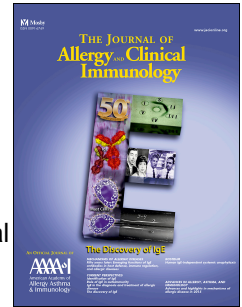


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Effect of endotoxin and alum adjuvant vaccine on peanut allergy

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Capsule Summary: Environmental endotoxin and alum-adjuvanted vaccines did not influence
peanut-allergy development in mice. Exposure to alum and CpG-adjuvanted vaccines indirectly
reduced allergic disease severity. Fecal metabolites differentiated mice based on disease
severity.

Keywords: Alum, CpG, MPL, Peanut Allergy, Endotoxin, Metabolomics, Vaccination,
C3H/HeOuJ Mice

To the Editor:

Environmental influences play a significant role in host immune responses and it is hypothesized that environmental changes may be responsible for enhanced allergy prevalence. The hygiene hypothesis proposed that smaller families and increased cleanliness reduces infectious diseases (and thereby endotoxin exposure) in a household and contributes to enhanced allergic disease¹. Vaccines can reduce microbial exposure by preventing infections and vaccine adjuvants, such as alum, may promote allergies in genetically-predisposed populations². Alum induces Th2 responses to co-administered antigens and potentially to unrelated environmental allergens, thus providing bystander (heterologous) responses that contribute to allergic disease³. Modification of childhood vaccines by the addition of Th1-enhancing toll-like receptor ligand adjuvants, such as monophosphoryl lipid A (MPL) or CpG, to alum may reduce the Th2-skewing activity of alum and balance host immunity to reduce the development of allergic disease.

In this study we utilized a mouse model of peanut allergy to test the hypothesis that environmental endotoxin and/or alum influences the severity of allergen-specific hypersensitivity. TLR4-responsive C3H/HeOuJ mice were housed in low (paper) or standard (corncob) endotoxin bedding for one month prior to alum-adjuvanted ovalbumin (OVA) immunization and peanut (PN) sensitization (A detailed experimental design can be found in the online repository and in **Table E1**). Although endotoxin was significantly lower in paper bedding compared to corncob before animal exposure, bedding endotoxin levels were similar after one day (**Figure E1A**). Multivariate analysis of the broad spectrum metabolomics data distinguished mice based on bedding type (**Figure E1B**) and the metabolites identified (**Table E2**) suggested that pathways related to amino acid metabolism and transport may be important in differentiating the type of bedding in the animal living environment (**Table E3**).

OVA/alum vaccines were administered subcutaneously to provide alum exposure prior to gastric PN-sensitization to evaluate alum's influence on allergen hypersensitivity. While Th1-inducing adjuvants used prophylactically or co-administered with allergens reduce the development of allergen-specific hypersensitivity reactions in mice, wide-spread prophylaxis with Th1-inducing adjuvants to reduce the development of allergy in humans is not practical. However, it may be practical to modify current vaccine formulations to contain alum and Th1-biased adjuvants to induce a balanced Th1:Th2 response and reduce the development of allergic disease. Therefore, we evaluated the Th1-inducing adjuvants MPL or CpG combined with alum for their ability to enhance OVA-specific Th1 responses and influence PN allergy outcomes. Antigen-specific serum antibodies were measured to evaluate adjuvant-modulated humoral immunity (**Figure 1A**). OVA-immunized mice developed anti-OVA IgG1 and IgG2a serum titers that were significantly greater than unvaccinated mice. Anti-OVA IgG1 was significantly greater in paper-housed, OVA/alum/CpG-vaccinated mice than corncob/OVA/alum or OVA/alum/CpG mice or paper/OVA/alum/MPL mice. Alum significantly increased OVA-IgE responses compared to PN 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 IgG2a titers in mice housed in paper (1:4.8 x10⁶) or corncob (1:1.8 x10⁶) compared to all other OVA-immunized mice. MPL neither enhanced OVA-specific Th1-associated IgG2a nor decreased Th2-associated IgE under the conditions tested and therefore, was removed from subsequent studies. Vaccine adjuvant's influence on PN-specific antibodies was also measured. PN-specific IgE was similar amongst all treatment groups; however, OVA/alum/CpG immunization decreased PN-IgG1 compared to corncob/OVA/alum mice.

Splenic T cell cytokine responses were measured to further assess the influence of the alum/CpG on Th-1 OVA immunity and evaluate bystander immunity to PN regardless of living environment since bedding type did not globally influence antibody responses (**Figure 1B**).

Alum enhanced OVA-induced Th2-associated IL-4, IL-5 and IL-13 compared to naïve, PN alone and OVA/alum/CpG-immunized mice. OVA-specific Th1-associated IFN γ was increased by alum/CpG compared to naïve, PN alone and OVA/alum. PN-induced cytokine responses were not changed by vaccine adjuvants.

