical manual (Illumina Inc., San Diego, CA, USA) using 0.5 µg of purified total RNA as a template for the reaction. The biotin-labelled cRNA samples were hybridized (16 h) 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 analysed using Illumina BeadStudio software. Additionally, the resulting digitized matrix was modified for the Illumina platform approach described previously [22, 23]. Power prediction analysis for minimum array requirements and microarray data analysis are described in 'Methods' of the supporting information. 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<sup>+</sup> T cells in three categories (medium only, cockroach antigen stimulation, and Bermuda grass antigen stimulation) at early (4 h) and late (48 h) 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 reverse transcriptase polymerase chain reaction*

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

#### *Flow-cytometric analysis of cell surface-expressed molecules*

The allergen-treated and untreated cells were harvested at 48 h, washed in phosphate-buffered saline once, and then incubated for 30 min 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, USA), or IgG1 isotype control antibodies (2.5 µg/mL). After washing, cells were analysed by fluorescence-activated cell sorting (Calibur, Becton-Dickinson, San Jose, CA, USA) [24].

#### *Single nucleotide polymorphism genotyping*

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

#### *Statistical analysis*

Statistical analysis was performed using SPSS (version 11; SPSS Inc., Chicago, IL, USA). The total cytokine mean levels between different groups were compared by means of one-way ANOVA. A *P*-value <0.05 was considered significant. The Cochran-Armitage trend test was used to test for the association between individual SNP and disease status using PLINK software [26, 27].

### **Results**

#### *Study population*

A final group of 18 atopic African-American subjects was selected for this study, including eight who were cockroach sensitized and 10 who were not cockroach sensitized. The clinical characteristics and demographics of these subjects are summarized in Table S1. 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 the total serum levels of IgE was observed between the cockroach-sensitized and the non-cockroach-sensitized group ( $2.73 \pm 0.45$  vs.  $2.76 \pm 0.42$ , *P* = 0.859).

#### *Cockroach antigen induced T-helper type 2 cytokine production*

To test the Th1/Th2 cytokine profile of co-cultured pDCs and CD4<sup>+</sup> T cells, Th1/Th2 cytokines were measured in the supernatants of co-cultured pDCs and CD4<sup>+</sup> T cells in the presence or absence of cockroach and Bermuda grass antigen. Spontaneous production of TNF-α at 4 h and IL-10 at 48 h was observed in resting DCs and CD4<sup>+</sup> T cells (Figs 1a and b, respectively). Significantly increased production was observed for TNF-α at 4 h, and IL-10 and IL-13 at 48 h when co-cultures were exposed to cockroach antigen, compared with resting cells isolated from all subjects without any differences between those with and without cockroach allergy. IL-13 production at 48 h was significantly higher in subjects with compared to without cockroach allergy (*P* = 0.039, Fig. 1c). A similar pattern was observed for Bermuda grass allergen. However, in

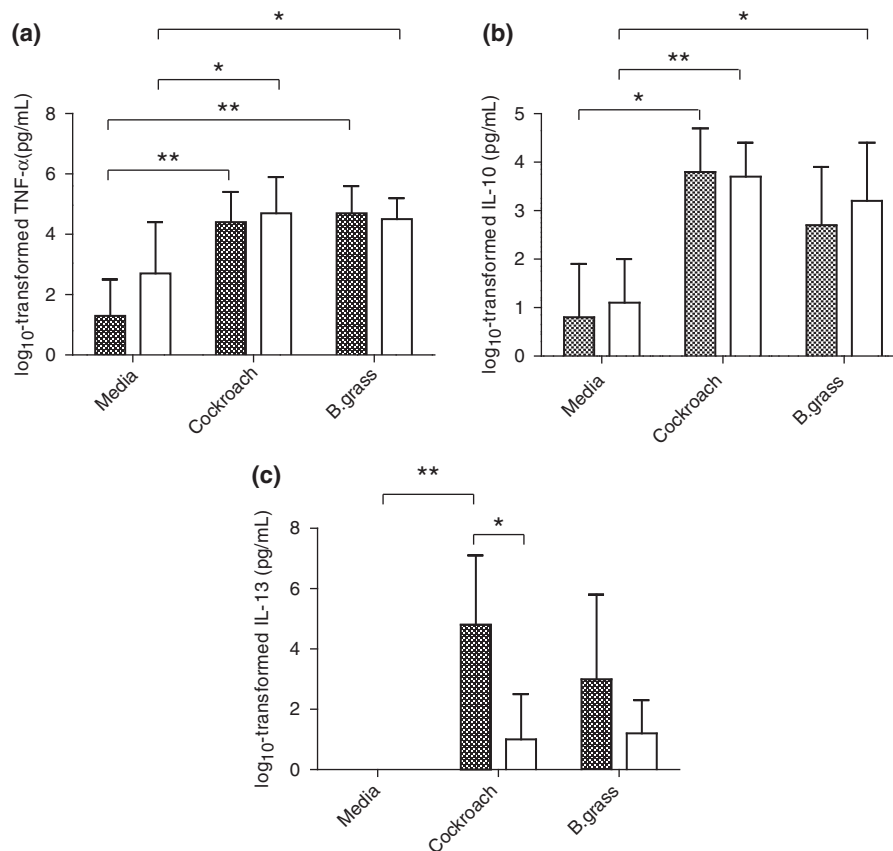


Fig. 1. Cockroach allergen induces the production of TNF- $\alpha$ , IL-10, and IL-13 in the supernatants of co-cultured plasmacytoid dendritic cells and CD4<sup>+</sup> T cells at 4 or 48 h. (a) The levels of TNF- $\alpha$  at 4 h. (b) The levels of IL-10 at 48 h. (c) The levels of IL-13 at 48 h. The black bars represent the cockroach-allergic subjects ( $n = 8$ ) and the white bars represent the cockroach non-allergic subjects ( $n = 10$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .

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- $\alpha$  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- $\alpha$ .

