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## Effect of endotoxin and alum adjuvant vaccine on peanut allergy

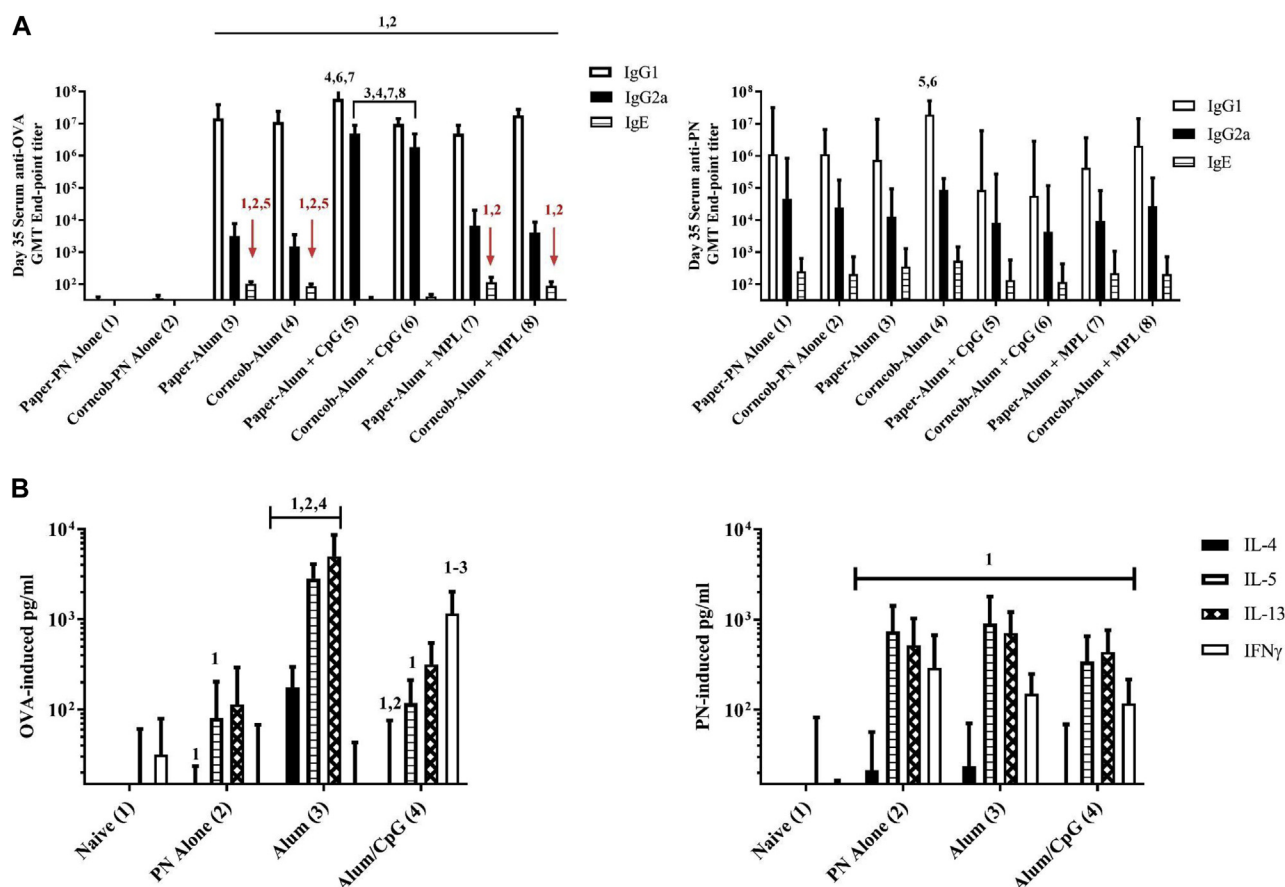


### To the Editor:

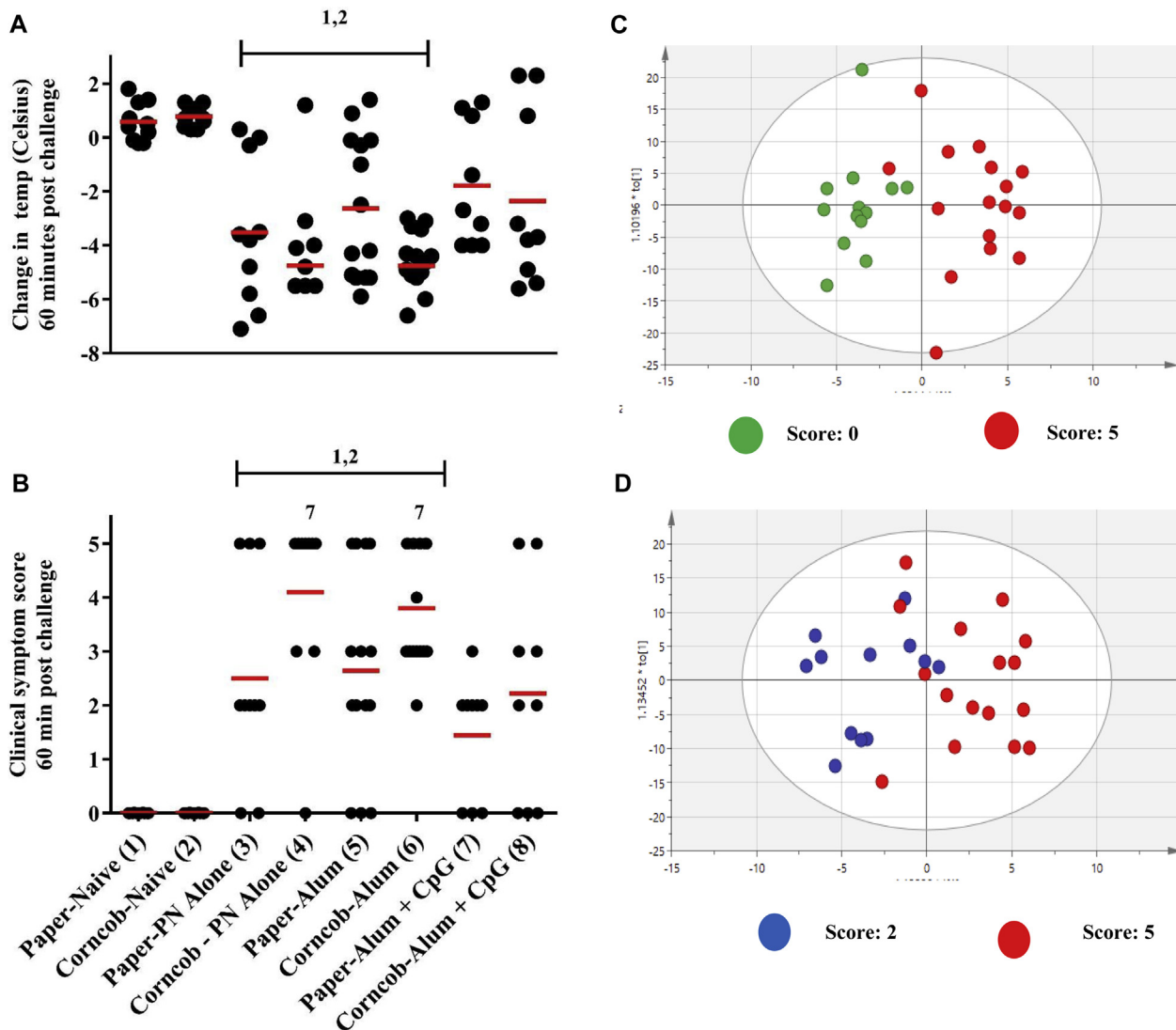
Environmental influences play a significant role in host immune responses, and it is hypothesized that environmental changes might be responsible for enhanced allergy prevalence. The hygiene hypothesis proposed that smaller families and increased cleanliness reduce infectious diseases (and thereby endotoxin exposure) in a household and contribute to enhanced allergic disease.<sup>1</sup> Vaccines can reduce microbial exposure by preventing infections, and vaccine adjuvants, such as alum, can promote allergies in genetically predisposed populations.<sup>2</sup> Alum induces T<sub>H</sub>2 responses to coadministered antigens and potentially to unrelated environmental allergens, thus providing bystander (heterologous) responses that contribute to allergic disease.<sup>3</sup> Modification of childhood vaccines by the addition of T<sub>H</sub>1-enhancing Toll-like receptor ligand adjuvants, such as

monophosphoryl lipid A (MPL) or CpG, to alum might reduce the T<sub>H</sub>2-skewing activity of alum and balance host immunity to reduce the development of allergic disease.

In this study we used a mouse model of peanut allergy to test the hypothesis that environmental endotoxin, alum, or both influence the severity of allergen-specific hypersensitivity. Toll-like receptor 4–responsive C3H/HeOuJ mice were housed in low (paper) or standard (corn cob) endotoxin bedding for 1 month before alum-adjuncted ovalbumin (OVA) immunization and peanut sensitization. (A detailed experimental design can be found in the [Methods](#) section and [Table E1](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).) Although endotoxin levels were significantly lower in paper bedding compared with those in corn cob bedding before animal exposure, bedding endotoxin levels were similar after 1 day (see [Fig E1, A](#), in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Multivariate analysis of the broad-spectrum metabolomics data distinguished mice based on bedding type (see [Fig E1, B](#)), and the metabolites identified (see [Table E2](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) suggested that pathways related to amino acid metabolism and transport might be important in differentiating the type of bedding in the animal's living environment (see [Table E3](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).



