Food Allergy Adjuvant and Early Immune Effects of Oral Exposure to Known and Suspected Gut Barrier Disruptors

Elena Drønen (elenakdr@outlook.com)  
Norwegian Institute of Public Health

Unni Nygaard  
Norwegian Institute of Public Health

Ellen Namork  
Norwegian Institute of Public Health

Hubert Dirven  
Norwegian Institute of Public Health

Research Article

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Abstract

**Background**: Exposure to adjuvants with a food allergen has been shown to promote sensitization and development of food allergy in animal models. Barrier disrupting capacities have been suggested to be one mechanism of adjuvant action. In this study, we investigated how gut barrier disrupting compounds affected food allergy development in a mouse model of peanut allergy. Sensitization and clinical peanut allergy in C3H/HEOuJ mice were assessed after repeated oral exposure to peanut extract together with cholera toxin (CT), the mycotoxin deoxynivalenol (DON), house dust mite (HDM) or the pesticide glyphosate (GLY). In addition, we investigated early effects 4 to 48 hours after a single exposure to the compounds, by assessing markers of intestinal barrier permeability, alarmin production, intestinal epithelial responses, and local immune responses.

**Results**: CT and DON exerted adjuvant effects on peanut allergy development assessed as clinical anaphylaxis in mice. The early markers were consistently only affected by DON, observed as increased IL-33 (interleukin 33) and thymic stromal lymphopoietin (TSLP) alarmin production in intestines and Interleukin 33 receptor ST2 in serum. DON also induced an inflammatory immune response in lymph node cells stimulated with lipopolysaccharide (LPS). HDM and GLY did not promote clinical food allergy and affected few (or none) of the early markers at the present doses.

**Conclusions**: Oral exposure to CT and DON promoted development of clinical anaphylaxis in the peanut allergy mouse model. DON, but not CT, affected the early markers measured in the present study, indicating that DON and CT have different modes of action at the early stages of peanut sensitization.

Background

The prevalence of food allergies appears to have increased in the western world over the past decades and is considered a major public health concern with considerable costs (1-4). Although the reason for this increase is largely unknown, environmental exposures and lifestyle factors have been suggested as a possible cause (5). Biological or chemical contaminants with adjuvant capacities may promote food allergy development through modulating and/or enhancing the immune response towards antigens present in food (6).

In animal models, exposure to adjuvants together with food allergens has been shown to promote, and also to be necessary, for sensitization and development of food allergy (7-9). The most commonly used adjuvant in these models, cholera toxin (CT), has also been shown to have gut barrier disrupting properties (10). Gut barrier disruptors are compounds capable of activating or damaging the intestinal epithelial barrier. This may lead to a leaky gut with increased uptake of allergens and other compounds from the intestinal lumen, into blood, resulting in more direct contact with the immune system. Furthermore, a damaged epithelial barrier may elicit responses resulting in production of endogenous danger signals such as alarmins. The cytokines interleukin 33 (IL-33), thymic stromal lymphopoietin (TSLP) and IL-25 act as alarmins released due to cell damage caused by infection or other cellular stress
These alarmins play important roles in maintaining gut homeostasis, but can also stimulate a pro-allergic microenvironment by typically activating T helper 2 and type 2 innate lymphoid cells (11-18).

The mycotoxin deoxynivalenol (DON) and house dust mite (HDM) extracts have been reported to have barrier disrupting capacities (19, 20) and to promote development of food and airway allergy, respectively, in animal models (9, 21). The HDM allergen Der p1 has been detected in human gut and been demonstrated to impair epithelial barrier both in vitro (human colonic biopsies) and in vivo (mice) (19). Presence of HDM allergen in breast milk is associated with an increased risk of atopic sensitization and respiratory allergy in children (22). Thus, we hypothesized that compounds able to affect the gut barrier integrity and function play a role in promoting development of (food) allergy. Our working hypothesis was further that compounds with allergy adjuvant potential can induce changes in barrier function early after exposure, and that markers for early changes could potentially be used to identify adjuvants promoting development of food allergy.

The objective of the present study was to investigate how known and suspected gut barrier disruptors affected food allergy development in a mouse model of peanut allergy. Sensitization and clinical peanut allergy development were assessed after repeated oral exposures to peanut extract together with one of the barrier disrupting compounds CT, DON and HDM, or the suspected barrier disruptor glyphosate (GLY). GLY has been reported to have effects on alarmin production (23) and effects on gut microbiota (24), but has unknown barrier disrupting capacities. Additionally, early effects of a single oral exposure to the compounds were investigated, including markers of intestinal integrity, alarmin production and local immune responses 4 to 48 hours after exposure.

Results

Food allergy development

The allergy-promoting capacity of the selected barrier disruptors was assessed in a food allergy mouse model. Mice were repeatedly exposed intragastrically (i.g.) to the barrier disruptors together with peanut extract (PE) as the food allergen and challenged with a high PE dose administered intraperitoneally (i.p.) to assess clinical anaphylaxis.