#### 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 unstimulated pDCs and CD4<sup>+</sup> T cells at 4 and 48 h using the 24 000 oligonucleotide human BeadChip (Illumina Inc.). Because we observed a very low response at 4 h, we limited our analysis to the immune responses at 48 h. A total of 13 512 genes were identified as being present in at least 75% of the samples. Using the SAM approach, with an FDR of 5% and a 1.5-fold cut-off, 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 (Fig. 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 IFN signalling ( $P < 0.000001$ ), nicotinate and nicotinamide metabolism ( $P < 0.0001$ ), and TLR signalling ( $P < 0.01$ ), followed by arginine and proline metabolism, lysine biosynthesis, and actin cytoskeleton signalling ( $P < 0.05$ ; Fig. 3).

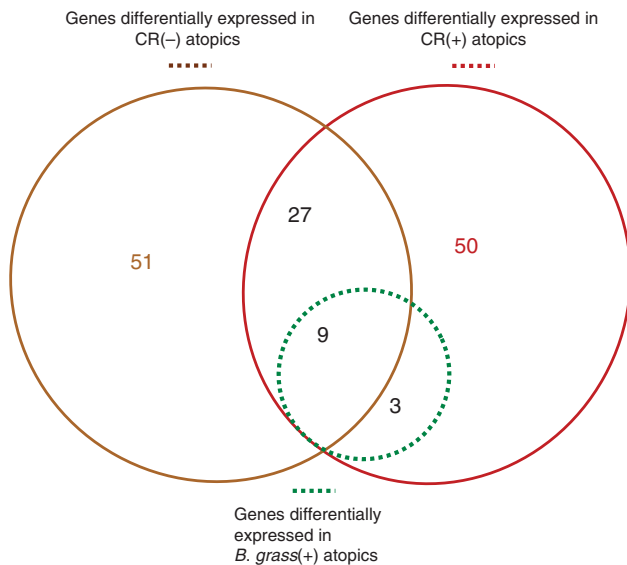


Fig. 2. Genes significant differentially expressed in subjects with ( $n=8$ ) and without cockroach allergen ( $n=10$ ). Gene expression profiles of cultures were analysed using GCOS 1.4 and SAM. Fifty genes ( $n=50$ , within dark red circle) were selected from differentially expressed genes in cockroach-allergic 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).

#### Microarray results were validated by reverse transcriptase polymerase chain reaction

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 as those used for the microarray study (Fig. 4). These eight genes were further validated in the human THP-1 cells, a widely used monocytic cell line, by RT-PCR (Fig. S2). The results showed that the mRNA levels of seven of the eight genes evaluated were increased after exposure to cockroach allergen extract (100 µg/mL) for 48 h, and the increased expression was higher compared with that exposed to Bermuda grass (100 µg/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 (Fig. 5a). The percentage of CD14<sup>+</sup> cells among BDCA2<sup>+</sup> cells was 3.2% in the presence of cockroach allergen (100 µg/mL), which was much higher compared with 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 (Fig. 5b). Additionally, soluble CD14 was significantly higher in the supernatants of co-cultured pDCs and CD4<sup>+</sup> T cells in exposure to cockroach antigen for 48 h, compared with resting cells isolated from all subjects ( $P=0.034$ ) or just from cockroach-allergic subjects ( $P=0.012$ ) (Fig. 5c).

#### The functional -260C/T variant in CD14 contributes to the differential gene expression

We examined whether the *CD14* functional polymorphism, *CD14* -260C/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 'Methods' of the supporting information, and the clinical characteristics are presented in Table S2. We observed a significant association for the *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 the *CD14* -260CC (wild-type) genotype ( $n=13$ ), CD14 gene expression was significantly increased in cells from cockroach-allergic subjects ( $n=6$ ) compared with those from non-allergic individuals ( $n=7$ ) at baseline ( $P=0.011$ ) (Fig. 6). Increased expression was even stronger when cultures were exposed to cockroach allergen for 48 h ( $P=0.002$ ). 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 that an interrelationship between cockroach allergen exposure and cockroach sensitization may depend partly on the *CD14* -260C/T functional variant.

#### Discussion

Previous studies on allergen-induced, immune responses have suggested a functional interaction between innate immunity and the 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 the expression of distinct repertoires of TLRs, and induce different types of innate

**Table 1.** Cockroach allergen-induced genes uniquely expressed in cells from subjects with cockroach allergy

| Symbol                 | Description                                                           | Fold change | P-value |
|------------------------|-----------------------------------------------------------------------|-------------|---------|
| <i>Up-regulation</i>   |                                                                       |             |         |
| 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                 | IFN-induced protein 44-like                                           | 4.82        | 0.031   |
| G1P2                   | IFN, $\alpha$ -inducible protein (clone IFI-15K)                      | 3.99        | 0.036   |
| IL24                   | IL-24                                                                 | 3.94        | 0.050   |
| MX1                    | Myxovirus (influenza virus) resistance 1, IFN-inducible protein p78   | 3.61        | 0.028   |
| S100A9                 | S100 calcium-binding protein A9 (calgranulin B)                       | 3.20        | 0.033   |
| G1P3                   | IFN, $\alpha$ -inducible protein (clone IFI-6-16) (G1P3)              | 3.06        | 0.032   |
| IFI44                  | IFN-induced protein 44                                                | 3.02        | 0.037   |
| RSAD2                  | Radical S-adenosyl methionine domain containing 2                     | 2.95        | 0.019   |
| LYN                    | V-yes-1 Yamaguchi sarcoma viral-related oncogene homologue            | 2.86        | 0.021   |
| IFIT1                  | IFN-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                   | IFN 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                  | IFN-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                 | IFN-induced transmembrane protein 3 (1-8 U)                           | 2.02        | 0.015   |
| VNN2                   | Vanin 2                                                               | 2.01        | 0.037   |
| PLSCR1                 | Phospholipid scramblase 1                                             | 2.00        | 0.001   |
| <i>Down-regulation</i> |                                                                       |             |         |
| 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<sup>+</sup> T cells of the cockroach-allergic group.

Among the 50 cockroach allergen-induced genes, 29 genes with twofold up-regulation and two genes with > 1.5-fold down-regulation are listed in this table.

pDC, plasmacytoid dendritic cells.