**FIG 1.** Antigen-specific immune responses induced by vaccination and allergen sensitization. Mice in paper or corn cob bedding were OVA naive or immunized with OVA/alum, OVA/alum/CpG, or OVA/alum/MPL. **A**, Antigen-specific serum antibodies were measured by means of ELISA ( $n = 9$ –15 mice per group). **B**, Splenic cytokine responses induced by peanut or OVA were measured by using the multiplex assay ( $n = 8$ –10 mice per group). Significant increases are indicated by numbers above bars ( $P < .05$ ). GMT, Geometric mean titer; PN, peanut.



**FIG 2.** Adjuvant combination of alum and CpG reduces allergic disease. **A** and **B**, C3H/HeOuJ mice were challenged with peanut and monitored for hypothermia (Fig 2, **A**) and clinical symptoms (Fig 2, **B**) based on prechallenge phenotype. Statistical differences between groups are indicated by numbers ( $P < .05$ ). Data were combined from 2 independent experiments ( $n = 9$ -15 per group). **C** and **D**, OPLS-DA scores plot associated with symptom scores comparing animals that exhibited (Fig 2, **C**) no change (score 0) and mild disease (score 2) (Fig 2, **D**) with severe disease (score 5). PN, Peanut.

OVA/alum vaccines were administered subcutaneously to provide alum exposure before gastric peanut sensitization to evaluate alum's influence on allergen hypersensitivity. Although  $T_H1$ -inducing adjuvants used prophylactically or coadministered with allergens reduce the development of allergen-specific hypersensitivity reactions in mice, widespread prophylaxis with  $T_H1$ -inducing adjuvants to reduce the development of allergy in human subjects is not practical. However, it might be practical to modify current vaccine formulations to contain alum and  $T_H1$ -biased adjuvants to induce a balanced  $T_H1/T_H2$  response and reduce the development of allergic disease. Therefore we evaluated the  $T_H1$ -inducing adjuvants MPL or CpG combined with alum for their ability to enhance OVA-specific  $T_H1$  responses and influence peanut allergy outcomes.

Vaccine antigen-specific serum antibody levels were measured to evaluate adjuvant-modulated humoral immunity (Fig 1, **A**).