Anaphylaxis

After the PE challenge, clinical anaphylaxis was assessed as anaphylactic score, drop in rectal temperature and presence of mast cell protease 1 (mMCP-1) in serum. The anaphylactic scores for mice challenged with 5 mg PE were clearly higher in the groups immunized with DON+PE and CT+PE, reaching statistical significance compared to three and two of the other groups, respectively, as shown in Fig. 1A. When including both the 2.5 and 5 mg challenge doses in the statistical analysis (Fig. 1B), thus doubling the number of data points per group, the CT+PE immunized group had significantly higher scores than all other groups. The increase in the DON+PE group, however, did not reach statistical significance (Fig. 1B), suggesting that the higher challenge dose of 5 mg PE was needed to elicit a clinical anaphylaxis score.
The rectal body temperature a strong decrease in the CT+PE group reaching statistically significance compared to all other groups (Fig. 1C). Four animals in the CT+PE group had a strong anaphylactic response (Fig. 1C) and serum samples for testing of mMCP-1 levels were not possible to collect from these animals. Still, the levels of mMCP-1 in serum were statistically significantly higher in the CT+PE immunized group compared to all other groups except for the CT-group (Fig. 1D). A weak but statistically significantly elevated mMPC-1 level was also observed in the CT group, as compared to the PE group. The group exposed to CT only and the groups immunized with HDM+PE and GLY+PE did not affect the anaphylaxis endpoints.

Allergic sensitization

The levels of Ara h2 specific-IgE in serum before PE challenge were below the assay detection limit for most samples (data not shown). As expected for the positive control group, the levels of total IgE were statistically significantly increased in the CT+PE group compared to most groups, except for DON+PE and CT (Fig. 1E). The CT group also reached statistically significantly higher levels of total IgE compared to all groups except CT+PE and DON+PE. The DON+PE group showed a (non-significant) tendency of increased total IgE levels in serum. This is supported by the observation that the DON+PE group was the only group not having statistically significantly lower total IgE levels compared to the CT and CT+PE groups (Fig. 1E). As the levels of total IgE were measured in blood sampled after challenge with PE, the levels were most likely decreased as a result of the anaphylactic reaction (25, 26), supporting that sensitization was induced in the DON+PE group.

Lymphoid immune cell responses

The lymphoid immune responses were assessed as release of the cytokines IL-1β, -2, -6, -10, -13, -17, IFNγ and TNF-α from spleen cells stimulated with PE ex vivo. All cytokines, except IL-1β and TNF-α, showed statistically significantly higher levels in the group immunized with CT+PE (Fig. 2). A similar, but weak trend (statistically significantly different from the PBS group) was observed for TNF-α, while no group differences were observed for IL-1β (data not shown). None of the other groups showed any cytokine release after PE stimulation (data not shown).

Short-term experiments

In the short-term experiments (exp. 1 and 2), mice were exposed to a single i.g. dose of PBS, PE or a barrier disruptor with (exp. 1, n=5) or without (exp. 2, n=8) PE. Responses were assessed after 4, 24 or 48 hours.

Intestinal epithelial responses: IL-33, ST2, TSLP and IL-25 in duodenum and ileum

Intestinal epithelial responses were measured as the release of the alarmins IL-33 and its receptor ST2, TSLP and IL-25 in homogenates from duodenum and ileum.
IL-33 levels were increased in the DON exposed group compared to the other groups after 4 and 24 hours, although only statistically significant at 24 hours in duodenum in exp. 2 with PE (Fig. 3D) and at 4 hours in ileum exp. 1 without PE (Fig. 3I). Exposure to CT alone only statistically significantly affected the level of IL-33 in ileum after 4 hours exp. 1 (Fig. 3K). For the other compounds tested, no statistically significant differences in levels of IL-33 were detected in duodenum, ileum or for any of the time-points (Fig. 3A, B, C, J and L).

The levels of the alarmin TSLP in duodenum were increased in the DON group after 4 hours (Fig. 3E and G). The TSLP levels showed no statistically significant differences in duodenum after 24 hours (Fig. 3F and H), or in ileum between any groups at any time point (Fig. 3M, N, O and P).

No clear effects of any of the compounds were observed for the soluble IL-33 receptor, ST2, for any time points in duodenum (data not shown). In ileum, the levels of ST2 were below the detection limit in both experiments.

A statistically significant difference was detected for IL-25 levels in duodenum between the DON+PE group and the HDM+PE group 48 hours after exposure, while no other effects of IL-25 concentrations in the intestines were detected after exposure to any of the compounds tested (data not shown).

ST2 in serum

Exposure to DON with PE resulted in statistically significantly increased levels of the IL-33 receptor ST2 in serum after 4, but not after 24 hours (Fig. 4A and B). ST2 levels after exposure to DON without PE were significantly increased both after 4 (only compared to the PE exposed group) and 24 hours (all groups except HDM) (Fig. 4C and D, respectively). No effects were seen for the other compounds tested, or for any components 48 hours after exposure (data not shown).