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 the risk of asthma [15]. Given that DCs have abilities to initiate immune response to common allergens and virus, we postulate that 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<sup>+</sup> T cells upon cockroach exposure. We observed an increased production of IL-13, IL-10, and TNF- $\alpha$  when co-cultures were exposed to cockroach allergen compared with resting cells, but detectable levels of

IL-12p70 and IFN- $\alpha$  were not observed. It is notable that IL-13 was higher in individuals with cockroach allergy compared with 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 favouring 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<sup>+</sup> T cells in exposure to cockroach allergen (data not shown). Interestingly, the increased expression was proportionate

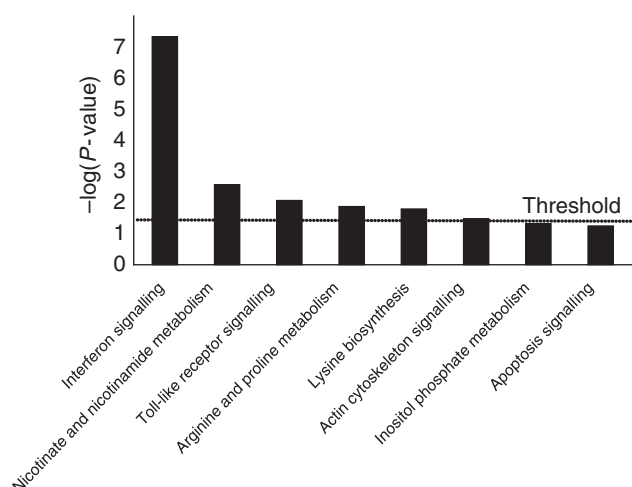


Fig. 3. Ingenuity Pathways Analysis of canonical pathways for target genes. The significance represents the degrees of our candidate genes involved in the represented function. The significance is expressed as a log-transformed  $P$ -value. The threshold line represents the significant  $P$ -value of 0.05.

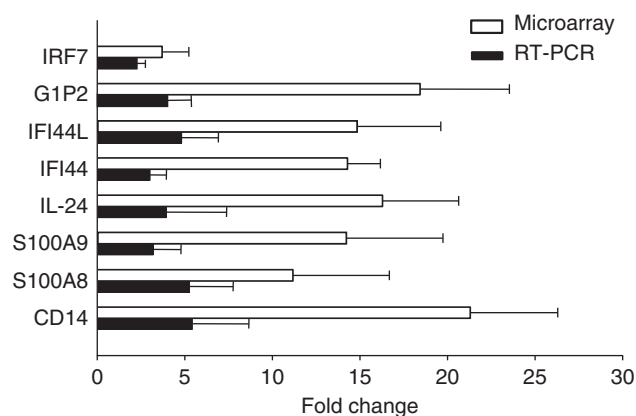


Fig. 4. Validation of the microarray results using RT-PCR. Each mRNA value in RT-PCR is expressed relative to *GAPDH* mRNA in five subjects ( $n=5$ ) and is expressed as a fold change. The filled bar represents fold change by microarray, and the open bar represents fold change by RT-PCR. Results are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

to the ratio of co-cultured pDCs and  $CD4^+$  T cells, suggesting that 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 leucocyte 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 that 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 the 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 h, 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 h 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 h, demonstrating a greater response in the cockroach antigen-treated cells at 48 h compared with 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 as those 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 a subset of blood mDCs. *CD14* serves as a receptor for LPS [41] and binding of LPS to *CD14* induces macrophage activation with the release of cytokines such as IL-6, IL-8, and TNF- $\alpha$  [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 whether 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