OVA-immunized mice had anti-OVA IgG<sub>1</sub> and IgG<sub>2a</sub> serum titers that were significantly greater than unvaccinated mice. Anti-OVA IgG<sub>1</sub> levels were significantly greater in paper-housed mice vaccinated with OVA/alum/CpG than in corncob/OVA/alum or OVA/alum/CpG mice or paper/OVA/alum/MPL mice. Alum significantly increased OVA IgE responses compared with peanut alone regardless of environment and in paper-housed mice vaccinated with alum/CpG. The addition of CpG to alum decreased OVA IgE responses; however, MPL did not. OVA/alum/CpG increased anti-OVA IgG<sub>2a</sub> titers in mice housed in paper ( $1.48 \times 10^6$ ) or corncob ( $1.18 \times 10^6$ ) compared with all other OVA-immunized mice. MPL neither enhanced OVA-specific  $T_H1$ -associated IgG<sub>2a</sub> levels nor decreased  $T_H2$ -associated IgE levels under the conditions tested and therefore was removed from subsequent studies. Vaccine adjuvant's influence on peanut-specific antibodies was also

measured. Peanut-specific IgE levels were similar among all treatment groups; however, OVA/alum/CpG immunization decreased peanut-specific IgG<sub>1</sub> levels compared with those in corn-cob/OVA/alum mice.

Splenic T-cell cytokine responses were measured to further assess the influence of alum/CpG on T<sub>H</sub>1 OVA immunity and evaluate bystander immunity to peanut, regardless of living environment, because bedding type did not globally influence antibody responses (Fig 1, B). Alum enhanced OVA-induced T<sub>H</sub>2-associated IL-4, IL-5 and IL-13 levels compared with those in naive, peanut-immunized, and OVA/alum/CpG-immunized mice. OVA-specific T<sub>H</sub>1-associated IFN- $\gamma$  levels were increased by using alum/CpG compared with naive, peanut alone, and OVA/alum mice. Peanut-induced cytokine responses were not changed by vaccine adjuvants.

Peanut-induced hypothermia and allergy symptoms were measured after completion of OVA vaccination and peanut sensitization. Naive mice exhibited no allergy symptoms or hypothermia. Mice vaccinated with peanut alone or OVA/alum displayed significant hypothermia and clinical symptoms compared with naive mice after challenge (Fig 2, A and B). Mice vaccinated with OVA/alum/CpG exhibited body temperatures not significantly different from those of peanut-naive mice. Exposure to OVA/alum/CpG in paper-housed mice significantly reduced clinical symptoms compared with peanut alone and OVA/alum mice living in corn-cob bedding. Although there is variability in allergic symptoms within treatment groups, our results suggest that CpG combined with alum indirectly influences host immunity to reduce anaphylaxis because alum/CpG adjuvants were administered on a different day and through a different route than peanut sensitization. In support of our observation, similar intragroup variability has been observed by others after treatment with allergen coadministered with CpG in nanoparticle immunotherapy.<sup>4</sup> No differences in allergic disease were observed based on living environment. Our results suggest that modification of childhood vaccine adjuvant formulations might provide a method to reduce the development/severity of allergic disease.

Multivariate analysis of the binned nuclear magnetic resonance (NMR) metabolomics data differentiated animals based on clinical symptom scores. Animals with a score of 5 were well differentiated from those with scores of 0 and 2 (Fig 2, C and D). The metabolites important to differentiating the groups are listed in Table E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Therefore this exploratory analysis suggests that fecal metabolomics might be useful in predicting the severity of the host response to allergens.

In this study we tested the influence of environmental endotoxin and alum adjuvants on development of allergic symptoms. Differences in anaphylactic symptoms were not observed based on bedding or OVA/alum compared with symptoms seen in control mice. Therefore we concluded that neither environmental endotoxin nor the presence of alum, under the conditions used, influenced allergic disease. However, alum/CpG-adjuvanted vaccines administered separately from peanut sensitization reduced allergic symptoms. Exploratory fecal metabolomics were able to identify unique profiles based on living environment and severity of allergic reactions.

According to the "old friends" hypothesis, increased microbial exposure inversely correlates with allergic disease.<sup>1</sup> We did not observe differences in allergic disease despite types of animal

bedding exhibiting different levels of endotoxin before animal exposure. However, there are still many uncertainties surrounding the influence of environmental endotoxin on allergic disease. Fecal metabolites related to the gut microbiome were identified that differentiated mice based on bedding type. It is possible that the gut microbiota influences host responses to endotoxin and regulates subsequent allergic sensitivity. Additional studies might need to completely separate animals from their feces or use mice receiving antibiotic therapy to reduce gastrointestinal microbial colonization to evaluate the contribution of environmental endotoxin to allergic disease.

Alum's ability to induce antigen-specific T<sub>H</sub>2 responses and create a bystander response that alters peanut immunity was evaluated. Despite the ability of alum to provide bystander immune activation to newly administered antigens<sup>3</sup> and our observation that alum enhanced OVA-induced T<sub>H</sub>2 responses, exposure to alum did not enhance allergy severity in our model. It is possible that our use of cholera toxin (CT) to sensitize mice to peanut might induce potent peanut-specific T<sub>H</sub>2 responses that cannot be further enhanced by other T<sub>H</sub>2-skewing adjuvants. Therefore additional work might be required to modify peanut sensitization regimens to allow for proper evaluation of the influence of environmental factors on allergic disease.

We believe that the immune responses modulated by alum/CpG observed in our study are examples of beneficial heterologous effects of vaccination.<sup>5</sup> Vaccine adjuvants induced unique innate cytokine responses 6 hours after immunization (see Fig E2, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Innate serum cytokines induced by gastric peanut sensitization were modified by the vaccine adjuvants administered subcutaneously on the previous day (see Fig E2, B). These data suggest that vaccine adjuvants might alter subsequent immune responses to newly introduced antigens and prospective allergens. Modulation of innate cytokine production might be the basis of vaccines providing heterologous immunity that influence allergic disease. Alum/CpG subsequently induced vaccine-specific T<sub>H</sub>1 immunity and allergic responses statistically similar to those of naive mice, suggesting that this combination reduced allergic disease severity. However, complete disease protection was not achieved in our study, and CpG might not improve allergy in human infants. Previous reports indicate that CpG does not enhance T<sub>H</sub>1 cytokines produced by neonatal human cells<sup>6</sup> or induce more IFN- $\gamma$  and IL-12p70 in newborn cells than infant or adult whole blood.<sup>7</sup> Therefore additional research is needed to identify safe and effective adjuvants that induce balanced T<sub>H</sub>1/T<sub>H</sub>2 responses in children and might provide indirect protection against allergy.

