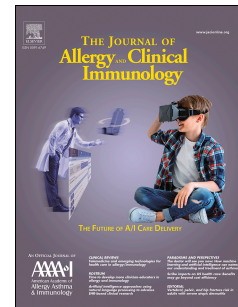


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Timing of exposure to environmental adjuvants is critical to mitigate peanut allergy

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PII: S0091-6749(20)31321-X

DOI: <https://doi.org/10.1016/j.jaci.2020.09.011>

Reference: YMAI 14756

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 10 April 2020

Revised Date: 27 August 2020

Accepted Date: 17 September 2020

Please cite this article as: Smeekens JM, Immormino RM, Kulis MD, Moran TP, Timing of exposure to environmental adjuvants is critical to mitigate peanut allergy, *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.09.011>.

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Timing of exposure to environmental adjuvants is critical to mitigate peanut allergy

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Word count: 1190

Figure count: 2

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Funding: JMS is funded by a T32 Allergy/Immunology Training Grant (AI007062) through Duke University and University of North Carolina at Chapel Hill. TMP is funded by the NIH/NIEHS (K08 ES029118). This work is supported by the American Academy of Allergy, Asthma and Immunology Foundation (TPM) and the UNC Center for Environmental Health and Susceptibility (TPM and MDK).

The authors have no conflicts of interest to declare.

Keywords: Airway sensitization, Food allergy, Peanut allergy, Anaphylaxis, Indoor dust, Toll-like receptors

Abbreviations:

Flagellin (FLA)

Human bronchial epithelial cell (HBEC)

Indoor dust (ID)

Lipopolysaccharide (LPS)

Pam3CSK4 (PAM)

Peanut (PN)

Peanut-specific IgE (PNsIgE)

Peanut-specific IgG1 (PNsIgG1)

Peanut-specific IgG2c (PNsIgG2c)

Toll-like receptor (TLR)

Capsule summary:

Toll-like receptor ligands in indoor dust act as environmental adjuvants to promote sensitization to inhaled peanut in mice.

The timing of exposure to environmental adjuvants relative to peanut is a determinant of peanut allergy development.

To the Editor:

Peanut (PN) allergy is a common and potentially life-threatening disease that typically arises during early childhood due to a combination of genetic and environmental factors. There is growing evidence that non-oral exposure to environmental PN in indoor dust (ID) via the respiratory tract¹ or skin² is a risk factor for PN allergy development. We previously reported that exposure to PN in combination with ID, but neither alone, induced PN-specific IgE and IgG1, resulting in anaphylaxis upon PN challenge¹. The components of ID responsible for its adjuvant activity remain unclear. Building on our previous findings, we aimed to define the environmental agents in ID that promote allergic sensitization to PN, and how the timing of adjuvant exposure influences PN allergy development.

To identify potential environmental adjuvants in ID³, we screened dust samples from four homes for various immunostimulatory agents, including proteases, β -glucans, endotoxin, and bacterial DNA. All of the immunostimulatory agents assayed were detected in IDs, albeit at variable levels between homes (Figure 1A). To determine if IDs differed in their adjuvant activity, we investigated the ability of ID to promote airway sensitization to PN in our mouse model. C57BL/6J mice (age 6-12 weeks) were exposed to PN with ID¹ (10 μ L of 100 mg/mL extract) or environmental adjuvants via oropharyngeal aspiration twice weekly for 14 days (see Supplemental Information and Table S1 for additional details). On day 14, serum was collected for measurement of PN-specific immunoglobulins (PNsIg)¹. To focus on the adjuvant activity of ID, we used IDs that contained low levels of ambient PN protein (<250 ng/mL), which alone were insufficient to induce PN sensitization¹. IDs varied in their ability to induce PNsIgE, PNsIgG1 and PNsIgG2c (Figure 1B), suggesting that the adjuvant activity of ID is household-dependent. To investigate if IDs have variable adjuvant activity in the human respiratory tract, we treated air-liquid interface cultures of primary human bronchial epithelial cells (HBECs) with different IDs. IDs induced differential production of the innate cytokine IL-1 α (Figure S1A), which is important for priming allergic responses in the airways⁴. Taken together, these findings indicate that the adjuvant activity of ID varies between households, possibly due to differing levels of environmental adjuvants within the home exposome.