PN-induced hypothermia and allergy symptoms were measured after completion of the OVA-vaccination and PN-sensitization. Naïve mice exhibited no allergy symptoms or hypothermia. PN-alone and OVA/alum-vaccinated mice displayed significant hypothermia and clinical symptoms compared to naïve mice after challenge (**Figure 2A-B**). Mice vaccinated with OVA/alum/CpG exhibited body temperatures not significantly different from PN-naïve mice. Exposure to OVA/alum/CpG in paper-housed mice significantly reduced clinical symptoms compared to PN-alone and OVA/alum mice living in corncob bedding. Although there is variability in allergy symptoms within treatment groups, our results suggest that CpG combined with alum indirectly influences host immunity to reduce anaphylaxis since alum/CpG adjuvants were administered on a different day and by a different route than PN-sensitization. In support of our observation, similar intragroup variability has been observed by others after treatment with the allergen co-administered with CpG in nanoparticle immunotherapy⁴. No differences in allergic disease were observed based on living environment. Our results suggest that modification of childhood vaccine adjuvant formulations may provide a method to reduce the development/severity of allergic disease.

Multivariate analysis of the binned NMR metabolomics data differentiated animals based on clinical symptom scores. Animals with score 5 were well differentiated from those with score 0 and score 2 (**Figure 2C-D**). The metabolites important to differentiating the groups are listed **Table E4**. Therefore, this exploratory analysis suggests that fecal metabolomics may be useful in predicting the severity of host response to allergens.

In this study we tested the influence of environmental endotoxin and alum adjuvants on the development of allergic symptoms. Differences in anaphylactic symptoms were not

observed based on bedding or OVA/alum compared to 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 separate from PN-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 disease¹. We did not observe differences in allergic disease despite using animal bedding exhibiting different levels of endotoxin prior to 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 may 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 Th2 responses and create a bystander response that altered PN-immunity was evaluated. Despite the ability of alum to provide bystander immune activation to newly administered antigens³ and our observation that alum enhanced OVA-induced Th2 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 PN may induce potent PN-specific Th2 responses that cannot be further enhanced by other Th2-skewing adjuvants. Therefore, additional work may be required to modify PN-sensitization regimens to allow for proper evaluation of the influence of environmental factors on allergic disease.

We believe that immune responses modulated by alum/CpG observed in our study is an example of beneficial heterologous effects of vaccination⁵. Vaccine adjuvants induced unique innate cytokine responses 6 hours after immunization (**Figure E2A**). Innate serum cytokines

induced by gastric PN-sensitization were modified by the vaccine adjuvants administered subcutaneously on the previous day (**Figure E2B**). These data suggest that vaccine adjuvants may alter subsequent immune responses to newly introduced antigens and prospective allergens. Modulation of innate cytokine production may be the basis of vaccines providing heterologous immunity that influence allergic disease. Alum/CpG subsequently induced vaccine-specific Th1-immunity and allergic responses statistically similar to naïve mice suggesting that this combination reduced allergic disease severity. However, complete disease protection was not achieved in our study and CpG may not improve allergy in human infants. Previous reports indicate that CpG does not enhance Th1 cytokines by neonatal human cells⁶ or induces more IFN γ and IL-12p70 in newborn cells than infant or adult whole blood⁷. Therefore, additional research is needed to identify safe and effective adjuvants that induce balanced Th1:Th2 responses in children that may provide indirect protection against allergy.

Broad spectrum metabolomics using feces collected prior to PN 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 humans⁸.

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

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Figure Legends

Figure 1. Antigen-specific immune responses induced by vaccination and allergen-sensitization. Mice in paper or corncob bedding were OVA-naïve or immunized with OVA/alum, OVA/alum/CpG or OVA/alum/MPL. (A) Antigen-specific serum antibodies were measured by ELISA (n= 9-15 mice/group). (B) Splenic cytokine responses induced by PN or OVA were measured by multiplex assay (n=8-10 mice/group). Significant increases are indicated by numbers above the bars ($p < 0.05$).

Figure 2. Adjuvant combination of alum + CpG reduces allergic disease.

C3H/HeO_uJ mice were challenged with PN and monitored for hypothermia (A) and clinical symptoms (B) based on pre-challenge phenotype. Statistical differences between groups are indicated by numbers ($p < 0.05$). Data combined from two independent experiments (n=9-15/group). OPLS-DA scores plot associated with symptom scores comparing the animals that exhibited (C) no change (score 0) and 2, (D) 2 and 5.