Broad-spectrum metabolomics using feces collected before peanut challenge provided additional information on disease severity. These metabolites include arginine, formate, glucose, glutamine, methanol, acetate, and methionine, which are consistent with a previous metabolomics study on asthma pathogenesis in human subjects.<sup>8</sup>

In summary, exposure to an alum/CpG-adjuvanted vaccine reduced the severity of peanut hypersensitivity in mice, whereas exposure to endotoxin and alum did not influence allergic symptoms. Fecal metabolomics can provide a method to predict the development, severity, or both of allergic reactions. Additional studies are needed to identify environmental contributions to human allergic disease, their mechanism of action, and prediction of allergy status before an allergic response.

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## Gene-environment interaction between an *IL4R* variant and school endotoxin exposure contributes to asthma symptoms in inner-city children



### To the Editor:

Gene-environment interactions are thought to play a critical role in determining asthma incidence and severity. A human IL-4 receptor alpha chain gene (*IL4R*) variant that results in a glutamine to arginine substitution at amino acid residue 576 (IL4Rα-Q576R polymorphism) is associated with asthma diagnosis and severity.<sup>1</sup> We have recently shown that the IL4Rα<sup>R576</sup> variant promotes severe airway inflammation by uniquely linking the IL4R to a growth-factor-receptor-bound protein 2 (GRB2)-dependent intracellular signaling pathway that destabilizes allergen-specific regulatory T cells to drive mixed T<sub>H</sub>2/T<sub>H</sub>17 cell inflammation.<sup>2</sup> School endotoxin exposure is associated with asthma morbidity.<sup>3</sup> High endotoxin exposure is associated with increased T<sub>H</sub>17 cell skewing,<sup>4</sup> leading us to test the hypothesis that there is an interaction between the IL4Rα-Q576R polymorphism, school endotoxin exposure, and asthma symptoms.

The School Inner-city Asthma Study was a single-center prospective cohort study conducted between 2008 and 2013 of children with persistent asthma attending inner-city elementary schools in a northeastern United States city (see this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Repeated assessment of asthma symptom days over a 14-day period was done by parental or caretaker survey at 3-month intervals. One-week classroom air samples were collected twice during the school year using charged particle samplers (Quadra; Sharper Image/Camelot Venture Group, Farmington Hills, Mich); endotoxin levels were assessed using the Limulus Amebocyte Lysate assay.<sup>3</sup> Genotyping of the *IL4R*<sup>Q576</sup> and *IL4R*<sup>R576</sup> alleles was carried out using the amplification resistance mutation screen PCR method<sup>2</sup> on DNA extracted from either whole blood (Gentra Puregene Blood Kit; Qiagen, Germantown, Md) or saliva (prepIT L2P; DNA Genotek, Ottawa, Ontario, Canada).

The IL4Rα-Q576R genotypes were modeled as a 3-group categorical variable (*Q/Q*, *Q/R*, or *R/R* genotypes, with the *Q/Q* genotype as the reference group). To test the hypothesis that the genotypes differed by self-reported race, Fisher exact tests were performed. To test the hypothesis that a gene-environment interaction was present, a multiplicative term between classroom endotoxin levels (continuous) and genotype (3-level categorical variable) was included in binomial family generalized estimating equations with a logit link and an overdispersion parameter. These models included only observations during the school year, and adjusted for age, sex, self-reported race, allergic sensitization, baseline asthma severity, use of asthma controller medications,



## METHODS

### Mice

Female 4- to 6-week-old C3H/HeOJ mice ( $n = 9$ -15 per group) were purchased from the Jackson Laboratory (Bar Harbor, Me; stock no. 000635) and housed in microisolator cages under specific pathogen-free conditions. Results were obtained from 2 independent experiments and combined. Animal procedures were approved by Duke's Institutional Animal Care and Use Committee.

### Experimental design

Mice were separated into 10 groups (Table E1). Five groups were housed in Certified/Irradiated Diamond Soft paper bedding (Harlan Teklad, Indianapolis, Ind), and 5 groups were housed in Enrich-o-cob corn cob bedding (Andersons Laboratory, Maumee, Ohio) for 1 month before initiation of vaccinations/allergic sensitization. Paper bedding was replaced 3 times a week to represent a low-endotoxin environment, and the standard endotoxin environment contained corn cob bedding that was replaced biweekly. Mice living in paper or corn cob bedding included naive mice and mice sensitized to peanut but no other vaccination (peanut alone); mice vaccinated with OVA and alum (alum); mice vaccinated with OVA, alum, and CpG (alum/CpG); or mice vaccinated with OVA, alum, and MPL (alum/MPL) before peanut sensitization. Vaccines were administered subcutaneously beginning 1 day before gastric peanut sensitization. All mice were challenged with peanut and monitored for hypersensitivity reactions on day 36.

### Endotoxin measurement

Endotoxin levels were measured in mouse bedding after autoclaving and before animal exposure. Bedding samples were processed, as previously described.<sup>E1</sup> Supernatants were analyzed with the Pierce LAL Chromogenic Endotoxin Quantification Kit (Thermo Scientific, Waltham, Mass). Data are presented as endotoxin units per milligram of bedding.

### Vaccinations

Subcutaneous vaccines containing low-endotoxin OVA (Endofit OVA, 10  $\mu$ g; InvivoGen, San Diego, Calif) and the following adjuvants were administered in 100- $\mu$ L volumes on days 0, 14, and 28 in sterile USP saline (APP Pharmaceuticals, Schaumburg, Ill): alum (0.5 mg of Al[OH]<sub>3</sub>; Alhydrogel, InvivoGen), alum/CpG-1826 (100  $\mu$ g; Vaccigrade, InvivoGen), or alum/MPL (25  $\mu$ g; Enzo Life Sciences, Farmingdale, NY).