We next sought to identify what immunostimulatory agents in ID were responsible for adjuvant activity in our airway sensitization model. Because ID1 demonstrated the greatest adjuvant activity based on IL-1 α production from HBECs and PN-specific immunoglobulins *in vivo*, we used this ID for all subsequent experiments. To determine if proteases were necessary for ID adjuvant activity, we heat-treated ID to abolish protease activity (Figure S2A). Co-exposure to heat-treated ID and PN induced slightly lower levels of PNsIgE but similar levels of PNsIgG1 (Figure S2B). Mice sensitized with heat-treated ID and PN also developed anaphylaxis (as indicated by decreased core body

temperature) after intraperitoneal challenge with PN, indicating that protease activity was not necessary for ID adjuvant activity. Next, the importance of β -glucans was investigated using *Clec7a*^{-/-} mice, which lack the β -glucan receptor Dectin-1. *Clec7a*^{-/-} and wild-type C57BL/6J mice sensitized with PN+ID demonstrated similar levels of PNsIgE, PNsIgG1 and anaphylaxis severity following PN challenge (Figure S2A). Furthermore, β -glucanase treatment of ID also failed to abolish adjuvant activity (Figure S2B), indicating that β -glucans were dispensable for ID adjuvant activity. Since endotoxin and bacterial DNA are present in dust and are Toll-like receptor (TLR) ligands, we investigated if TLR signaling was necessary for the adjuvant activity of ID. Because all TLRs except TLR3 signal through the adapter protein MyD88, we examined the ability of ID to induce sensitization in *Myd88*^{-/-} mice. *Myd88*^{-/-} mice sensitized with PN+ID did not produce PNsIgE or PNsIgG1 and failed to react upon PN challenge (Figure 1C), indicating that the adjuvant activity of ID is MyD88-dependent.

To determine if MyD88-dependent TLR ligands were sufficient to induce sensitization to PN, we co-exposed wild-type mice to PN and 5 μ g unmethylated CpG dinucleotides (CpG, TLR9 agonist), 100 ng lipopolysaccharide (LPS, TLR4 agonist), 1 μ g Pam3CSK4 (PAM, TLR1/2 agonist) or 1 μ g flagellin (FLA, TLR5 agonist). Co-exposure to PN and either LPS, PAM or FLA resulted in significantly increased PNsIgE levels compared to PN alone (Figure 2A). PN with LPS or FLA also led to increased PNsIgG1. Co-exposure to PN and CpG did not significantly induce PNsIgE and PNsIgG1, but stimulated robust production of PNsIgG2c (Figure 2A). Despite differences in the types of PN-specific immunoglobulins induced, all mice developed anaphylaxis upon PN challenge (Figure 2A), indicating that co-exposure to TLR ligands and PN was sufficient to induce airway sensitization to PN. While we cannot exclude the possibility that impaired IL-1/IL-33 signaling in *Myd88*^{-/-} mice also contributed to decreased PN sensitization, our results suggest that MyD88-dependent TLR ligands in ID are an important source of adjuvant activity.