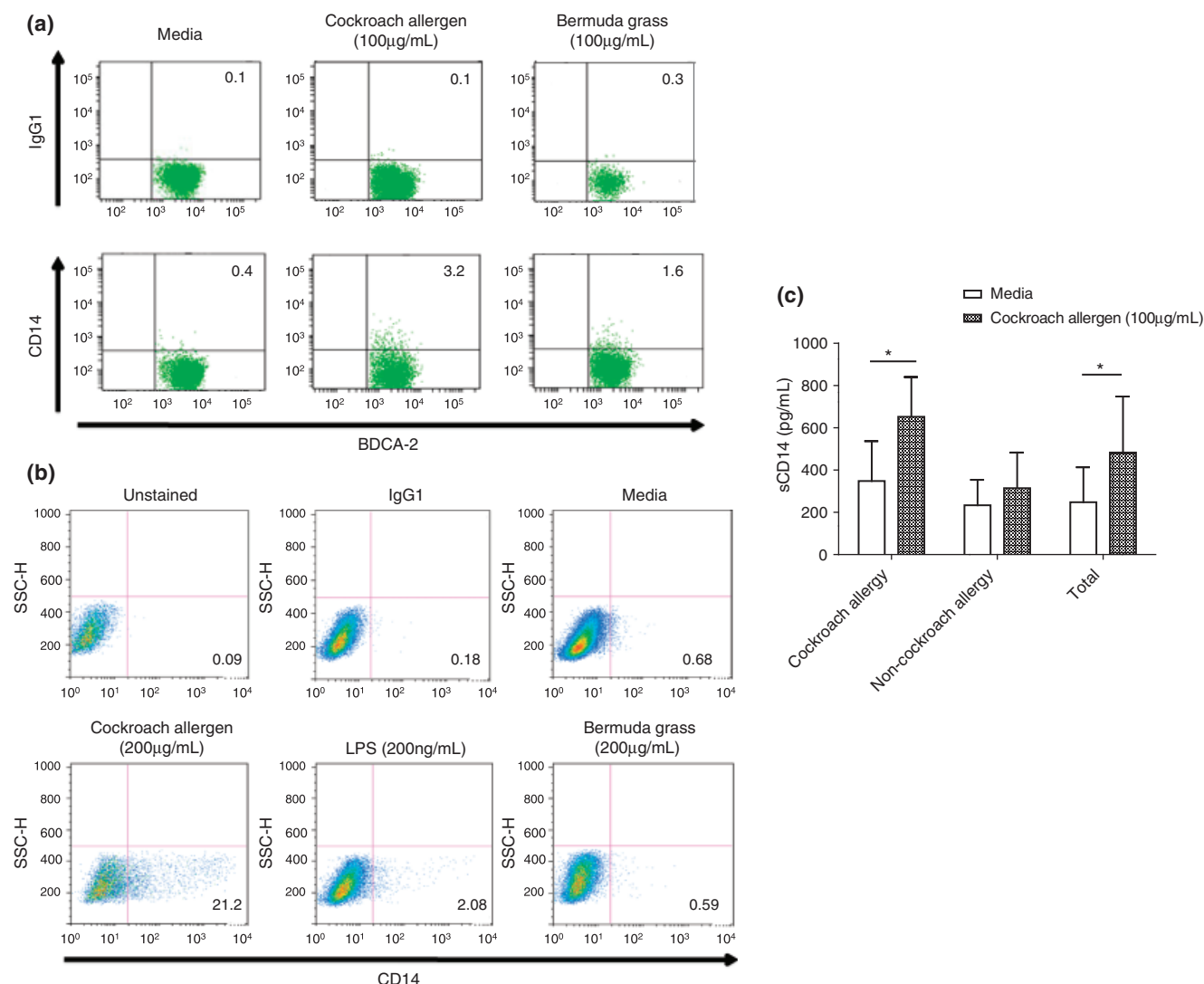


**Fig. 5.** CD14 expression was detected by fluorescence-activated cell sorting. (a) Detection of CD14 expression on purified plasmacytoid dendritic cells (pDCs). pDCs were exposed to media, cockroach allergen extract (100 µg/mL), and Bermuda grass (100 µg/mL) for 48 h and then stained with BDCA2, CD14, and isotype antibodies. (b) Detection of CD14 expression on human THP-1 cells. THP-1 cells were exposed to media, cockroach allergen extract (200 µg/mL), lipopolysaccharide (LPS) (200 ng/mL), and Bermuda grass (200 µg/mL) for 48 h and then stained with CD14. Numbers in (a) and (b) indicate the percentage of CD14<sup>+</sup> cells, and data in both (a) and (b) represent one of three independent experiments. (c) The levels of soluble CD14 were detected by ELISA in the supernatants of co-cultured pDCs and CD4<sup>+</sup> T cells from cockroach-sensitized (black bar, *n* = 8) and cockroach non-sensitized (open bar, *n* = 8) individuals. \**P* < 0.05.

**Table 2.** Association between the *CD14* -260C/T variant and asthma in an African-American case-control population

| Characteristic | No. of cases | Asthmatic, <i>n</i> (%) | Non-asthmatic, <i>n</i> (%) | OR 95% CI        | <i>P</i> -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 single nucleotide polymorphism was not significantly associated with cockroach sensitization.

OR, odds ratio.

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 maximum up-regulation was observed in cockroach allergen extract-treated

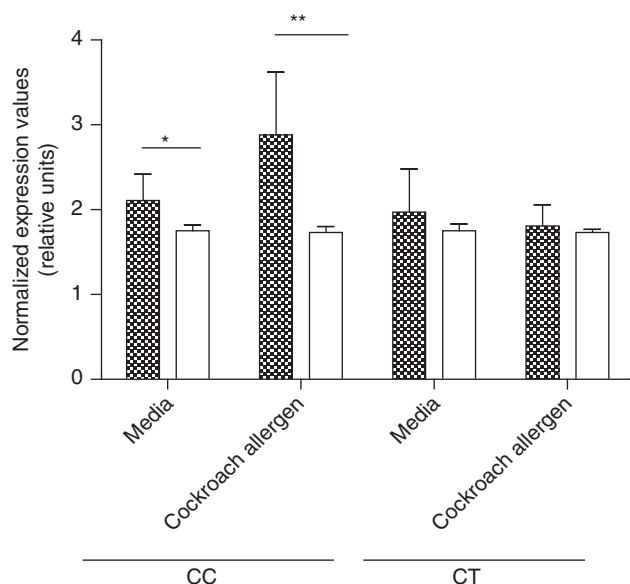


Fig. 6. *CD14* gene expression was compared between individuals with ( $n=8$ ) and without cockroach allergy ( $n=10$ ) stratified by cockroach exposure and *CD14* -260 genotype (CC,  $n=13$ , and CT,  $n=5$ ). The mRNA expression was determined by signal densities (mean  $\pm$  SD). Results represent normalized expression values relative units. \* $P < 0.05$ , \*\* $P < 0.01$ .

cells compared with cells treated with the amount of LPS typically present in the cockroach extract (1.14 ng/ $\mu$ L LPS) and Bermuda grass extract. These observations suggest that cockroach allergen – not the LPS present in cockroach extract – may induce CD14 expression on pDCs and subsequently lead to an allergic inflammation.

A functional SNP, *CD14* (-260C/T), is one of the most replicated genetic associations with asthma and associated traits [44, 45]. Recent studies suggest that this functional polymorphism in the promoter of *CD14* may modulate specific responses to environmental aeroallergens, at least among individuals pre-disposed to atopy [46, 47]. Our results in this study supported the previous evidence of association between the *CD14* -260C allele or the CC genotype and increased asthma, and further demonstrated that 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 that 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 the 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 a risk to cockroach allergy.

IPA analyses identified six major canonical pathways mediating cockroach allergen-induced immune responses, including two of the most common pathways: IFN signalling and TLR signalling 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 the augmented production of type I IFN [50]. Surface expression of PDC-TREM requires both TLR and IFN signalling. It would be of interest to investigate whether cockroach allergen can bind to either PDC-TREM or TLRs on pDCs. We speculate that cockroach allergen may activate TLR and IFN signalling pathways and lead to an augmented production of IFNs. Indeed, among the differentially expressed genes identified here for cockroach sensitization, several of the top genes are IFN-related genes, including *IFI44* (IFN-induced protein 44), its ligand *IFI44L*, *G1P2* (IFN,  $\alpha$ -inducible protein), *G1P3*, and *IFIT1*. This finding was unexpected, because it is known that type I IFN is mainly produced by pDCs, and plays an important role in DC activation, maturation, and T-cell differentiation towards Th1 type immune response [51]. Thus, the results in this study appear to be contradictory, wherein a strong Th2 response and a dominant type I IFN response were observed when co-cultured pDCs and CD4<sup>+</sup> T cells were exposed to cockroach allergen. We speculate that there may be cross-regulation by type I IFN between pDCs and CD4<sup>+</sup> T cells after a 48-h culture. In this instance, a feedback mechanism might exist, whereby CD4<sup>+</sup> 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 under co-cultured conditions is forthcoming.