### Peanut sensitization

Crude peanut extract was obtained, as previously described.<sup>E2</sup> Peanut sensitization was achieved by means of gastric gavage of peanut (1 mg) and CT (10  $\mu$ g; catalog no. 100B; List Biologicals, Campbell, Calif) in 200  $\mu$ L of USP saline on days 1, 8, 15, and 22.

### Peanut provocation

Temperature transponders (BioMedic Data Systems, Seaford, Del) were injected subcutaneously before allergen challenge and used to monitor body temperature after peanut challenge. On day 36, mice were challenged intraperitoneally with peanut (250  $\mu$ g). Body temperature and clinical symptoms were monitored before and every 20 minutes after peanut challenge for 1 hour. Clinical symptoms were reported by using a 0- to 5-point scoring system, as previously described.<sup>E3</sup> The following score system was used to grade hypersensitivity reactions in mice after challenge: 0, no symptoms; 1, repetitive scratching with hind legs near the ear canal or mouth; 2, decreased activity, self-isolation, or swollen snout; 3, motionless for greater than 1 minute or laying prone on stomach; 4, no response to whisker stimulation or decreased or no activity to prodding; and 5, convulsion, death, or temperature decrease of greater than 5°C.

### Sample collection

Blood was collected on day 35 by using the submandibular lancet method,<sup>E4</sup> and serum antibody levels were measured by using ELISA. Day 35 fecal pellets were frozen at -80°C for metabolomics.

### ELISA

Antigen-specific log<sub>2</sub> end point antibody titers were determined by means of ELISA. ELISA plates were coated with 2  $\mu$ g/mL peanut or OVA and completed as previously described.<sup>E5</sup> Antigen-specific IgE ELISAs were coated with 5  $\mu$ g/mL anti-mouse IgE-unlabeled (SouthernBiotech, Birmingham, Ala), and biotinylated antigens were used to determine antigen specificity at 2  $\mu$ g/mL. The end point titer was calculated as the last sample dilution with a signal 3-fold greater than that of a naive sample at the same dilution.

### Innate cytokine analysis

Two groups of mice ( $n = 10$ ) were immunized subcutaneously with OVA vaccines adjuvanted with alum or alum/CpG on day 0. One group of 8 mice remained OVA naive. Serum was collected from each animal 6 hours after vaccination. Serum was also collected from naive mice at the same time as immunized mice. Five mice from each vaccine group, including naive mice, were sensitized gastrically with peanut/CT 24 hours after OVA vaccination. Serum was collected for a second time from all mice 6 hours after administration of the peanut sensitization dose and analyzed for cytokine responses by using a Luminex multiplex assay (Bio-Rad Laboratories, Hercules, Calif).

### Antigen-induced cytokine analysis

Naive, peanut alone, alum, or alum/CpG mice living in the corn cob environment because the living environment did not affect humoral immunity were analyzed for peanut- and OVA-induced T-cell cytokine responses after OVA immunization and peanut sensitization. On day 36, spleens were harvested and cultured *in vitro* with media alone, peanut (200  $\mu$ g/mL), or OVA (200  $\mu$ g/mL) for 96 hours by using similarly described methods.<sup>E6</sup> Supernatants were analyzed for cytokine responses by using a Luminex multiplex assay.

### Immunology statistical analysis

The unpaired Student *t* test was used to determine statistical differences between endotoxin levels in bedding samples. ANOVA with Tukey multiple comparisons was used to compare antibody and challenge results. Antigen-induced cytokine responses were calculated by subtracting the picogram per milliliter value from wells that contained cells in media from the picogram per milliliter values of wells stimulated with antigen (peanut or OVA) and then log<sub>10</sub>-transformed for normalization before analysis with 1-way ANOVA with Tukey multiple comparisons. GraphPad Prism 7 software (GraphPad Software, La Jolla, Calif) was used for all analyses.

### Broad-spectrum NMR metabolomics

A subset of animals was chosen from immunologic studies for the exploratory broad-spectrum metabolomics analysis, and sample preparation and data collection and analysis were as described previously.<sup>E7</sup> Briefly, fecal pellets were homogenized in ceramic bead tubes (MagNA Lyser) and D<sub>2</sub>O (Sigma-Aldrich, St Louis, Mo). Each NMR sample consisted of 50 mg/mL fecal extract with 10% Chenomx ISTD solution (Chenomx, Edmonton, Alberta, Canada) containing 5 mmol/L 4,4-dimethyl-4-silapentane-1-sulfonic acid (chemical shift indicator), 100 mmol/L imidazole (pH indicator), and 0.2% NaN<sub>3</sub> (to inhibit bacterial growth). NMR spectra were processed by using the ACD NMR Processor 12.0 (ACD Labs, Toronto, Calif) and binned (0.10-9.00 ppm) into 226 integrated segments (NMR bins), excluding water (4.68-4.88 ppm) and imidazole (7.10-7.50 ppm) by using intelligent bucket

integration with a 0.04-ppm bucket width and 50% looseness. Binned data were normalized to the total integral of each spectrum. Before multivariate analysis, normalized binned data were mean centered and Pareto scaled. All of the metabolomics data generated from this study and associated metadata have been uploaded to the public accessible NIH Common Fund Metabolomics Data Repository (<http://www.metabolomicsworkbench.org>).