Figure 1

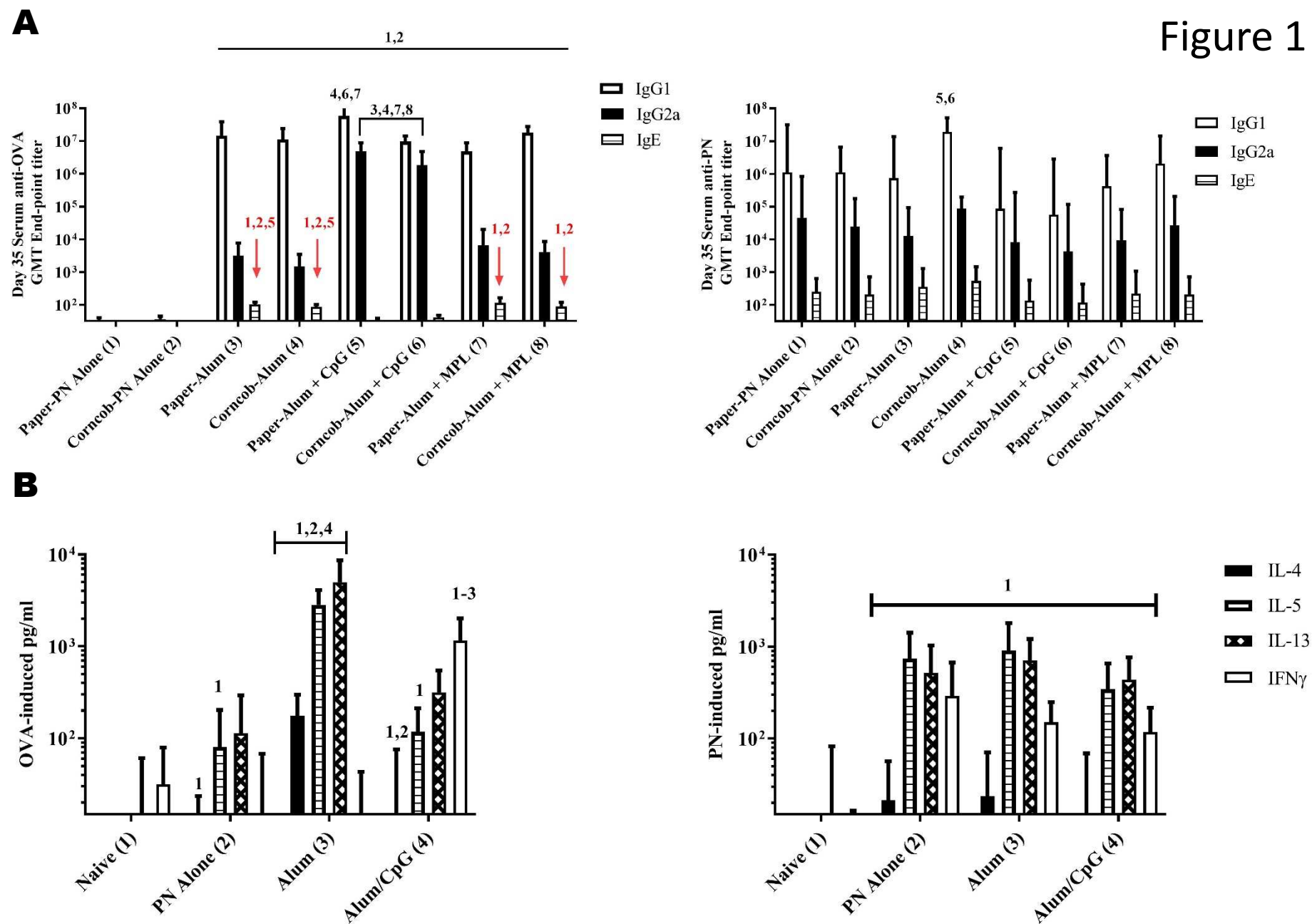
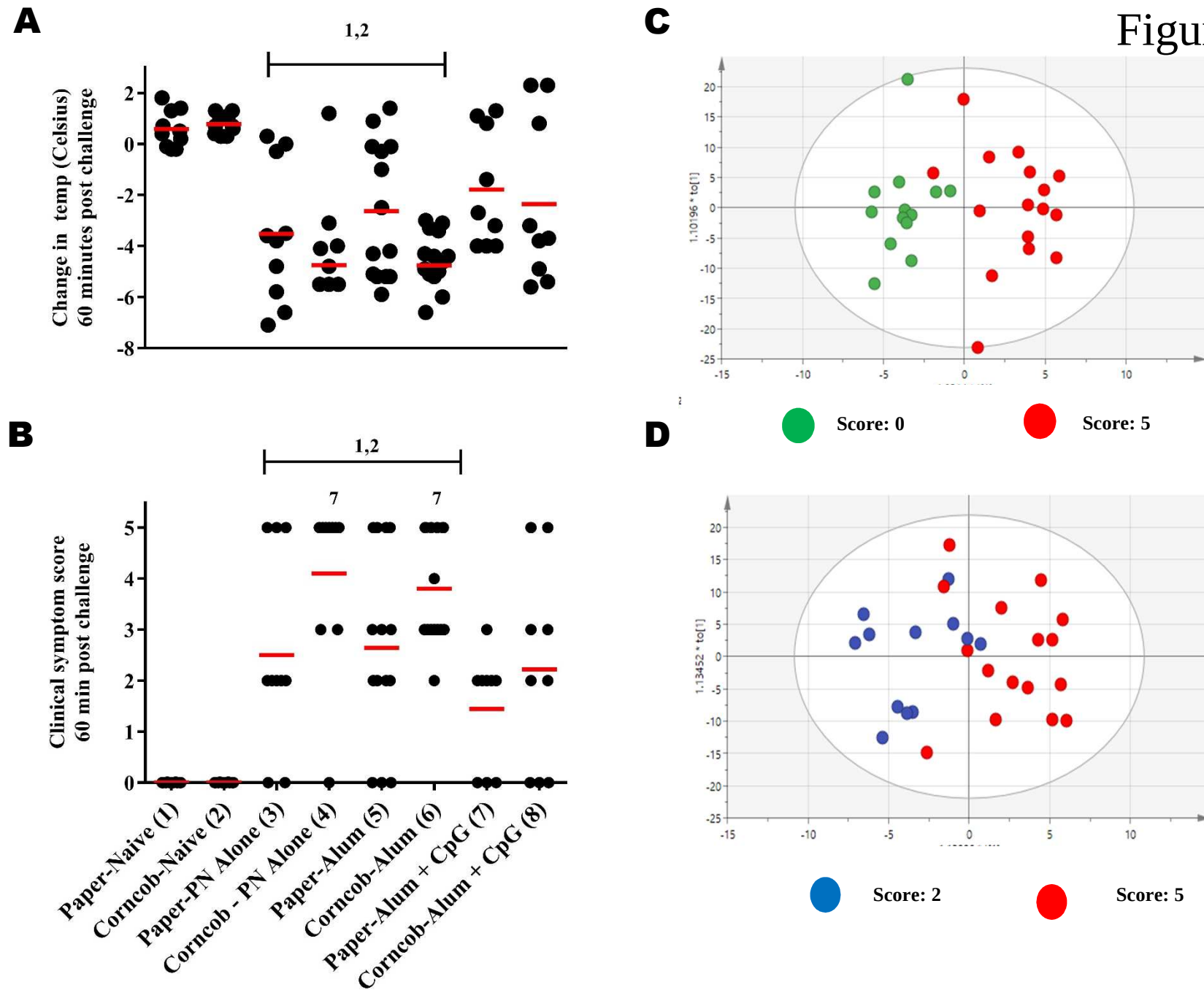