There are conflicting reports regarding the role of microbial exposure and atopy. Epidemiological studies have suggested that exposure to endotoxin and other microbial products confers protection against asthma and atopy⁵. However, LPS and other TLR ligands have also been reported to promote allergic sensitization to inhaled antigens^{6,7}. To determine if a “high microbe” environment was protective against sensitization to peanut, we added LPS (1 μ g) or CpG (5 μ g) to ID and delivered this with PN to the airways of mice. Remarkably, LPS enhanced ID-induced PNsIgE and PNsIgG1 production and anaphylaxis upon PN challenge (Figure 2B), indicating that co-exposure to PN and microbial products promoted allergic sensitization to PN. Interestingly, the addition of CpG trended towards decreased PNsIgE and PNsIgG1, but was not statistically significant (Figure 2C). Previous studies have shown that exposure to LPS can induce

regulatory mechanisms that suppress allergic responses upon subsequent exposure to house dust mite allergen⁵. To determine if the timing of microbial exposure similarly impacted systemic sensitization to PN, we first exposed mice to LPS (100 ng) or CpG (3 µg) three times weekly for 14 days, which was followed by sensitization to PN+ID as described above. Pre-treatment with LPS or CpG prior to PN+ID exposure significantly decreased serum PNsIgG1 and anaphylaxis severity (Figure 2D). Thus, the timing of microbial exposure relative to PN appears to be an important determinant of PN sensitization.

Overall, these results demonstrate that co-exposure to environmental PN and TLR ligands in ID promotes allergic sensitization to PN. In contrast, exposure to TLR ligands in the absence of PN protects against allergic sensitization upon subsequent PN exposure. Thus, the timing of exposure to TLR ligands appears to determine whether exposure to environmental food antigens leads to tolerance or allergy. Our findings may help explain why some studies show either no or even contradictory effects of endotoxin exposure on risk for food sensitization or allergy^{8,9}. For example, endotoxin levels in household dust were inversely associated with egg and overall food allergy, but not peanut allergy, in an inner city cohort⁸. In contrast, a large cross-sectional study found that household endotoxin levels were positively associated with sensitization to egg and milk, but not peanut⁹. These studies and our findings highlight the complex role of the environment in food allergy development, which likely depends not only on the levels of TLR ligands and food allergens within the indoor exposome, but also the route and relative timing of exposure to these agents during childhood development. Since TLR ligands are ubiquitous in ID, our findings suggest that reducing environmental PN levels prior to dietary introduction may decrease the likelihood of PN sensitization in at-risk infants. Future studies should investigate if reduction of environmental PN levels in the home is a feasible and effective strategy for mitigating PN allergy development in children.

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Acknowledgements: We acknowledge cell and media provision by the Marsico Lung Institute Tissue Procurement and Cell Culture Core supported by NIH grant DK065988 and Cystic Fibrosis Foundation grant BOUCHE15R0.

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Figure legends

Figure 1. The adjuvant activity of ID is MyD88-dependent. **A:** Measurement of environmental adjuvants in ID (n=4). **B:** Immunoglobulin levels in C57BL/6J mice (n=6 per group) sensitized to inhaled PN and ID from four homes. **C:** Immunoglobulin levels and PN challenge outcomes in WT or *Myd88*^{-/-} mice (n=6-7 per group) sensitized with PN and ID. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Comparisons in B were performed by one-way ANOVA. Comparison of immunoglobulin levels in C were performed by Student's t test. Comparisons of peanut challenge outcomes in C were performed by 2-way ANOVA. *PN*, peanut, *ID*, indoor dust

Figure 2. Co-exposure to TLR ligands and peanut promotes sensitization whereas pre-exposure to TLR ligands protects against peanut allergy. **A:** Immunoglobulin levels and PN challenge outcomes in C57BL/6J mice (n=5 per group) sensitized to inhaled PN and either CpG (5 µg), Pam3CSK4 (PAM, 1 µg), flagellin (FLA, 1 µg) or LPS (100 ng). Comparisons are between each group and PN. **B-C:** Immunoglobulin levels and PN challenge outcomes in C57BL/6J mice (n=6 per group) sensitized to inhaled PN and ID alone or supplemented with either LPS (1 µg, **B**) or CpG (5 µg, **C**). **D:** Immunoglobulin levels and PN challenge outcomes in C57BL/6J mice (n=5-9 per group) pretreated for 2 weeks with PBS, LPS (100 ng) or CpG (3 µg) and then sensitized to inhaled PN and ID. Statistical comparisons are between each group and PBS/PN+ID. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Comparisons of immunoglobulin levels were performed by one-way ANOVA. Comparison of PN challenge results were performed by two-way ANOVA. *PN*, peanut, *ID*, indoor dust