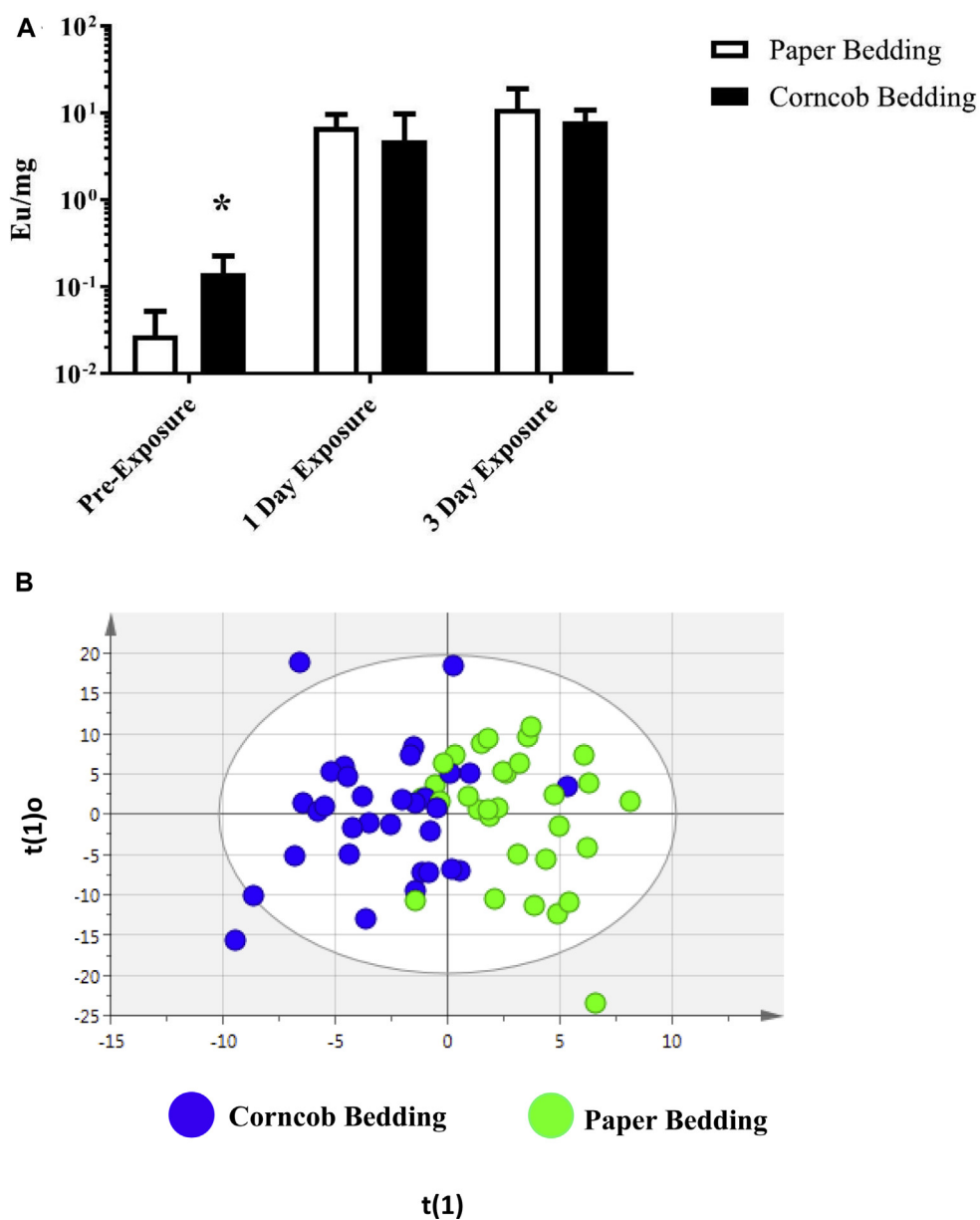
## Multivariate and statistical analysis of metabolomics data

Descriptive statistics and 2-sided hypothesis tests using continuously binned NMR-binned data were conducted with SAS 9.4 software (SAS Institute, Cary, NC). Hypothesis testing was conducted by using the *t* test with the Satterthwaite correction for unequal variances. Multivariate data analysis methods (eg, principal component analysis and orthogonal partial least-square discriminant analysis [OPLS-DA]) were used to reduce the dimensionality and enable the visualization of the separation of study groups (SIMCA 14; Umetrics, Umeå, Sweden). These pattern recognition methods are used commonly to analyze high-dimensional multicollinear data, such as metabolomics.<sup>E8,E9</sup> OPLS-DA is a supervised analysis for categorical outcomes and was used to determine the NMR bins that were important for differentiating the phenotypic groups. Loadings plots and variable importance for projection (VIP) plots were inspected along with the VIP statistic, which summarizes the importance of the bin in differentiating phenotypic groups in a supervised multivariate analysis.<sup>E8</sup> Model statistics are reported for OPLS-DA models, including the proportion of variance in the metabolomics data associated with the phenotype (R2X), the proportion of variance in the phenotype explained by the model (R2Y), and the measure of predictive variation of the model assessed by means of 7-fold cross validation (Q2). Bins with either a VIP of 1.0 or greater with a jack-knife CI that did not include 0, a *P* value of less than .1, or a magnitude of fold change of greater than 2 were determined to be important to differentiating the study groups. Chemomx