We acknowledge that co-cultured pDCs and CD4<sup>+</sup> T cells may lead to cell heterogeneity (pDCs and CD4<sup>+</sup> T cells) for microarray analysis; however, the results from this study can be further validated on purified pDCs at both mRNA and protein levels. We also acknowledge that a 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]. Using a 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 the 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 a dose of endotoxin equivalent to that found in cockroach extract.

In summary, we have demonstrated a strong Th2 response when co-cultured pDCs and CD4<sup>+</sup> 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 that both IFN signalling pathway and TLR signalling pathways are two of the most significant canonical pathways. As part of our continuing efforts, these differentially expressed genes and genes within the identified signalling 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 with those governing the overall inflammatory response.

## Acknowledgements

We would like to thank Tracey Hand and Monica Campbell of the Johns Hopkins Bayview Genetics Research Facility, Yu-chi Chen and Tonya Watkins of the Johns Hopkins Bayview Lowe Family Genomics Core, and Beverly Plunkett, Maria Stockton, Cassandra Foster, and Patricia Oldewurtel, Johns Hopkins University, for technical support.

**Grant funding:** This research was supported by National Institutes of Health (NIH) grants AI50024, HL72455, and HL087699, an Asthma & Allergy Foundation of America New Investigator Award Grant (K. C. B.) and Johns Hopkins Internal Research Grant (P. Gao). K. C. B. was supported in part by the Mary Beryl Patch Turnbull Scholar Program.

## References

- 1 Salo PM, Arbes SJ Jr, Crockett PW, Thorne PS, Cohn RD, Zeldin DC. Exposure to multiple indoor allergens in US homes and its relationship to asthma. *J Allergy Clin Immunol* 2008; 121: 678–84, e2.
- 2 Pomes A. Intrinsic properties of allergens and environmental exposure as determinants of allergenicity. *Allergy* 2002; 57:673–9.
- 3 Rosenstreich DL, Eggleston P, Kattan M *et al*. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N Engl J Med* 1997; 336:1356–63.
- 4 Leaderer BP, Belanger K, Triche E *et al*. Dust mite, cockroach, cat, and dog allergen concentrations in homes of asthmatic children in the northeastern United States: impact of socioeconomic factors and population density. *Environ Health Perspect* 2002; 110:419–25.
- 5 Helm RM, Squillace DL, Jones RT, Brenner RJ. Shared allergenic activity in Asian (*Blattella asahinai*), German (*Blattella germanica*), American (*Periplaneta americana*), and Oriental (*Blatta orientalis*) cockroach species. *Int Arch Allergy Appl Immunol* 1990; 92:154–61.
- 6 Ledford DK. Indoor allergens. *J Allergy Clin Immunol* 1994; 94 (Part 2):327–34.
- 7 Sporik R, Squillace SP, Ingram JM, Rakes G, Honsinger RW, Platts-Mills TA. Mite, cat, and cockroach exposure, allergen sensitization, and asthma in children: a case-control study of three schools. *Thorax* 1999; 54:675–80.
- 8 Wunschmann S, Gustchina A, Chapman MD, Pomes A. Cockroach allergen Bla g 2: an unusual aspartic proteinase. *J Allergy Clin Immunol* 2005; 116:140–5.
- 9 Pomes A, Chapman MD, Vailes LD, Blundell TL, Dhanaraj V. Cockroach allergen Bla g 2: structure, function, and implications for allergic sensitization. *Am J Respir Crit Care Med* 2002; 165:391–7.
- 10 Togias A, Horowitz E, Joyner D, Guydon L, Malveaux F. Evaluating the factors that relate to asthma severity in adolescents. *Int Arch Allergy Immunol* 1997; 113:87–95.
- 11 Banchereau J, Briere F, Caux C *et al*. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000; 18:767–811.
- 12 Guernonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 2002; 20:621–67.
- 13 Bratke K, Lommatzsch M, Julius P *et al*. Dendritic cell subsets in human bronchoalveolar lavage fluid after segmental allergen challenge. *Thorax* 2007; 62:168–75.
- 14 Masten BJ, Olson GK, Tarleton CA *et al*. Characterization of myeloid and plasmacytoid dendritic cells in human lung. *J Immunol* 2006; 177:7784–93.
- 15 Upham JW, Zhang G, Rate A *et al*. Plasmacytoid dendritic cells during infancy are inversely associated with childhood respiratory tract infections and wheezing. *J Allergy Clin Immunol* 2009; 124:707–13, e2.
- 16 Wohlfahrt JG, Kunzmann S, Menz G *et al*. T cell phenotype in allergic asthma and atopic dermatitis. *Int Arch Allergy Immunol* 2003; 131:272–82.
- 17 Hansel NN, Cheadle C, Diette GB *et al*. Analysis of CD4<sup>+</sup> T-cell gene expression in allergic subjects using two different microarray platforms. *Allergy* 2008; 63:366–9.