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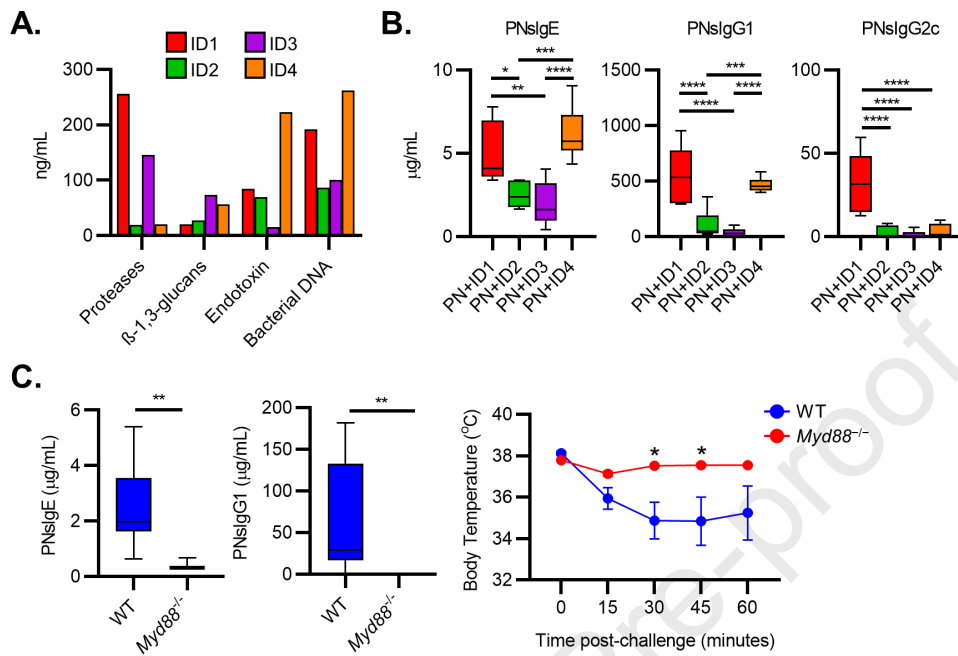
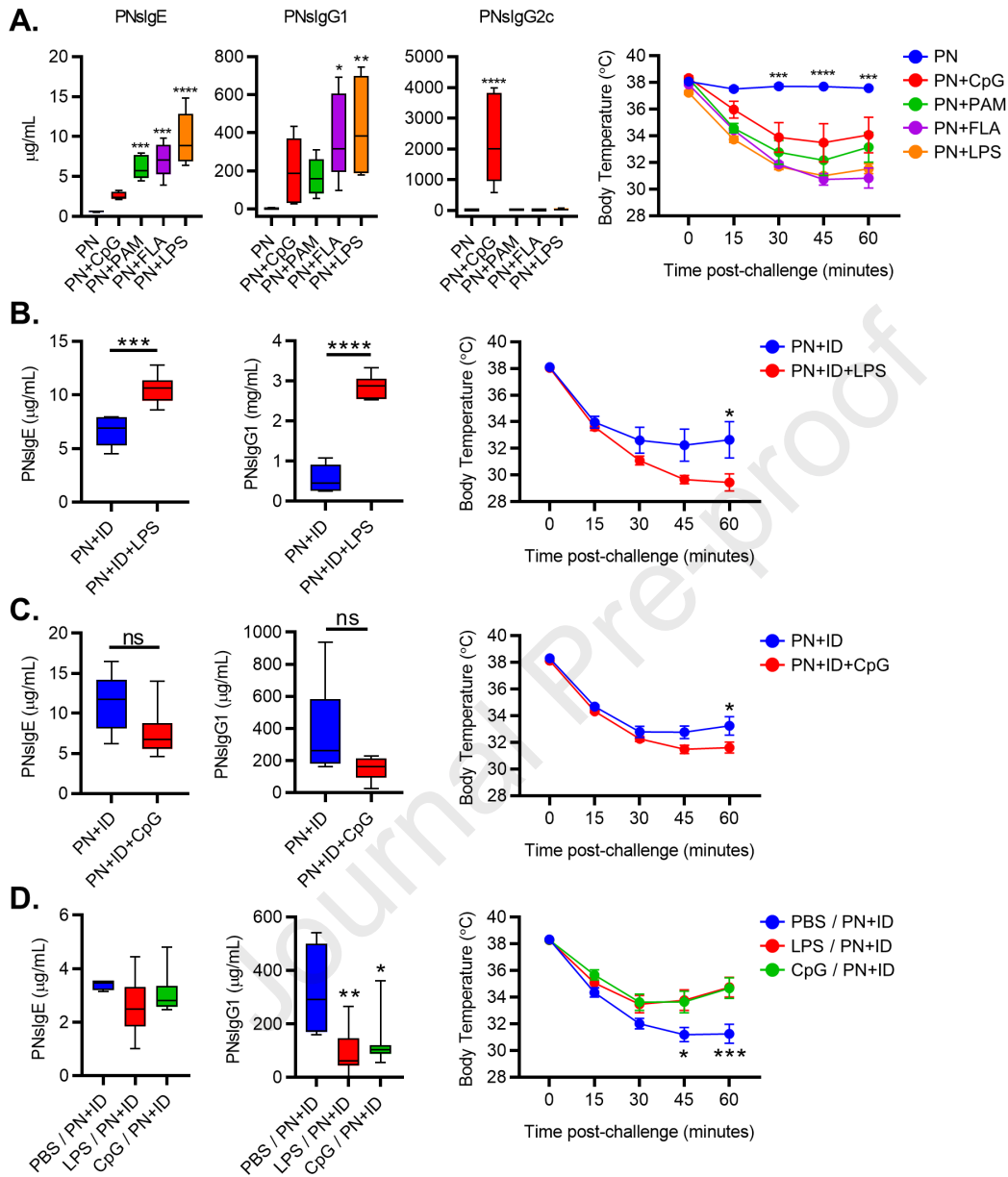
Figure 1