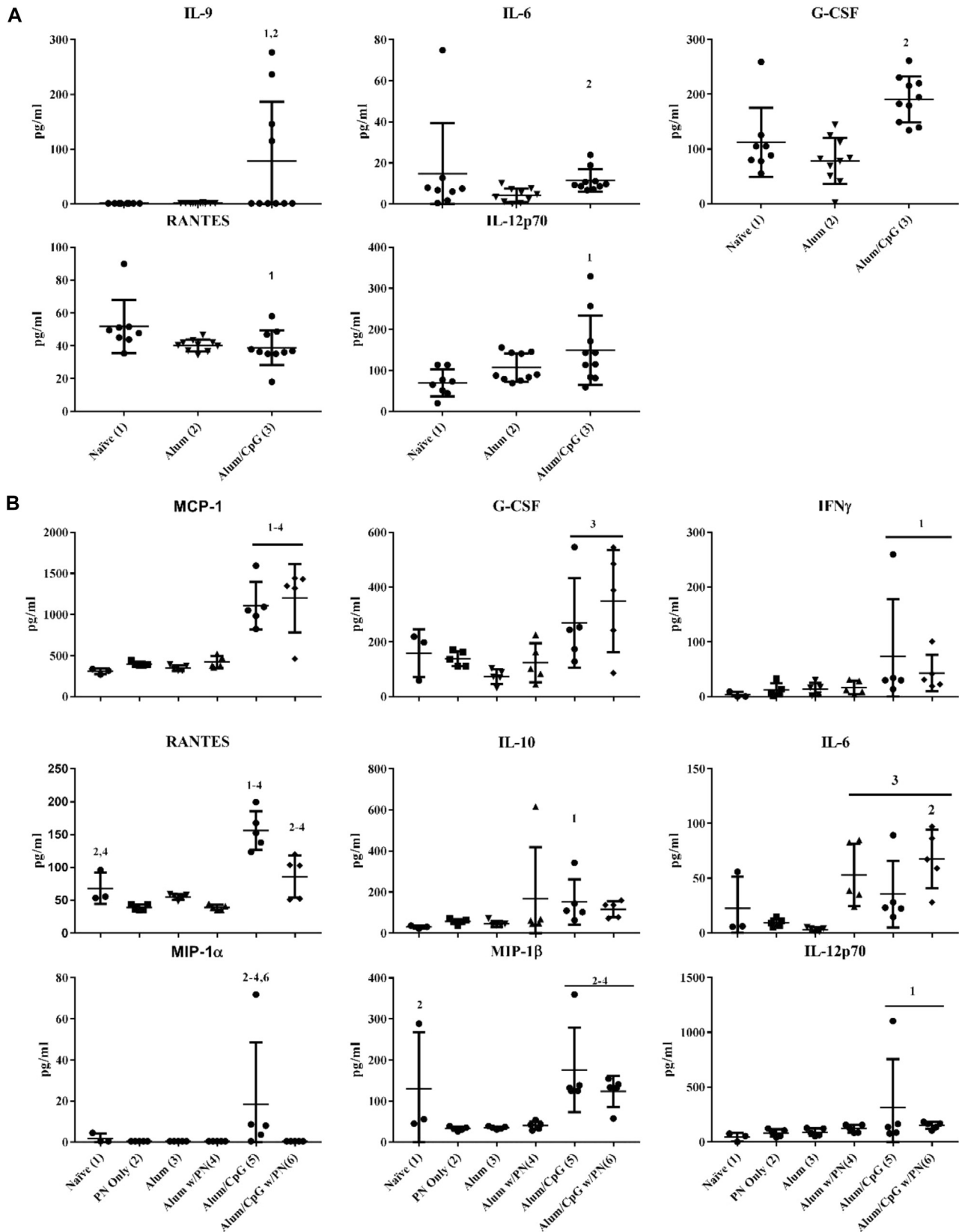
NMR Suite 8.1 Professional software, which has a concentration library of approximately 350 compounds, was used to match the signals in the bins important to differentiating study groups to metabolites. NMR signals that could not be library matched were classified as unknown. Metabolic pathway analysis was performed by using the MetaCore module in GeneGo software (GeneGo, Philadelphia, Pa).

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**FIG E1.** Endotoxin levels are lower in paper compared with corncob bedding. **A**, Endotoxin levels in bedding before animal exposure and 1 or 3 days after animal exposure. \*Significant increase in endotoxin units per milligram compared with paper bedding, as determined by using the unpaired Student *t* test ( $P = .0002$ ). **B**, OPLS-DA score plot showing separation of animals that lived on corncob bedding (*blue*) from those that lived on paper bedding (*green*;  $n = 53$ ,  $R^2X = 0.504$ ,  $R^2Y = 0.597$ ,  $Q^2 = 0.301$ ).



**FIG E2.** Vaccine adjuvant influences innate cytokine response. Mice were vaccinated subcutaneously with alum or alum/CpG on day 0. Half of the immunized mice were sensitized with peanut plus CT (PN) 24 hours after immunization (day 1). **A**, Serum cytokine levels measured 6 hours after subcutaneous vaccination. **B**, Serum cytokine levels measured 6 hours after sensitization. Cytokines were measured by using the Luminex assay. Numbers indicate significant differences between groups by using 1-way ANOVA ( $P < .05$ ).



**TABLE E1.** Experimental design

| TABLE 1. Experimental design |                 |  |  |            |                  |
|------------------------------|-----------------|--|--|------------|------------------|
| Bedding                      | Treatment group |  |  |            |                  |
|                              | NA              |  | Subcutaneous vaccination: days 0, 14, and 28 |            |                  |
|                              | NA              | Gastric sensitization: days 1, 8, 15, and 22 |  |            |                  |
|                              |                 | Intraperitoneal challenge: day 36            |  |            |                  |
|                              |                 | Naive  | Peanut alone                                 | OVA + alum | OVA + alum + CpG |
| Paper                        | 10              |  |  |            |                  |
| Corncob                      | 10              |  |  |            |                  |
| Paper                        |                 | 10   |  |            |                  |
| Corncob                      |                 | 10   |  |            |                  |
| Paper                        |                 |  | 14   |            |                  |
| Corncob                      |                 |  | 15   |            |                  |
| Paper                        |                 |  |  | 9          |                  |
| Corncob                      |                 |  |  | 9          |                  |
| Paper                        |                 |  |  |            | 10               |
| Corncob                      |                 |  |  |            | 10               |

C3H/HeOuJ mice were housed in paper or corncob bedding. Mice were naive to peanut, sensitized to peanut only, or peanut sensitized and immunized with OVA/alum, OVA/alum/CpG, or OVA/alum/MPL. A total of 2 independent experiments were performed with 9 to 15 mice per group. Environmental acclimation occurred 4 weeks before immunization. Peanut-induced anaphylaxis was measured 2 weeks after completion of peanut sensitization.

NA, Not applicable.