Figure 2



Online Repository:**Methods and Materials:**

Mice: Female 4-6-week-old C3H/HeOuJ mice (n= 9-15 per group) were purchased from Jackson Laboratory (Stock No: 000635, Bar Harbor, ME) and housed in micro-isolator cages under specific-pathogen free conditions. Results 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, IN) and 5 groups were housed in Enrich-o-cob corncob bedding (Andersons Laboratory; Maumee, OH) for one month prior to initiation of vaccinations/allergic sensitization. Paper bedding was replaced three times a week to represent a low endotoxin environment and the standard endotoxin environment contained corncob bedding that was replaced biweekly. Mice living in paper or corncob bedding included naïve mice, mice sensitized to PN but no other vaccination (PN alone), vaccinated with OVA and alum (Alum), vaccinated with OVA, alum and CpG (Alum/CpG) or OVA, alum and MPL (Alum/MPL) prior to PN-sensitization. Vaccines were administered subcutaneously beginning one day prior to gastric PN-sensitization. All mice were challenged with PN and monitored for hypersensitivity reactions on day 36.

Endotoxin Measurement: Endotoxin was measured in mouse bedding after autoclaving and before animal exposure. Bedding samples were processed as previously described^{E1}. Supernatants were analyzed using Pierce LAL Chromogenic Endotoxin Quantification Kit (Thermo Scientific; Waltham, MA). Data is presented as Endotoxin units (Eu)/milligram of bedding.

Vaccinations: Subcutaneous vaccines containing low endotoxin ovalbumin (OVA) (Endofit OVA 10 µg, Invivogen; San Diego, CA) and the following adjuvants in sterile USP saline (APP

Pharmaceuticals; Schaumburg, IL): alum (0.5 mg of $\text{Al}(\text{OH})_3$) (Alhydrogel, Invivogen), alum/CpG-1826 (100 μg) (Vaccigrade, Invivogen) or alum/MPL (25 μg) (Enzo Life Sciences; Farmingdale, NY) were administered in 100 μl volumes on days 0, 14 and 28.

Peanut-Sensitization: Crude peanut extract was obtained as previously described^{E2}. PN-sensitization was achieved by gastric gavage of PN (1 mg) and cholera toxin (CT, 10 μg , Cat. #100B List Biologicals; Campbell, CA) in 200 μl of USP saline on days 1, 8, 15 and 22.