FABP2 and Ara h2 in serum

Serum levels of fatty acid-binding protein 2 (FABP2) and peanut allergen component Ara h2 were measured as markers for gut barrier permeability. After a single exposure to the compounds with PE in exp. 1, the serum levels of FABP2 were low (data not shown), and levels of Ara h2 were below the detection limit and therefore not analyzed in exp. 2.

Lymphoid immune cell responses

The lymphoid responses were measured as cytokine release from immune cells stimulated with LPS or Concanavalin A (ConA) \textit{ex vivo}. In supernatants from LPS-stimulated mesenteric lymph node (MLN) cells, the cytokines TNF-\(\alpha\), IFN\(\gamma\), IL-1\(\beta\) and IL-6 were analyzed. The groups exposed to DON, both with or without PE, showed increased levels of TNF-\(\alpha\), IL-6 and IFN\(\gamma\) after 4 hours in both experiments (Fig. 5A-F).

IFN\(\gamma\) also showed a strong and statistically significant increase 24 hours after DON exposure, but not 24 hours after exposure to DON with PE (Fig. 5H and G). No statistically significant group differences were
observed for TNF-α and IL-6 at the two other time-points in any of the two experiments, except a trend for elevated TNF-α levels after 48 hours (Kruskal-Wallis test p-value of 0.0159, data not shown). The levels of IL-1β were below the detection limit in exp. 1 and low in exp. 2, but the group exposed to DON showed statistically significantly higher levels of IL-1β 4 hours after exposure (data not shown).

The cytokine concentrations in supernatants from LPS-stimulated Peyer’ patches (PP) cells (assessed only in exp. 2) 24 hours after exposure were low and fluctuated close to the detection limit for all four cytokines. No statistically significant group differences were observed (data not shown).

ConA-stimulated MLN and PP cells

In supernatants from ConA-stimulated MLN cells, the cytokines IL-5 and 13 were analyzed in both experiments, as well as IL-10, -17 and IFNγ in exp. 1 and IL-1β, -2, -4, and -6 in exp. 2. With two exceptions, no statistically significant group differences were seen at any time point in any of the experiments (data not shown). The exceptions were an overall significant increase for the levels of IL-5 at 24 hours after exposure, and at 48 hours after exposure for IL-17 (p-values of 0.0245 and 0.0374 from the Kruskal-Wallis test, respectively). No statistically significant pairwise group differences were found. The levels of IL-4 and IL-1β were both below the assay detection limit.

**Discussion**

By assessing effects of known and suspected barrier disruptors, we found that CT and DON exerted an adjuvant effect on development of peanut allergy, while HDM and GLY did not show convincing effects at the tested doses. Increased anaphylactic score and a trend of increased levels of total IgE in the DON+PE group indicate that DON may promote development of peanut allergy in our model. This is supported by the finding that oral DON exposure acted as an adjuvant in a mouse model of whey allergy reported by Bol Schoemakers et al. (9). Our results suggest that CT was a stronger adjuvant than DON, since doses of CT were much lower (15 µg CT versus 100 µg of DON), also in agreement with Bol-Schoenmakers et. al (9).

Regarding early effects, the most prominent results were seen in the groups exposed to DON, with or without the PE allergen. Levels of the alarmins IL-33 and TSLP in intestinal tissue, the IL-33 receptor ST2 in serum, and the immune response markers TNF-α, IL-6 and IFNγ released from LPS-stimulated MLN cells were all increased in DON-exposed groups 4 or 24 hours after a single exposure in both short-term experiments. Few of these markers were convincingly affected after a single CT exposure. HDM and GLY did not clearly affect any of these markers. Both DON and CT promoted development of clinical anaphylaxis in the peanut food allergy model, but only DON showed a clear pattern of effects on the early markers (short-term experiments). Thus, the present study indicates that the underlying mechanisms of adjuvanticity of CT and DON differ.

Considering the different short-term effects after exposure to DON and CT, it is of interest to note that the two groups also responded differently after PE challenge. Strong and weak effects of DON and CT,
respectively, on intestinal alarmin production were also reported in the whey allergy model by Bol-Schoenmakers et al (9). They also reported that CT most strongly affected other early markers such as upregulation of costimulatory molecules and MHC class II in MLN cells, supporting our suggestion of different mechanistic pathways adjuvant activity of DON and CT.

As alarmins rise in response to damaged epithelial cells (11, 12), it could be anticipated that the levels of alarmins in the intestine would rise early after exposure to barrier disruptors and drive pro-allergenic responses. In both short-term experiments, the intestinal levels of IL-33, but not IL-25 or ST2, were increased after DON exposure. Together with the simultaneous increase in serum levels of ST2, the soluble receptor IL-33, suggest that DON exert effects on the IL-33 pathway. TSLP levels were weakly (non-significantly) but consistently increased in both experiments 4 hours after DON exposure. Our findings are in agreement with earlier studies suggesting that IL-33 play a central role, while TSLP seem to be associated with, but being non-essential, during allergen sensitization (27, 28).