**TABLE E2.** Metabolites that distinguished animals living in corn cob bedding from animals living in paper environments

| Library-matched metabolites                            | VIP        | P value      | Fold change  | Library-matched metabolites           | VIP        | P value      | Fold change  |
|--|------------|--------------|--------------|---------------------------------------|------------|--------------|--------------|
| 3-Hydroxyphenylacetate                                 | 0.4        | .030         | 1.1          | Glucose (3 bins)                      | 0.7 to 1.7 | .007 to .088 | −1.2 to −1.1 |
| Acetamide  | 0.7        | .051         | 1.1          | Glucose, arabinose                    | 2.3        | .081         | −1           |
| Acetate  | 5.5        | .16          | 1.1          | Glucose, arabinose, betaine, fructose | 1.7        | .139         | −1           |
| Acetoin  | 2.2        | .056         | 1.2          | Glucose, leucine                      | 1.5        | .04          | −1.1         |
| Acetone, acetoin                                       | 2.5        | .026         | 1.2          | Glutamate                             | 1.7        | .015         | 1.1          |
| Alanine, unknown                                       | 1.3        | .219         | 1.1          | Glutamate, N-acetyl amino acids       | 1          | .021         | 1.1          |
| Arabinose  | 0.9        | .025         | −1.1         | Glutamate, arabinose, glucose         | 2.2        | .036         | −1.1         |
| Arabinose, fructose                                    | 1.5        | .123         | −1.1         | Histamine, uridine                    | 0.4        | .052         | −1.2         |
| Arabinose, glucose (3 bins)                            | 1 to 1.5   | .016 to .068 | −1.2 to −1.1 | Isovalerate, isoleucine, leucine      | 1.5        | .582         | 1            |
| Arabinose, xylose, glucose                             | 1.9        | .174         | −1.1         | Lactate (2 bins)                      | 2 to 3.2   | .046 to .047 | −1.2 to −1.1 |
| Arginine, glucose, lysine, alanine, leucine, glutamate | 2.3        | .136         | −1           | Lysine                                | 2.1        | .041         | 1.1          |
| Arginine, unknown                                      | 2.6        | .059         | 1.1          | Phenol                                | 0.5        | .004         | 1.1          |
| Azelate, butyrate                                      | 2.5        | .621         | −1           | Tryptophan                            | 0.5        | .003         | −1.2         |
| Betaine, TMAO, xylose, glucose                         | 1.5        | .024         | −1.1         | Tyrosine, xanthurenate                | 0.5        | .024         | 1.1          |
| Betaine, TMAO, xylose, glucose, arginine               | 2          | .041         | −1.1         | Uridine                               | 0.4        | .005         | −1.7         |
| Butyrate (3 bins)                                      | 1.8 to 3.1 | .59 to .813  | −1.1 to −1   | Valine, propionate, isobutyrate       | 1.6        | .671         | −1           |
| Butyrate, methionine, methylsuccinate                  | 1.4        | .564         | 1            | Xylose (2 bins)                       | 0.9 to 1   | .089 to .091 | −1.1 to −1.1 |
| Asparagine   | 2.8        | .057         | 1.1          | Xylose, methanol                      | 1.2        | .092         | −1.1         |
| Fructose (4 bins)                                      | 1.1 to 2.5 | .057 to .449 | −1.1 to −1   | Unknown (23 bins)                     | 0.1 to 2.7 | .008 to .725 | −2.1 to 1.2  |
| Fructose, xylose                                       | 1          | .174         | −1           |                                       |            |              |              |

Library-matched metabolites that differentiate animals living in corn cob environments (n = 27) from those living in paper environments (n = 26) are shown. *P* values are based on *t* tests (assuming unequal variances), and a positive fold change indicates mean peak intensity of corn cob bedding is greater than mean peak intensity of paper bedding.

**TABLE E3.** Potential pathways that differ between living in corncob versus paper environments

| No. | Endogenous networks   | <i>P</i> value | False discovery rate |
|-----|---|----------------|----------------------|
| 1   | Glutamic acid pathways and transport  | 3.010E-12      | 1.475E-10            |
| 2   | L-glutamate pathways and transport  | 8.667E-09      | 2.123E-07            |
| 3   | (L)-proline pathways and transport  | 3.545E-08      | 5.790E-07            |
| 4   | Tyrosine pathway  | 2.895E-05      | 3.275E-04            |
| 5   | L-serine pathways and transport   | 3.342E-05      | 3.275E-04            |
| 6   | (L)-alanine pathways and transport  | 5.543E-05      | 3.880E-04            |
| 7   | (L)-phenylalanine pathways and transport  | 1.141E-04      | 6.833E-04            |
| 8   | Methionine pathways and transport   | 1.255E-04      | 6.833E-04            |
| 9   | Amino acid metabolism: asparagine, aspartic acid, arginine metabolism and transport | 1.470E-04      | 7.205E-04            |
| 10  | L-ornithine pathways and transport  | 1.727E-04      | 7.691E-04            |

The top 10 endogenous networks identified in differentiating the fecal metabolites associated with paper and corncob environments in which the *P* value is based on distribution of the endogenous metabolite network and the false discovery rate is less than .001 are shown.

**TABLE E4.** Comparison of metabolites important to differentiating allergic disease severity

| Metabolites important to differentiating both score 0 vs 5 and score 52 vs score 5 | Metabolites unique to differentiating score 0 (n = 13) vs score 5 (n = 16) | Metabolites unique to differentiating score 2 (n = 12) vs score 5 (n = 16) |
|--|--|--|
| 3-Hydroxyphenylacetate   | 3,4-Dihydroxymandelate   | 3-Hydroxybutyrate  |
| 5-Aminopentanoate  | Acetate  | Acetoin  |
| Arabinose  | Betaine  | Acetone  |
| Arginine   | Homocysteine   | Alanine  |
| Azelate  | Isoleucine   | Fumarate   |
| Butyrate   | Isobutyrate  |  |
| Formate*   | Isovalerate  |  |
| Fructose   | Methionine   |  |
| Glucose  | Methylamine  |  |
| Glutamate  | Methylguanidine  |  |
| Glutamine  | N-acetyl amino acids   |  |
| Guanine  | Nicotinate*  |  |
| Homovanillate  | Propionate   |  |
| Lactose  | Propylene glycol   |  |
| Leucine  | Sarcosine  |  |
| Lysine   | Thymol   |  |
| Methanol   | TMAO   |  |
| Phenol   | Valine   |  |
| Tryptophan   |  |  |
| Xylose   |  |  |

Library-matched metabolites that differentiate the severity of an allergen-induced reaction are shown. Metabolite profiles were identified based on comparisons of allergy symptom scores between mice that displayed symptom scores of 0 and 5 and 2 and 5, regardless of environmental risk factors, including living environment and vaccinations.

\*Fold change of 2 or greater.