Peanut-Provocation: Temperature transponders (BioMedic Data Systems; Seaford, DE) were injected subcutaneously before allergen challenge and used to monitor body temperature after PN challenge. On day 36, mice were challenged intraperitoneally with PN (250 μg). Body temperature and clinical symptoms were monitored prior to and every 20 minutes after PN-challenge for one hour. Clinical symptoms were reported using a 0-5 scoring system as previously described^{E3}. The following score system was used to grade hypersensitivity reactions in mice after challenge: 0; no symptoms, 1; repetitive scratching with hind legs near 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 $> 5^\circ\text{C}$.

Sample Collection: Blood was collected on day 35 using the submandibular lancet method^{E4} and serum antibodies were measured by ELISA. Day 35 fecal pellets were frozen at -80°C for metabolomics.

ELISA: Antigen-specific $\log_2$ endpoint antibody titers were determined by ELISA. ELISA plates were coated with 2 $\mu\text{g}/\text{ml}$ of PN or OVA and completed as previously described^{E5}. Antigen-specific IgE ELISAs were coated with 5 $\mu\text{g}/\text{ml}$ of anti-mouse IgE-unlabeled (Southern Biotech; Birmingham, AL) and biotinylated antigens were used to determine antigen-specificity at 2 $\mu\text{g}/\text{ml}$. The endpoint titer was calculated as the last sample dilution with a signal 3-fold greater than a naïve 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-naïve. Serum was collected from each animal 6 hours after vaccination. Serum was also collected from naïve mice at the same time as immunized mice. Five mice from each vaccine group, including naïve, were gastrically sensitized with PN/CT 24-hours after OVA-vaccination. Serum was collected for a second time from all mice 6 hours after administration of the PN-sensitization dose. Serum was analyzed for cytokine responses by a multiplex-luminex assay (Bio-Rad; Hercules, CA).

Antigen-induced Cytokine Analysis: Naïve, PN alone, Alum or Alum/CpG mice living in the corncob environment since living environment did not impact humoral immunity were analyzed for PN- and OVA-induced T cell cytokine responses after OVA-immunization and PN-sensitization. On day 36, spleens were harvested and cultured in vitro with media alone, PN (200 µg/ml) or OVA (200 µg/ml) for 96-hours using methods similarly described^{E6}. Supernatants were analyzed for cytokine responses by a multiplex-luminex assay.

Immunology Statistical Analysis: An unpaired Student's T-test determined statistical differences between the endotoxin levels in the bedding samples. Analysis of Variance (ANOVA) with Tukey's multiple comparisons was used to compare antibody and challenge results. Antigen-induced cytokine responses were calculated by subtracting the pg/ml value from wells that contained cells in media from the pg/ml values of wells stimulated with antigen (PN or OVA) then log₁₀-transformed for normalization before analysis with One-way ANOVA with Tukey's multiple comparisons. GraphPad Prism 7 (San Diego, CA) software was used for all analyses.

Broad Spectrum NMR Metabolomics: A subset of animals was chosen from the immunological studies for the exploratory broad spectrum metabolomics analysis, and the sample preparation and data collection and analysis was as described previously^{E7}. Briefly,

fecal pellets were homogenized in ceramic bead tubes (MagNA Lyser) and D₂O (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 5mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS, chemical shift indicator), 100 mM imidazole (pH indicator), and 0.2% NaN₃ (to inhibit bacterial growth). NMR spectra were processed using ACD NMR Processor 12.0 (ACD Labs, Toronto, CA) 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) using intelligent bucket integration with a 0.04 ppm bucket width and 50% looseness. The binned data were normalized to total integral of each spectrum. Prior to multivariate analysis, the 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 two-sided hypothesis tests using the continuous binned NMR binned data were conducted using SAS 9.4 (SAS Institute Inc, Cary, NC). Hypothesis testing was conducted using the t-test with the Satterthwaite correction for unequal variances. Multivariate data analysis methods (e.g., Principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA)) were used to reduce the dimensionality and to enable the visualization of the separation of the study groups (SIMCA 14; Umetrics, Umeå Sweden). These pattern recognition methods are commonly used to analyze high dimensional multicollinear data such as metabolomics^{E8, 9}. 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 projections (VIP) plots were inspected along with the VIP statistic, which summarizes the importance of the bin in differentiating the phenotypic

groups in a supervised multivariate analysis^{E8}. Model statistics are reported for OPLS-DA models including the proportion of the variance in the metabolomics data associated with the phenotype (R2X), the proportion of the variance in the phenotype explained by the model (R2Y), and the measure of predictive variation of the model assessed by 7-fold cross validation (Q2). Bins that had either a VIP ≥ 1.0 with a jack-knife confidence interval that did not include 0, p-value < 0.1 , or magnitude of fold change > 2 were determined to be important to differentiating the study groups. Chenomx 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 using the MetaCore® module in GeneGo software (GeneGo, Philadelphia, PA).