MLN cells were stimulated with ConA or LPS to activate T cells or B cells and monocytes, respectively. ConA-induced cytokine release from MLN cells were not affected by treatment with any of the compounds. This may be due to the short time periods studied after exposure (4 -48 hours), as lymph node responses in other allergy models have been shown to be at its maximum 5-8 days after immunization with an allergen together with an adjuvant (29). However, we observed that MLNs stimulated with LPS were more sensitive to reflect responses at these early time points, and induced strong TNF-α, IL-6 and IFNγ production in cells from DON-exposed mice. Inflammatory cytokines may promote allergic sensitization, as demonstrated for TNF-α, acting as an allergy adjuvant in airway models (30). Taken together with the observed increase of alarmins in the intestines after exposure to DON and previous reports of DON-induced inflammation in the intestines (31), this suggests that early stage inflammation may have contributed to the observed adjuvant effect of DON on food allergy development.

Exposure to HDM or GLY did not show adjuvant effects on food allergy development or effects on the early markers in the present experiment. However, HDM allergens have demonstrated allergy-promoting capacities for airway allergic disease (21, 27, 32). To our knowledge, we are the first to investigate HDM adjuvant potential after oral exposure, inspired by the observations of HDM presence in human gut and breast milk and Der p1s capacity of epithelial barrier disruption (19, 33). Our data showed that two of the twelve mice exposed to HDM+PE had high anaphylactic scores after PE challenge. One of these mice had strongly elevated mMCP-1 levels in serum and a dramatic drop in body temperature, while the other had a strongly increased production of PE-induced inflammatory cytokines TNF-α, IL-6 and IFNγ. No significant effects were, however, detected on group level, although the group median value for the anaphylactic score seemed to be somewhat increased. From the present and previous studies in food allergy mouse models, we have experienced that high and low responders exist, even within positive control groups receiving CT with allergen (9, 34-36). Although the two responding mice may have been cases of natural variability, our data may indicate that HDM have the capacity to act as an allergy adjuvant after oral exposure. The HDM groups did not demonstrate any clear effects, and the adjuvant capacity of HDM in
intestine and food allergy development remains speculative. However, further investigations covering a dose-range of HDM in the gut is encouraged.

As Ara h2-IgE levels were below the detection limit and PE-specific IgE could not be detected due to methodological challenges (see methods section), total IgE was the only parameter reflecting sensitization in the present experiment. In addition to the effects on the total IgE in the CT+PE and DON+PE groups, we also observed a trend of elevated levels in the group exposed to CT alone. This may suggest that the CT exposed mice had become sensitized to proteins such as soy, wheat or barley in their feed, as this was the only source of food proteins in the group exposed to CT without PE. This is supported by the weak but statistically significantly elevated serum levels of mMCP-1 and a tendency of a temperature-drop in this group. A cross-reaction between sensitization to soy in the feed and PE as the challenging food allergen may have contributed to these observations (37-39).

Considerations such as reducing the number of animals, and practicalities such as workload, influenced the experimental design. Thus, screening several markers of endpoints such as intestinal permeability, alarmin production and local immune responses, were favored over choosing to administer more than one dose of the barrier disruptors. Consequently, lack of different doses of the barrier disruptors is one of the limitations of the present study.

Conclusions

Among the known and suspected barrier disrupting compounds tested in the present mouse model, CT and DON acted as adjuvants by promoting development of peanut food allergy. Only DON affected the investigated early markers, by activating an intestinal epithelial (IL-33 and ST2) and an inflammatory response in lymph node cells. As both DON and CT were found to promote clinical food allergy, but the early markers were only clearly affected by DON, our results suggest that DON and CT have different modes of action at the early stages of sensitization. In general, little is known about how adjuvants or barrier disruptors may influence or modulate food allergy development. A combination of a wider set of dose-ranges and markers reflecting different modes of action is necessary to predict compounds with food allergy adjuvant capacity. As DON is a common mycotoxin present in grains such as wheat and oat, human exposure to DON through daily life may represent a real-life contributor to food allergy development.

Methods

The allergy-promoting capacity of the selected barrier disruptors was assessed in a food allergy mouse model where mice were repeatedly exposed to the barrier disruptors together with a food allergen. Two short-term mouse experiments were also carried out, to investigate the early effects on the gut barrier after a single exposure to the same barrier disruptors with (exp. 1) or without (exp. 2) the allergen.
The levels of the alarmins TSLP, IL-25 and IL-33 and the IL-33 receptor ST2 were determined in intestinal tissue homogenate supernatants (see below). ST2 was also analyzed in serum, as a measure of altered barrier function (40, 41). As markers of altered intestinal barrier permeability, serum concentrations of the stable peanut allergen Ara h2 and FABP2 were examined (42). As markers of lymphoid tissue immune responses, the cytokines TNF-α, IFNγ, IL-1β, -2, -4, -5, -6, -10, -13, and -17 were assessed after ex vivo stimulation of cells from the mesenteric lymph nodes (MLNs), Peyer’s patches (PP) or spleen. Cells were stimulated with ConA to activate T cells or LPS to activate B cells and monocytes.