Figure 2



Supplemental Information

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Word count: 1148

Figure count: 1

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Funding: JMS is funded by a T32 Allergy/Immunology Training Grant (AI007062) through Duke University and University of North Carolina at Chapel Hill. TMP is funded by the NIH/NIEHS (K08 ES029118). This work is supported by the American Academy of Allergy, Asthma and Immunology Foundation (TPM) and the UNC Center for Environmental Health and Susceptibility (TPM and MDK).

The authors have no conflicts of interest to declare.

Keywords: Airway sensitization, Peanut allergy, Anaphylaxis, Indoor dust, Toll-like receptors

Methods

Mice

C57BL/6J mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and bred at the University of North Carolina (UNC) at Chapel Hill. *Clec7a*^{-/-} mice (B6.129S6) were obtained from Jackson Laboratories (Bar Harbor, ME), and *Myd88*^{-/-} mice (B6.129P2) were obtained from the Doerschuk lab at UNC. All mice were housed in specific pathogen-free conditions, raised on standard mouse chow free of any peanut ingredients, and kept on a 12:12-hour light/dark cycle. *MyD88*^{-/-} mice additionally received sterile chow and sterile acidified water. Female and male mice between 6 and 12 weeks of age were used for experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill under protocol 18-074.

Reagents

Unmethylated CpG DNA, Pam3CSK4 (PAM) and flagellin (FLA) were from Invivogen (San Diego, CA); lipopolysaccharide (LPS) was from Sigma (St. Louis, MO). Each TLR ligand was purchased as a lyophilized powder. Stock solutions were prepared with PBS and stored at -20°C. For animal treatments, the TLR ligands were diluted under sterile conditions and mixed with peanut (PN) and/or indoor dust (ID) as indicated below. In all cases, treatment solutions were warmed before oropharyngeal (o.p.) administration.