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Table E1: Experimental Design					
Bedding	Treatment Group				
	N/A		Subcutaneous Vaccination: Days 0, 14 and 28		
	N/A	Gastric Sensitization: Days 1, 8, 15 and 22			
	Intraperitoneal Challenge: Day 36				
	Naïve	PN Alone	OVA +Alum	OVA + Alum + CpG	OVA + Alum + MPL
	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 naïve to peanut, sensitized to peanut only, PN-sensitized and immunized with OVA/alum; OVA/alum/CpG or OVA/alum/MPL. A total of 2 independent experiments were performed with 9-15 mice/group. Environmental acclimation occurred 4 weeks prior to immunization. PN-induced anaphylaxis was measured 2 weeks after completion of PN-sensitization.					

Table E2: Metabolites that Distinguished Animals Living in Corncob from Animals Living in Paper Environments

Library-Matched Metabolites	VIP	p-value	FC	Library-Matched Metabolites	VIP	p-value	FC
3-Hydroxyphenylacetate	0.4	0.030	1.1	Glucose (3 bins)	0.7 - 1.7	0.007 - 0.088	-1.2 -- 1.1
Acetamide	0.7	0.051	1.1	Glucose, Arabinose	2.3	0.081	-1
Acetate	5.5	0.16	1.1	Glucose, Arabinose, Betaine, Fructose	1.7	0.139	-1
Acetoin	2.2	0.056	1.2	Glucose, Leucine	1.5	0.04	-1.1
Acetone, Acetoin	2.5	0.026	1.2	Glutamate	1.7	0.015	1.1
Alanine, unknown	1.3	0.219	1.1	Glutamate, N-acetyl amino acids	1	0.021	1.1
Arabinose	0.9	0.025	-1.1	Glutamate, Arabinose, Glucose	2.2	0.036	-1.1
Arabinose, Fructose	1.5	0.123	-1.1	Histamine, Uridine	0.4	0.052	-1.2
Arabinose, Glucose (3 bins)	1 - 1.5	0.016 - 0.068	-1.2 -- 1.1	Isovalerate, Isoleucine, Leucine	1.5	0.582	1
Arabinose, Xylose, Glucose	1.9	0.174	-1.1	Lactate (2 bins)	2 - 3.2	0.046 - 0.047	-1.2 -- 1.1
Arginine, Glucose, Lysine, Alanine, Leucine, Glutamate	2.3	0.136	-1	Lysine	2.1	0.041	1.1
Arginine, Unknown	2.6	0.059	1.1	Phenol	0.5	0.004	1.1
Azelate, Butyrate	2.5	0.621	-1	Tryptophan	0.5	0.003	-1.2
Betaine, TMAO, Xylose, Glucose	1.5	0.024	-1.1	Tyrosine, Xanthurenate	0.5	0.024	1.1
Betaine, TMAO, Xylose, Glucose, Arginine	2	0.041	-1.1	Uridine	0.4	0.005	-1.7
Butyrate (3 bins)	1.8 - 3.1	0.59 - 0.813	-1.1 - -1	Valine, Propionate, Isobutyrate	1.6	0.671	-1
Butyrate, Methionine, Methylsuccinate	1.4	0.564	1	Xylose (2 bins)	0.9 - 1	0.089 - 0.091	-1.1 -- 1.1
Asparagine	2.8	0.057	1.1	Xylose, Methanol	1.2	0.092	-1.1
Fructose (4 bins)	1.1 - 2.5	0.057 - 0.449	-1.1 - -1	Unknown (23 bins)	0.1 - 2.7	0.008 - 0.725	-2.1 - 1.2
Fructose, Xylose	1	0.174	-1				

Library-matched metabolites that differentiate the animals living on corncob environments (n=27) and those living on paper environments (n=26). The p-values are based on t-test (assuming unequal variances), and a positive fold change (FC) indicates mean peak intensity of corncob bedding > mean peak intensity of paper bedding.

Table E3: Potential Pathways that Differ between living in Corncob vs. Paper Environments

#	Endogenous Networks	p-value	FDR
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 corn cob environments, where the p-value is based on the distribution of the endogenous metabolites network and the false discovery rate (FDR) are < 0.001.

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 5-Aminopentanoate Arabinose Arginine Azelate Butyrate Formate* Fructose Glucose Glutamate Glutamine Guanine Homovanillate Lactose Leucine Lysine Methanol Phenol Tryptophan Xylose	3,4-Dihydroxymandelate Acetate Betaine Homocystine Isoleucine Isobutyrate Isovalerate Methionine Methylamine Methylguanidine N-acetylaminoacids Nicotinate* Propionate Propylene glycol Sarcosine Thymol TMAO Valine	3-Hydroxybutyrate Acetoin Acetone Alanine Fumarate
*Fold Change (FC) ≥ 2 The library-matched metabolites that differentiate the severity of an allergen-induced reaction. The 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		