- 18 Ketloy C, Engering A, Srichairatanakul U *et al.* Expression and function of Toll-like receptors on dendritic cells and other antigen presenting cells from non-human primates. *Vet Immunol Immunopathol* 2008; **125**:18–30.
- 19 Schroeder JT, Bieneman AP, Xiao H *et al.* TLR9- and FcepsilonRI-mediated responses oppose one another in plasmacytoid dendritic cells by down-regulating receptor expression. *J Immunol* 2005; **175**:5724–31.
- 20 Moseman EA, Liang X, Dawson AJ *et al.* Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells. *J Immunol* 2004; **173**:4433–42.
- 21 Fonteneau JF, Larsson M, Beignon AS *et al.* Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *J Virol* 2004; **78**:5223–32.
- 22 Grigoryev DN, Mathai SC, Fisher MR *et al.* Identification of candidate genes in scleroderma-related pulmonary arterial hypertension. *Translational Res* 2008; **151**:197–207.
- 23 Grigoryev DN, Howell MD, Watkins TN *et al.* Vaccinia virus-specific molecular signature in atopic dermatitis skin. *J Allergy Clin Immunol* 2010; **125**:153–9, e28.
- 24 Gao PS, Shimizu K, Grant AV *et al.* Polymorphisms in the sialic acid-binding immunoglobulin-like lectin-8 (Siglec-8) gene are associated with susceptibility to asthma. *Eur J Hum Genet* 2010; **18**:713–9.
- 25 Harris ML, Darrah E, Lam GK *et al.* Association of autoimmunity to peptidyl arginine deiminase type 4 with genotype and disease severity in rheumatoid arthritis. *Arthritis Rheum* 2008; **58**:1958–67.
- 26 Purcell S, Neale B, Todd-Brown K *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81**:559–75.
- 27 Gao PS, Rafaels NM, Hand T *et al.* Filaggrin mutations that confer risk of atopic dermatitis confer greater risk for eczema herpeticum. *J Allergy Clin Immunol* 2009; **124**:507–13, 513 e1–7.
- 28 LeVan TD, Bloom JW, Bailey TJ *et al.* A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. *J Immunol* 2001; **167**:5838–44.
- 29 Zambelli-Weiner A, Ehrlich E, Stockton ML *et al.* Evaluation of the CD14/–260 polymorphism and house dust endotoxin exposure in the Barbados Asthma Genetics Study. *J Allergy Clin Immunol* 2005; **115**:1203–9.
- 30 Bellinghausen I, Brand U, Knop J, Saloga J. Comparison of allergen-stimulated dendritic cells from atopic and nonatopic donors dissecting their effect on autologous naive and memory T helper cells of such donors. *J Allergy Clin Immunol* 2000; **105**:988–96.
- 31 Hammad H, Lambrecht BN. Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation. *J Allergy Clin Immunol* 2006; **118**:331–6.
- 32 Lipscomb MF, Masten BJ. Dendritic cells: immune regulators in health and disease. *Physiol Rev* 2002; **82**:97–130.
- 33 Charbonnier AS, Hammad H, Gosset P *et al.* Der p 1-pulsed myeloid and plasmacytoid dendritic cells from house dust mite-sensitized allergic patients dysregulate the T cell response. *J Leukocyte Biol* 2003; **73**:91–9.
- 34 Kadowaki N. The divergence and interplay between pDC and mDC in humans. *Front Biosci* 2009; **14**:808–17.
- 35 Wills-Karp M, Luyimbazi J, Xu X *et al.* Interleukin-13: central mediator of allergic asthma. *Science* 1998; **282**:2258–61.
- 36 Zhu Z, Homer RJ, Wang Z *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, sub-epithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999; **103**:779–88.
- 37 Hammad H, Charbonnier AS, Duez C *et al.* Th2 polarization by Der p 1-pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood* 2001; **98**:1135–41.
- 38 Ghaemmaghami AM, Gough L, Sewell HF, Shakib F. The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. *Clin Exp Allergy* 2002; **32**:1468–75.
- 39 Cobb JP, Mindrinos MN, Miller-Graziano C *et al.* Application of genome-wide expression analysis to human health and disease. *Proc Natl Acad Sci USA* 2005; **102**:4801–6.
- 40 Xin Y, Yu L, Chen Z *et al.* Cloning, expression patterns, and chromosome localization of three human and two mouse homologues of GABA(A) receptor-associated protein. *Genomics* 2001; **74**:408–13.
- 41 Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; **249**:1431–3.
- 42 Dentener MA, Bazil V, Von Asmuth EJ, Ceska M, Buurman WA. Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages. *J Immunol* 1993; **150**:2885–91.
- 43 Virchow JC Jr, Julius P, Matthys H, Kroegel C, Luttmann W. CD14 expression and soluble CD14 after segmental allergen provocation in atopic asthma. *Eur Respir J* 1998; **11**:317–23.
- 44 Woo JG, Assa'ad A, Heizer AB, Bernstein JA, Hershey GK. The –159 C → T polymorphism of CD14 is associated with non-atopic asthma and food allergy. *J Allergy Clin Immunol* 2003; **112**:438–44.
- 45 Ober C, Tsalenko A, Parry R, Cox NJ. A second-generation genomewide screen for asthma-susceptibility alleles in a founder population. *Am J Hum Genet* 2000; **67**:1154–62.
- 46 Baldini M, Lohman IC, Halonen M, Erickson RP, Holt PG, Martinez FD. A Polymorphism in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am J Respir Cell Mol Biol* 1999; **20**:976–83.
- 47 Jackola DR, Basu S, Liebler CL *et al.* CD14 promoter polymorphisms in atopic families: implications for modulated allergen-specific immunoglobulin E and G1 responses. *Int Arch Allergy Immunol* 2006; **139**:217–24.
- 48 Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. *Nat Immunol* 2004; **5**:1219–26.
- 49 Asselin-Paturel C, Trinchieri G. Production of type I interferons: plasmacytoid dendritic cells and beyond. *J Exp Med* 2005; **202**:461–5.
- 50 Watarai H, Sekine E, Inoue S, Nakagawa R, Kaisho T, Taniguchi M. PDC-TREM, a plasmacytoid dendritic cell-specific receptor, is responsible for augmented production of type I interferon. *Proc Natl Acad Sci USA* 2008; **105**:2993–8.

- 51 Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 2005; 23:275–306.
- 52 Chapman MD, Vailes LD, Hayden ML, Benjamin DC, Platts-Mills TA, Arruda LK. Structural and antigenic studies of cockroach allergens and their relevance to asthma. *Adv Exp Med Biol* 1996; 409:95–101.

### Supporting Information

Additional supporting information may be found in the online version of this article:

**Figure S1.** The levels of TNF- $\alpha$  were detected by ELISA in the supernatant of cultured human pDCs of cockroach sensitized subjects ( $N = 3$ ) in the presence of medium alone and different concentrations of cockroach extract and/or poly-IC (50  $\mu\text{g/mL}$ ).

**Figure S2.** RT-PCR confirmed that human monocytic cells (THP-1) show similar patterns when compared with

co-cultured pDCs and CD4<sup>+</sup> T cells after cells were exposed to cockroach allergen (100  $\mu\text{L/mL}$ ) for 48 h. The most up-regulated was noted for seven of eight genes evaluated in cockroach allergen extract-treated cells as compared to those treated with LPS (100 ng/mL) and Bermuda grass extract (100  $\mu\text{g/mL}$ ). Data represent mean fold changes of three experiments.