Peanut extract was prepared from roasted de-fatted peanut flour (Golden Peanut, Alpharetta, GA) by aqueous extraction in PBS as described previously¹. ID extracts were prepared as previously described². Briefly, bulk vacuum dust samples were collected from single-family homes. Dust samples were passed through a coarse sieve (425µm, #40), weighed and then extracted at 100 mg/mL with PBS at 4°C with mild agitation for 16 hours. The samples were centrifuged to remove insoluble debris, and supernatants were sterilized by passage through a 0.22 µm filter.

Endotoxin was measured by ToxinSensor Endotoxin Detection System (GenScript, Piscataway, NJ); bacterial DNA by qPCR using primers specific for bacterial ribosomal DNA³; β-1,3-glucans by GlucateLL® Kit (Associates of Cape Cod Inc., East Falmouth, MA); and proteases by Fluoro™ Protease Assay (measured as trypsin equivalents, G Biosciences, St. Louis, MO). Indoor dust extracts were aliquoted, stored at -80°C, and discarded after more than two freeze-thaw cycles. Total peanut protein in dust samples was measured using the Veratox for Peanut Allergen Assay (Neogen, Lansing, MI).

Separate batches of ID were used to create protease or β -glucans depleted IDs. To remove protease activity extracted dust was heated to 95°C for 30 minutes. To degrade β -glucans extracted dust was treated at room temperature with 100 U of endo- β -(1,3)-glucanase (Megazyme; Bray, Ireland) and subsequently heated to 65°C for 30 minutes to inactivate the endo- β -(1,3)-glucanase. As a control, heat inactivated endo- β -(1,3)-glucanase (65°C for 30 minutes) was used to similarly treat an ID sample. In each case following heating, IDs were centrifuged to remove insoluble precipitants.

Peanut airway sensitization and challenge model

Mice were lightly anesthetized with isoflurane inhalation and given 0.05-50 μ g of PN with ID, or environmental adjuvants (as outlined in Table S1) in a total volume of 50 μ L by o.p. aspiration twice weekly for two weeks as previously described². Briefly, mice were lightly anesthetized with isoflurane and then vertically suspended by the mandible on a specialized rodent intubation device. The tongue was then extended with forceps and the agent was deposited in the oral cavity using a pipette. The tongue was kept extended until the animal inhaled several times, resulting in dissemination of the agent in the peribronchial and alveolar regions of the respiratory tract. A limitation of this method is the minor possibility that antigen can be taken up in the oral cavity rather than inhaled into the lungs. For pretreatment experiments, mice were administered CpG or LPS (o.p.) three times per week for two weeks prior to the two week sensitization with PN+ID.

Serum was collected three days after the final sensitization by submandibular bleed and used for immunoglobulin quantification. One week after the final sensitization, mice were challenged to peanut (6-8 mg protein) by intraperitoneal injection. Core body temperatures were monitored every 15 minutes for one hour with a rectal thermometer (Physitemp, Clifton, NJ).

ELISA protocol for PNsIgE, PNsIgG1 and PNsIgG2c

Peanut-specific IgE, IgG1 and IgG2c were quantified via ELISA as described previously¹. Briefly, 96-well plates were coated with 20 μ g/mL HSA-DNP (for standard curves) or peanut extract (for samples) and subsequently blocked with 2% BSA in PBS-0.5% Tween. Samples were diluted 1:20 for peanut-specific IgE ELISAs, 1:20,000 for peanut-specific IgG1 ELISAs 1:10,000 for peanut-specific IgG2c ELISAs. Standard curves were generated with mouse IgE anti-DNP, IgG1 anti-DNP or IgG2a anti-DNP (Accurate Chemicals, Westbury, NY) ranging from 0.002 to 2 μ g/mL. IgE