Test solutions

We tested the environmental contaminants with known or suspected barrier disrupting capacities, cholera toxin (CT), deoxyvalenol (DON) and house dust mite (HDM) (9, 10, 21). We defined CT, DON and HDM as known barrier disruptors, and the pesticide Glyphosate (GLY) as a suspected barrier disruptor. The doses for CT (EMD Biosciences Inc., San Diego, CA, USA), DON (Sigma Aldrich, St. Louis, MO, USA), HDM (Stallergenes Greer, London, UK), and GLY (SUPELCO, Bellefonte, PA, USA), were all based on literature where exposure to these doses gave biological effects (doses and references are stated in Table 1). Phosphate buffered saline (PBS, 10 mM 0.9% NaCl pH 7.4) was given to groups as negative control and was used as vehicle. Peanut extract (PE, Stallergenes Greer, London, UK) was used as the allergen, as peanut is one of the most potent food allergens and a widely used ingredient in foods.
<table>
<thead>
<tr>
<th>Test compounds and dose per animal (in 250 µL PBS)</th>
<th>Effects demonstrated at this dose level in earlier studies</th>
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<tbody>
<tr>
<td><strong>exp. 1</strong></td>
<td><strong>exp. 2</strong></td>
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<tr>
<td><strong>PBS: vehicle ctr.</strong></td>
<td>PBS</td>
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<tr>
<td><strong>PE wo adjuvant: neg. ctr.</strong></td>
<td>1 mg PE</td>
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<tr>
<td><strong>CT: pos. adjuvant ctr.</strong></td>
<td>15 µg CT</td>
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<td></td>
<td>1 mg PE</td>
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<tr>
<td><strong>DON</strong></td>
<td>100 µg DON</td>
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<td></td>
<td>1 mg PE</td>
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<tr>
<td><strong>HDM</strong></td>
<td>20 µg HDM</td>
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<td></td>
<td>1 mg PE</td>
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<tr>
<td><strong>GLY</strong></td>
<td>20 µg GLY</td>
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<td></td>
<td>1 mg PE</td>
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<tr>
<td><strong>CT wo allergen: neg. ctr.</strong></td>
<td>15 µg CT</td>
</tr>
</tbody>
</table>

*single exposure
# repeated exposures
§Roundup® herbicide contains approximately 40% GLY (Monsanto Company, St. Louis, MO, USA).

Abbreviations: Pos positive, Neg negative, ctr control, wo without

Animals
Female C3H/HeOuJ mice were purchased from Charles River Laboratories (Sulzfeld, Germany), and Jackson Laboratory (Bar Harbor, ME, USA) for exp. 2 due to supply issues. The mice were 4 to 5 weeks old at arrival and randomly allocated in groups and housed on Nestpack bedding (Datesand Ltd, Manchester, UK) at room temperature of 21±2°C, humidity of 55±10 % and exposed to 12 hours light/dark cycle. The mice were given pelleted feed (RM1, SDS, Essex, UK) and tap water ad libitum, and were acclimatized for at least one week before dosing. The experiments with live animals were performed in conformity with the laws and regulations in Norway and were approved by the Experimental Animal Board under the Ministry of Agriculture in Norway (FOTS ID 11121).

Food Allergy Model

Immunization, challenge and anaphylaxis assessment

The present peanut food allergy model was based on the mouse model for lupine allergy (Vinje, Larsen (8), and the mouse model for peanut anaphylaxis developed by Li, Serebrisky (7). Twelve mice in each of the 7 groups were immunized i.g. at day 0, 1, 2, 7, 21, 28 and 35 with PE alone or mixed with the different barrier disruptors, including a group given CT alone. CT with PE was used as a positive control for food allergy development as it has strong adjuvant effects (46), while negative control groups were the vehicle-exposed (PBS) and non-sensitized mice (PE or CT alone).

A challenge of a high dose of PE was given at the end of the experiment to induce anaphylaxis and thereby to confirm clinically relevant signs of food allergy. Due to practical considerations, half of the mice in each group were challenged at day 42 and 43. Prior to challenge, the mice were weighed, 100 µl blood sampled from the vena saphena in Microvette tubes without heparin (Sarstedt, Nümbrecht, Germany) and serum stored at -20°C for detection of Ara h2 specific IgE (see below). The mice were challenged with an intraperitoneal (i.p.) injection of 5 mg PE in 250 µL of PBS (7). Due to dilution errors on one of the days, challenge with 2.5 mg PE in 250 µL PBS was given to approximately half of each groups of mice. Temperature measurements (at 0, 15 and 30 minutes) and clinical anaphylaxis score were recorded and reported as previously described (35). After 30 minutes, all challenged mice were exsanguinated, and serum was collected. We did not manage to collect serum from four and two mice in the PE+CT and GLY+PE groups, respectively. Serum was prepared and stored at -20°C until detection of total IgE and the anaphylaxis marker mMCP-1 (see below). Spleens were collected and kept in HBSS containing 2 % fetal bovine serum and 1 % penicillin/streptomycin on ice.