**Table S1.** Demographic characteristics of African American atopic participants with and without cockroach sensitization.

**Table S2.** Clinical characteristics of the African American subjects for genetic association study.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## ONLINE DATA SUPPLEMENT

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

Peisong Gao<sup>1</sup>, Dmitry N. Grigoryev<sup>1</sup>, Nicholas M. Rafaels<sup>1</sup>, Deguang Mu<sup>1</sup>, Jerry M. Wright<sup>2</sup>, Beverly Plunkett<sup>1</sup>, Christopher Cheadle<sup>1</sup>, Alkis Togias<sup>1</sup>, Terri H. Beaty<sup>3</sup>, Rasika A. Mathias<sup>1,4</sup>, John T. Schroeder<sup>1</sup>, Kathleen C. Barnes<sup>1</sup>

<sup>1</sup>Division of Allergy and Clinical Immunology, Department of Medicine, The Johns Hopkins University, Baltimore, MD 21224; <sup>2</sup>Department of Physiology, JHU, Baltimore, MD 21205;

<sup>3</sup>Department of Epidemiology, Bloomberg School of Public Health, JHU, Baltimore, MD 21205;

<sup>4</sup>Division of General Internal Medicine, Department of Medicine, The Johns Hopkins University, Baltimore, MD 21287

## **METHODS**

### ***Study population:***

Pediatric and adult asthmatics self-reporting as African American and participating in the consortium on Genomic Research on Asthma in the African Diaspora (GRAAD) were included in the genetic association studies. A diagnosis of asthma was based on both a reported history of asthma and a documented history of physician-diagnosed asthma. A standardized questionnaire based on either the American Thoracic Society or The International Study of Asthma & Allergy in Childhood (ISAAC) was administered by a clinical coordinator. All controls were recruited separately and had no history of asthma. 464 asthma cases (45.5% male, aged  $23.8 \pm 17.9$ ) and 471 control subjects (41.4% male, aged  $35.2 \pm 16.5$ ) were included in the final analysis. Detailed clinical characteristics of these subjects are presented in Table E2. Atopic status was determined

by a positive skin prick test (ST; wheal  $\geq 3$  mm greater than the saline control) and/or positive specific IgE ( $\geq 0.35$  kU/L) to one of 12 common aeroallergens (Greer Laboratories, Lenoir, NC, USA) including dust mite mix (*D. farinae* and *D. pteronyssinus*), eastern oak pollen mix (Black, Red and White Oak), grass pollen mix (Kentucky, Orchard, Red Top Timothy), cat hair, dog hair, cockroach mix (German and American), ragweed pollen (giant, short), *Alternaria tenuis*, mouse and rat, and two controls (histamine and glycerine). Cockroach sensitization was classified as either a positive ST or specific IgE measurement to either *B. germanica* or *P. Americana* in blood using radioallergosorbent test (RAST).

#### *Cell isolation*

A total of 60 ml of blood was drawn and anticoagulated with 10 mM EDTA. PBMCs were isolated from peripheral blood using a 61% isotonic Percoll (Amersham, Buckinghamshire, UK) gradient. Both pDCs and CD4<sup>+</sup> T cells were isolated from human PBMCs using commercially available immunomagnetic microbead cell isolation kits, BDCA-4 and CD4<sup>+</sup>, with LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's instructions. Purity of pDCs preparation was determined by flow cytometry using CD123-APC and BDCA2-FITC.

#### *Power prediction analysis for minimum array requirements:*

Sample size determination for class comparisons in the microarray experiments was employed as previously described(1). We generated five gene expression profiles for healthy volunteers and z-transformed expression data applying log<sub>10</sub>. Identified average standard deviation of z-transformed expression values of all probe sets ( $\sigma=0.163$ ) was submitted to the microarray sample size identifying formula accepting fold change  $\pm 1.5$  ( $\log_{10}(1.5)=0.176$ ), a 5% false discovery rate (significance level  $\alpha=0.05$ ) and imposing 90% of detecting power ( $1-\beta$ ). The

power.t.test function of R2.3.1 program ([www.r-project.org](http://www.r-project.org)) was implemented: `power.t.test(n=NULL,delta=0.176,sd=0.163,sig.level=0.05,power=0.90,type="two.sample",alternative="two.sided")`. These calculations identified  $n=7.87$ . We concluded that filtering gene expression data using a 1.5-fold change and 5% false discovery rate cutoffs would identify significant transcriptional changes in the setting of seven subjects per clinical condition.

#### *Microarray Data Analysis:*

Hybridization signals of 24,351 transcripts were analyzed using BeadStudio version 1.5.0.34 software (Illumina, Inc.). Resulting digitized matrix was processed by modified for Illumina platform approach described previously(1). Briefly, the detectability (BeadStudio detection>0.95) of hybridization signals was tested and detected (Present) and non-expressed (Absent) transcripts identified. The chip background and brightness were computed using high quartile and whole set of “Absent” hybridization signals, respectively. The expression data was stratified by experimental conditions ( $n=7$ ) and hybridization of each transcript was evaluated for each cluster. The transcripts that were “Present” and produced hybridization signal which was at least twice as high as that of background in at least 4 out of 7 hybridizations in any given patient group were considered expressed. The signal intensity values of these transcripts from each chip were increased by corresponding to a given chip background value (background adjustment) and divided by a chip brightness coefficient (normalization). Computational identification of allergen-affected candidate genes was conducted using Significance Analysis of Microarrays (SAM 2.20) with 1,000 permutations without application of arbitrary restrictions. Genes with the highest deviation from the background (top 70% of differentially expressing genes) were analyzed using t-test statistics. Genes with a false discovery rate (FDR) <5% were considered significantly affected by allergens.

## References

- E1. Grigoryev, DN, Mathai SC, Fisher MR, Girgis RE, Zaiman AL, Houston-Harris T, Cheadle C, Gao L, Hummers LK, Champion HC, Garcia JGN, Wigley FM, Tudor RM, Barnes KC, Hassoun PM. Identification of candidate genes in scleroderma-related pulmonary arterial hypertension. *Transl Res*. 2008. 151(4):197-207.