plates were detected using the following antibodies in succession: sheep IgG anti-mouse IgE (0.5 µg/mL, The Binding Site, Birmingham, UK), biotinylated donkey anti-sheep IgG (0.5 µg/mL, Accurate Chemicals), and NeutrAvidin-HRP (0.5 µg/mL, Pierce Biotechnology, Waltham, MA). IgG1 plates were detected with HRP-goat anti-mouse IgG1 (1:40,000 Southern Biotech). IgG2c plates were detected with either HRP-goat anti-mouse IgG2a for standard curves (1:20,000, Southern Biotech, Birmingham, AL) or HRP-goat anti-mouse IgG2c for samples (1:20,000, Southern Biotech). All plates were developed using TMB (SeraCare, Milford, MA), stopped using 1% HCl (SeraCare) and read at 450 nm using a microplate spectrophotometer (BioTek, Winooski, VT).

Human bronchial epithelial cell (HBEC) culture

Primary human bronchial epithelial cells (HBECs) from five normal donors (non-smokers and non-asthmatics) were obtained from the Marsico Lung Institute Tissue Procurement and Cell Culture Core following one passage (UNC IRB 03-1396). Cells were expanded further on collagen coated T75 plates in bronchial epithelial growth media (PneumaCult-EX Stemcell Vancouver, Canada) for one passage then moved to culture inserts (24 mm diameter, polyester 0.4 µm pore size; Costar 3450, Fisher Scientific). While submerged, the HBECs were allowed to expand to confluence then shifted to air-liquid interface (ALI) conditions. ALI cultures were maintained until the HBECs were fully differentiated as judged by the ability to visualize cilia driven movement of the mucus layer under a light microscope, typically >2 weeks. To assess the innate cytokine response, 10% (v/v) ID was applied to the apical cell surface of fully differentiated HBEC cultures from each of the five donors. Four hours later, apical washes were collected for cytokine analysis. Measurement of human cytokines was performed using IL-1α ELISA kits (R&D Systems).

Statistical analysis

GraphPad Prism version 8 was used for all data analysis. Student's *t* tests, and one- or two-way ANOVA tests were performed, and a *P* value < 0.05 was considered significant.

Figure legends

Supplemental Figure 1. A: IL-1 α production by HBECs (n=3-5 donors per ID) stimulated with ID. Comparisons are between each ID and PBS.

Supplemental Figure 2. A: Protease activity in ID and heat-treated ID. **B:** Immunoglobulin levels and PN challenge outcomes in C57BL/6J mice (n=6 per group) sensitized with inhaled PN and ID or heat-treated ID to abolish protease activity.

Supplemental Figure 3. A: Immunoglobulin levels and PN challenge outcomes in wild-type (WT) or *Clec7a*^{-/-} mice (n=6-8 per group) sensitized with inhaled PN and ID. **B:** Immunoglobulin levels and PN challenge outcomes in C57BL/6J mice (n=6 per group) sensitized to inhaled PN and either ID or glucanase-treated ID. *PN*, peanut, *ID*, indoor dust

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Table S1. Sensitization details for each experiment.

Mouse strain	Pretreatment	Treatment	Peanut	Figure
C57BL/6J	-	PN+ID1, 2, 3, or 4	500 ng	1B
C57BL/6J	-	PN+ Heated ID or β -glucanase treated ID	50 μ g	S2B, S3B
C57BL/6J or <i>Clec7a</i> ^{-/-}	-	PN+ID1	50 μ g	S3A
C57BL/6J or <i>Myd88</i> ^{-/-}	-	PN+ID1	500 ng	1C
C57BL/6J	-	PN+CpG (5 μ g), PAM (1 μ g), FLA (1 μ g), or LPS (100 ng)	50 ng	2A
C57BL/6J	-	PN+ID1+CpG (5 μ g) or LPS (1 μ g)	50 μ g	2B, 2C
C57BL/6J	CpG (3 μ g) or LPS (100 ng)	PN+ID1	50 ng	2D