In blood collected after challenge and exsanguination, levels of mMCP-1 were determined using mouse CPT-1 (mMCP-1) ELISA kit according to the manufacturer’s protocol (ELISA Ready-SET-Go! ® Invitrogen by Thermo Fisher).

Spleen cell preparation, PE stimulation and detection of released cytokines

Spleen yields an abundant cell number compared to LNs and were chosen to assess expression of cytokines reflecting systemic changes to the immune system reported in food allergy models (7, 8). Cells
counts are presented as the total cell number \(10^6\) per animal. Cell-suspensions were adjusted to \(3 \times 10^6\) cells/mL in culture medium (RPMI 1640, Gibco®, Thermo Fisher Scientific) containing 10% FBS and 1% penicillin/streptomycin. In a total volume of 200 µl per well in Microtest plates (Microtest Plate 96 Well, C, Sarstedt). Spleen cells were stimulated for 5 days \textit{in vitro} with PE at a final concentration of 1 mg/mL to assess the cytokine responses mainly in allergen-specific cells. After incubation at 37 °C in 5% CO\(_2\) for 5 days, the plates were centrifuged at \(180 \times g\) for 5 min at RT and the supernatants were collected and stored at \(-80\ °C\).

After thawing, supernatants were collected and stored, and TNF-α, INF-γ, IL-1β, -2, -6, -10, -13, and -17 cytokine concentrations were detected using BD Cytometric bead array (CBA) Mouse Soluble Protein Flex Sets, measured on a BD LSR II flow cytometer. The cytokines were analyzed using the FCAP Array software (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

Measures of peanut sensitization – total IgE and Ara h2-specific IgE

As described for other food allergen extracts (35), development of ELISAs for PE-specific IgE was challenging (unpublished data) due to high background signals from the PE extract and costs if combining all recombinant peanut allergens. Thus, as predictors of peanut allergy sensitization, the levels of the peanut component Ara h2 specific IgE and total IgE in serum were analyzed. IgE specific to the most important peanut allergen Ara h2 is considered a reliable marker of severe peanut allergy in humans (47, 48). Total IgE are reflecting specific IgE in animal models with controlled allergen exposures (35, 49).

To assess sensitization, Ara h2-specific IgE was assessed in serum samples collected immediately prior to challenge, since specific IgE passes from the blood to the tissues during anaphylaxis. The ELISA assay was developed in our lab. 100 µL of monoclonal rat anti-mouse IgE (2 µg/mL, Experimental Immunology unit, University of Louvain, Brussels, Belgium) in 0.05 M bicarbonate buffer (pH 9.6) was added in each well plate (Nunc MaxiSorp™ flat-bottom 96 well plate, Thermo Fisher Scientific) and incubated 1 hour at 21 °C following an over-night incubation at 4°C. The plates were then washed five times with TBS/Tween (50 mM Tris/HCL-buffer pH 8.0 with 0.05% Tween 20) using an automatic plate washer (405 LS microplate washer, Biotek), blocked with 300 µL of 4% BSA (Bovine Serum Albumin A7930-100G, Sigma Aldrich) in PBS (BSA/PBS) and incubated for 1 hour at 21°C. Plates were washed as previously described. 100 µL of diluted Ara h2 specific IgE from a mouse serum pool containing anti-Ara h2 from PE-immunized mice previously prepared in the lab (The Norwegian Institute of Public Health) was added as doublets for a standard curve. Dilutions were 1:1, following a 2-fold titration to 1:64 in 4% BSA/PBS. 4% BSA/PBS in doublets were added as blanks. Serum samples were diluted 1:5 with 4% BSA/PBS. 100 µL of diluted serum sample was added per well and incubated at 4°C over-night. Plates were then washed as earlier described and 100 µL of biotin-labeled Ara h2 (3 µg/mL, Biotin Natural Ara h2, B1-AH2-1, Indoor Biotechnologies) in 4% BSA in PBS was added. Plates were incubated 1 hour at 21 °C and then washed. Poly-HRP-strepatividin (Poly-HRP-strepatividin N200, Thermo Fisher Scientific) was diluted 1:40 000 in 4% BSA/PBS and 100 µL added to each well. Plates were incubated for 1 hour at 21 °C, washed, before addition of 100 µL of TMB solution (TMB Stabilized Chromogen SB01, Life Technologies Europe B.V.,
Bleiswijk, Netherlands) and incubated at RT for 15 minutes. The reaction was stopped by 50 µL of sulfuric acid 95-97% (Merck Milipore, Burlington, MA, USA) in each well. Plates were read at 450 nm in a BioTek microplate-reader.