## Figure Legends

**Figure E1.** The levels of TNF- $\alpha$  were detected by ELISA in the supernatant of cultured human pDCs of cockroach sensitized subjects (N=3) in the presence of medium alone and different concentrations of cockroach extract and/or poly-IC (50  $\mu$ g/ml).

**Figure E2.** RT-PCR confirmed that human monocytic cells (THP-1) show similar patterns when compared with co-cultured pDCs and CD4<sup>+</sup> T cells after cells were exposed to cockroach allergen (100  $\mu$ l/ml) for 48 hours. The most up-regulated was noted for seven of eight genes evaluated in cockroach allergen extract-treated cells as compared to those treated with LPS (100 ng/ml) and Bermuda grass extract (100 $\mu$ g/ml). Data represent mean fold changes of three experiments.

**Table EI:** Demographic characteristics of African American atopic participants with and without cockroach sensitization

| Characterization                 | Cockroach sensitization |              | Total        |
|----------------------------------|-------------------------|--------------|--------------|
|                                  | Positive (+)            | Negative (-) |              |
| No. of subjects                  | 8                       | 10           | 18           |
| Age (years)                      | 37.75 ± 11.08           | 35.30 ± 8.55 | 36.39 ± 9.53 |
| Gender (male%)                   | 50.00%                  | 30.00%       | 38.89%       |
| Atopy                            | 100%                    | 100%         | 100%         |
| Asthma                           | 75%                     | 50%          | 61.1%        |
| Total serum IgE                  | 2.73 ± 0.45             | 2.76 ± 0.42  | 2.75 ± 0.42  |
| (log <sub>10</sub> -transformed) |                         |              |              |
| Specific IgE (KIU/L)             |                         |              |              |
| German cockroach                 | >0.35                   | <0.35        |              |
| Bermuda grass                    | >0.35                   | >0.35        |              |

**Table E2.** Clinical characteristics of the African American subjects for genetic association study

| Characteristic        | Total         | Cases         | Controls      |
|-----------------------|---------------|---------------|---------------|
| Subjects (n)          | 935           | 464           | 471           |
| Males; N (%)          | 406 (43.4%)   | 211(45.5%)    | 195 (41.4%)   |
| Age; Mean (SD)        | 29.55 (18.10) | 23.78 (17.85) | 35.23 (16.51) |
| Total IgE             | 213.7         | 315.6         | 143.3         |
| (95% CI) <sup>†</sup> | (191.5-238.4) | (270.4-368.3) | (123.8-165.8) |
| Atopy; N (%)          | 641 (75.2%)   | 369 (85.2%)   | 272 (64.9%)   |

<sup>†</sup>Geometric mean serum levels of total IgE

Figure E1.

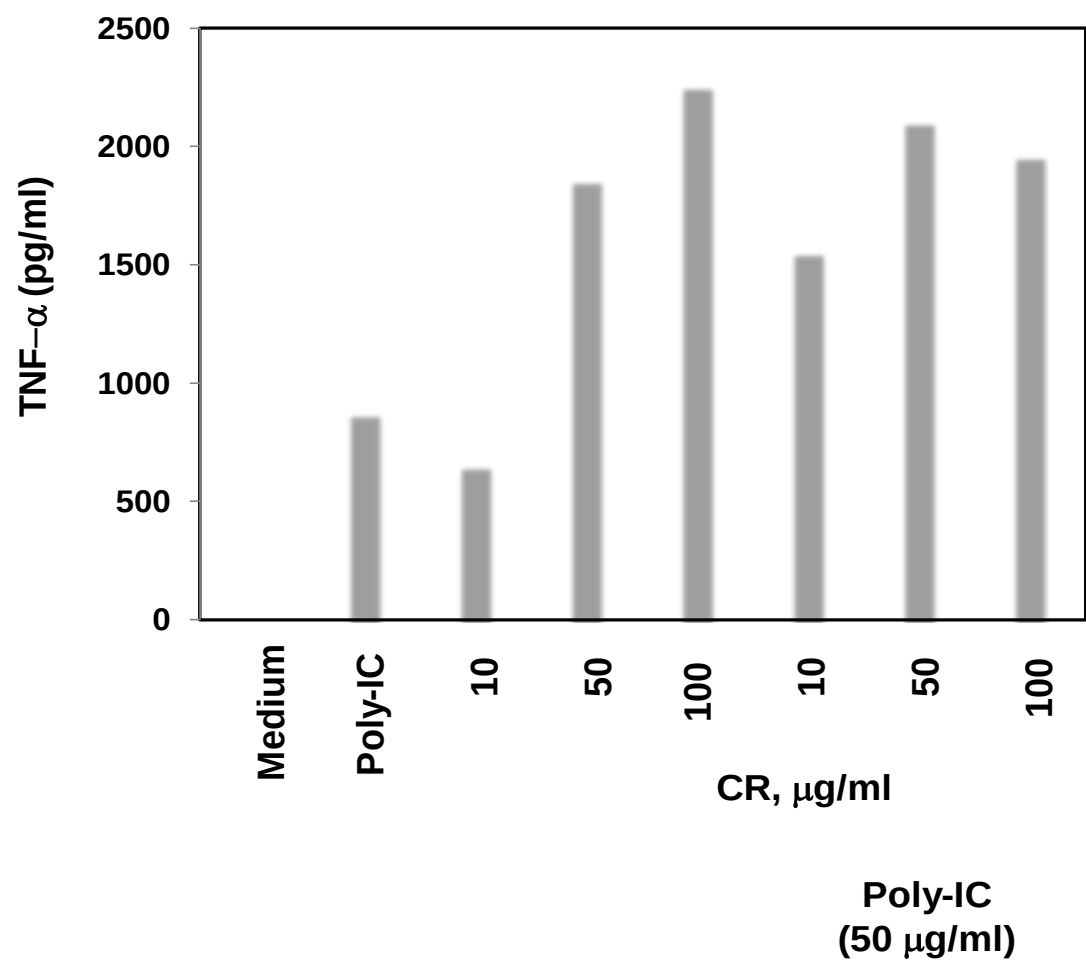


Figure E2.