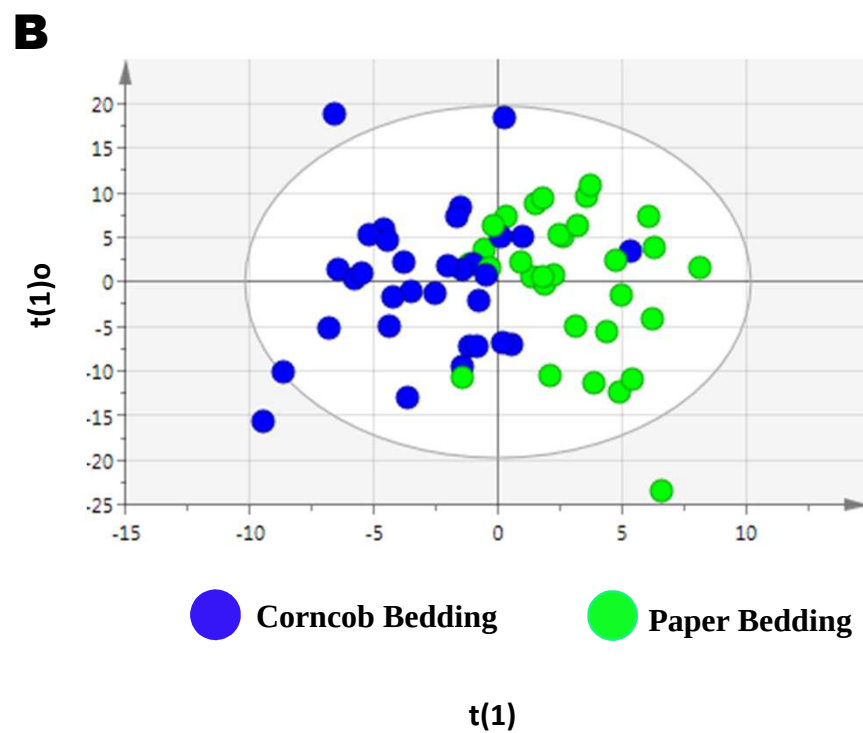
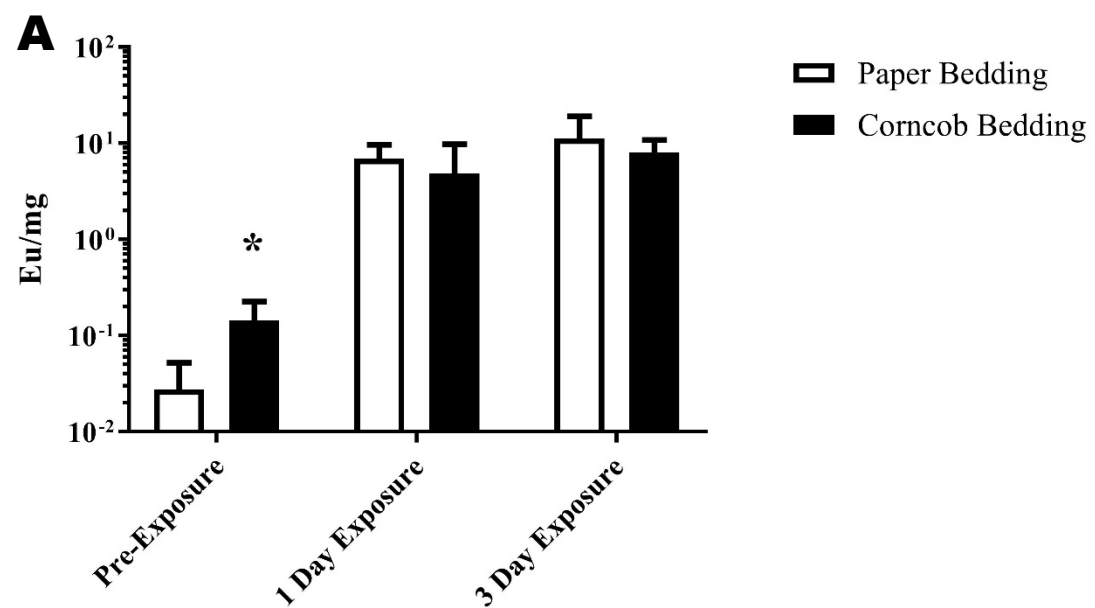
Supplemental Figure Legend**Figure E1. Endotoxin levels are lower in paper bedding compared to corncob bedding.**

(A) Endotoxin levels in bedding pre-animal exposure and 1 or 3 days post-animal exposure). * Significant increase in EU/mg compared to paper bedding as determined by an unpaired Student's T-test ($p=0.0002$). (B) OPLS-DA scores plot showing the separation of animals that lived on corncob bedding (green) from those that lived on paper bedding (blue) [$n=53$, $R^2X=0.504$, $R^2Y=0.597$, $Q^2=0.301$].

Figure 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 PN+CT (/PN) 24 hours after immunization (Day 1). (A) Serum cytokines measured 6 hours after subcutaneous vaccination. (B) Serum cytokines measured 6 hours post-sensitization. Cytokines were measured by luminex assay. Numbers indicate significant differences between groups using One-way ANOVA ($p < 0.05$).