Serum levels of the allergic sensitization marker total IgE was determined using IgE Mouse uncoated ELISA kit according to the manufacturer’s protocol (Invitrogen by Thermo Fisher Scientific). This was assessed in serum collected after challenge and exsanguination due to limited amounts of serum from the sample collected before challenge.

Short-term experiments

Experiment 1: early effects 4, 24 and 48 hours after a single exposure

Five groups of mice, 5 in each group, were exposed once by intragastric (i.g.) gavage to 250 µL of test solutions according to table 1. At 4, 24 and 48 hours after exposure, the mice were anesthetized with isoflurane gas administered as a 3.5% mixture with medical O₂ in a coaxially ventilated open mask and exsanguinated by heart puncture before cervical dislocation. The collected blood samples were kept for 1-3 hours at RT before the samples were prepared and stored at – 80 °C until further use.

The mesenteric lymph nodes (MLNs) were excised and kept on ice in 5 mL Meinecker tubes with 1 mL HBSS (Hanks’ Balanced Salt Solution, Gibco® by Thermo Fisher Scientific, Waltham, MA, USA) containing 2% fetal bovine serum (FBS superior, Biochrom, Cambridge, UK) and 1% penicillin/streptomycin (10 000 units/mL penicillin, 10 mg/mL streptavidin, PAA Laboratories Inc, Etobicoke, ON, Canada), to ensure cell viability.

The small intestine was excised, and the duodenum and ileum were placed on cold metal plates to minimize degradation of proteins. The duodenum was defined as the top 7 cm of the small intestine closest to the stomach and the ileum as the lower 7 cm closest to the caecum. Duodenum and ileum were flushed thoroughly with cold PBS using a syringe, and visceral adipose tissue was removed. Two 1 cm sections from each of the 7 cm segments were snap frozen in 1.8 ml cryo tubes in liquid nitrogen and stored at – 80 °C until homogenization.

Homogenization of intestinal tissue

Immediately after thawing the intestinal samples, the 1 cm pieces of the 2 cm intestinal segments were weighed in 1.5 mL tubes (Micro Tubes, Brand Scientific Equipment Pvt. Ltd., Wertheim, Germany). The weights of 1 cm duodenum and ileum were noted and 0.6 mL of RIPA lysis buffer with 0.04% protease-inhibitor was added to the tubes to avoid the tissue from drying and to reduce protein degradation (9).

The tissue samples were transferred to 2 mL CK28-R tubes containing ceramic beads (Bertin Instruments) and homogenized using Precellys 24 tissue homogenizer (Bertin Instruments) at 6000 rpm (2x30 seconds, 30 seconds pause). After homogenization, the tubes were left to cool on ice for up to 30 minutes
to diminish frothing. Samples were centrifuged at 1970 x g for 5 minutes, and the supernatants collected and stored at −80 °C until analysis.

ELISA analysis

Samples from serum or tissue homogenate supernatants were analyzed using ELISA kits according to the manufacturer’s protocol; TSLP, IL-25, IL-33, and the IL-33 receptor ST2 (Invitrogen, Thermo Fisher Scientific), FABP2 (MyBioSource, San Diego, CA, USA) and Ara h2 (Indoor biotechnologies, Charlottesville, VA, USA). The Ara h2 concentration was determined using an anti-peroxidase-conjugated Fab fragment for detection (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Optical density (OD) values were measured using a Microplate Reader (Elx808 Absorbance Reader, BioTek, Shoreline, WA, USA) with software Gen5™ (BioTek). Protein concentrations were determined based on a standard curve generated on each microwell plate provided in the kits. For results from the intestinal homogenates, all concentrations were normalized by dividing by the respective tissue weight.

Lymph node cell preparation, stimulation and determination of cytokine release

Preparation of MLNs was performed as described for spleen cells. To assess the cytokine responses, MLN cells were stimulated for 48 hours with either 5 µg/mL ConA (Concanavalin A from Canavalia ensiformis, Sigma-Aldrich), or 10 µg/mL LPS (lipopolysaccharides from Escherichia coli 026: B6, Sigma-Aldrich), or culture medium alone. Supernatants were collected and stored, and TNF-α, IFNγ, IL-1β, -5, -6, -10, -13 and -17 cytokine concentrations were determined as described in the food allergy model.

Experiment 2: effects 4 and 24 hours after a single exposure

In exp. 2, mice were exposed to the same test-solutions as in exp. 1, as well as the pesticide GLY, but without co-exposure to PE (Table 1). All adjustments and changes in exp. 2 were based on results from exp. 1. Since few exposure-related changes were observed at the time-point 48 hours, only the two earliest time-points 4 and 24 hours were chosen. The number of mice was increased to 8 per group at each time-point.

Furthermore, the whole length (7 cm) of intestinal tissue was used for homogenization. In addition to MLNs, up to seven PPs, primarily sampled from the ileum were collected, but also from the jejunum and duodenum if needed. PPs from each group were pooled, processed and prepared as described for MLNs. Cytokine release from stimulated MLNs and PPs were determined as described for exp. 1. However, since the levels of the cytokines IL-17, -10 and -5 were below the detection limit in exp. 1, these were excluded in exp. 2, while assessment of IL-2 and the Th2 cytokine IL-4 was included.

Due to low intestinal levels of FABP2 and Ara h2 and low levels of IL-25 in both intestines and serum samples in exp.1, these analyzes were excluded in exp. 2. All ELISA analyzes in exp. 2 were incubated overnight on microwell plates at +4°C to increase the sensitivity.

Statistical analysis
For most of the endpoints, the assumptions of equal variance and a normality distribution in our data sets were violated, thus non-parametric statistics were used: A Kruskal-Wallis test was initially performed, and if a p-value $\leq 0.05$ was detected, we conducted a Dunn's multiple comparison test to identify which groups were different. Correlation of anaphylactic score and body temperature drop in the food allergy model was performed by Spearman rank-order correlation test. All analyzes were performed in GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA).

**List Of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ara h2</td>
<td><em>Arachis hypogaea</em> 2 (a major peanut allergen)</td>
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<tr>
<td>Ara h2-IgE</td>
<td>Ara h2 antibody specific IgE</td>
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<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
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<tr>
<td>Der p1</td>
<td><em>Dermatophagoies pteronyssinus</em> 1 (a house dust mite allergen)</td>
</tr>
<tr>
<td>DON</td>
<td>Deoxynivalenol</td>
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<tr>
<td>FABP2</td>
<td>Fatty acid-binding protein 2</td>
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<tr>
<td>GLY</td>
<td>Glyphosate</td>
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<tr>
<td>HDM</td>
<td>House dust mite</td>
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<tr>
<td>I.g.</td>
<td>intragastrical</td>
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<tr>
<td>I.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
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<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IL-...</td>
<td>Interleukin ...</td>
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<tr>
<td>LN</td>
<td>Lymph nodes</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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<tr>
<td>MLN</td>
<td>Mesenteric lymph nodes</td>
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<tr>
<td>mMCP-1</td>
<td>mast cell protease 1</td>
</tr>
<tr>
<td>PE</td>
<td>Peanut extract</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer's patches</td>
</tr>
<tr>
<td>ST2</td>
<td>suppression of tumorigenicity 2 (Interleukin 33 receptor)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
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Declarations

Ethics approval statement

The study was approved by the Experimental Animal Board under the Ministry of Agriculture in Norway (FOTS ID 11121). The experiments were performed in conformity with the laws and regulations for experiments with live animals in Norway.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Competing interests

None of the authors have any competing interests to report.

Funding

The experiments were funded by the Norwegian Institute of Public Health.

Authors’ contributions

EKD performed the animal experiments, and all laboratory assays with contributions from EN and UCN and prepared the manuscript. EN and UCN were major contributors in the design of the experiments and the writing of the manuscript. HD contributed to the design of the experiment. All authors read and approved the final manuscript.

Acknowledgments

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References


44. Jasper R, Locatelli GO, Pilati C, Locatelli C. Evaluation of biochemical, hematological and oxidative parameters in mice exposed to the herbicide glyphosate-Roundup((R)). Interdiscip Toxicol.


47. Koppelman SJ, Wensing M, Ertmann M, Knulst AC, Knol EF. Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2004;34(4):583-90.


**Figures**
Figure 1

Anaphylaxis and sensitization after repeated immunizations with peanut extracts. A) Anaphylactic score after i.p. challenge of 5 mg PE (PBS, PE, PE+DON, PE+GLY, CT, all n=8), CT+PE and PE+HDM (n=4). B) Anaphylactic score after i.p. challenge of 2.5 or 5 mg PE (n=12). C) mMCP-1 concentrations in serum collected 30 minutes after PE challenge (PBS, PE, PE+DON, CT, all n=12), PE+CT (n=8), GLY+PE (n=10). Dotted line represents the upper assay detection limit. D) Temperature-change over the first 30 minutes.
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**Figure 2**

Cytokine release from PE-stimulated spleen cells ex vivo. Mice were repeatedly immunized and received a PE challenge (n=12), followed by spleen cell harvesting. Brackets denote statistically significant differences (*p<0.05, **p<0.001) between two groups. Columns denote the group median value while each symbol denote individual animals.
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IL-33 and TSLP concentrations per mg tissue in duodenum and ileum from mice. Figure shows levels at 4 and 24 hours after a single i.g. dose of PBS, PE or a barrier disruptor with (exp. 1, n=5) or without (exp. 2, n=8) PE. Brackets denote statistically significant differences (*p<0.05, **p<0.001) between two groups. Columns denote the group median value while each symbol denotes the individual animals. Inserted box: p value for the Kruskal-Wallis test.
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