Figure E1



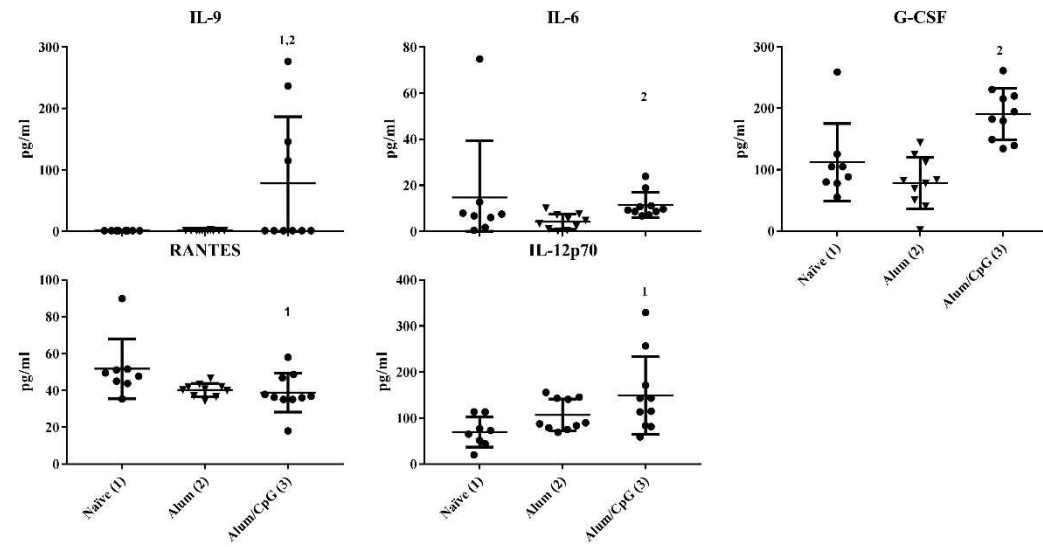
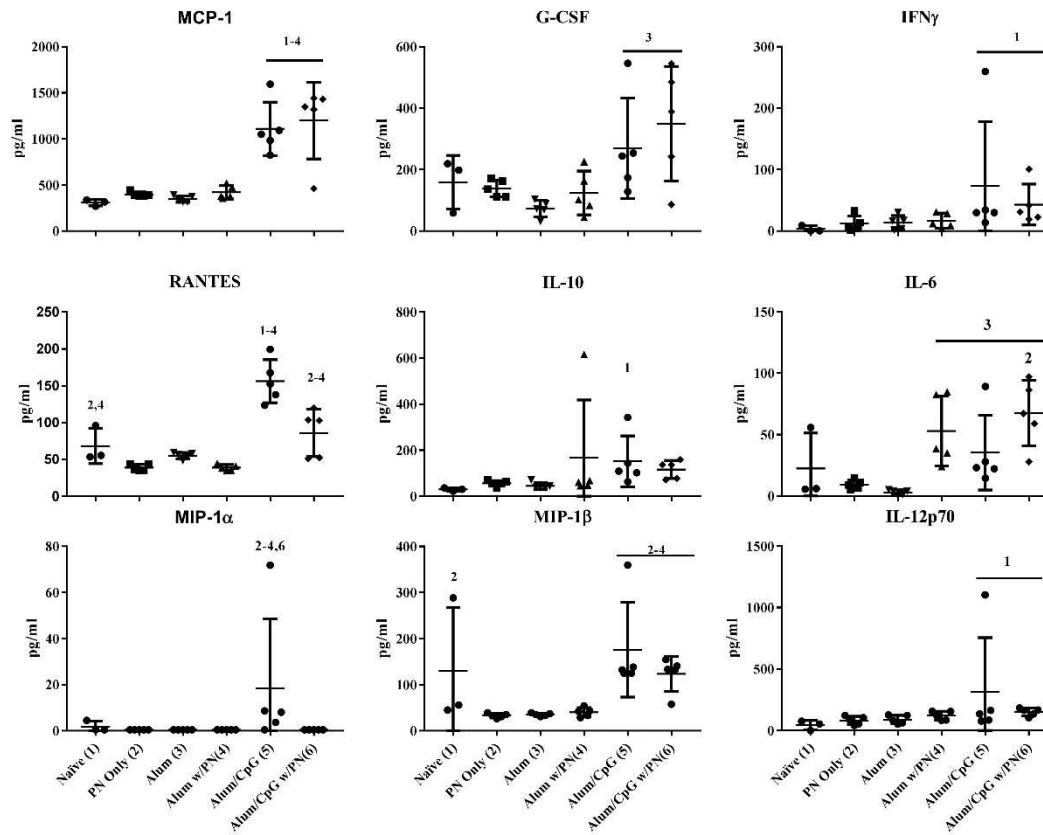
A**B**

Figure E2