Do environmental contaminants with barrier disruptor capacities promote food allergy development?

Detecting early markers of an altered gut barrier function

Elena Klåpbakken Drønen

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Department of Biosciences
The Faculty of Mathematics and Natural Sciences

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Abstract

Food allergy is considered a major public health concern. Why and how some people develop allergic disease is largely unknown, but exposure to environmental contaminants with barrier disruptor effects has been linked to allergy development. We hypothesize that barrier disruptors alter the gut barrier function in such a way that food allergy development is promoted, either by increasing the permeability and uptake of allergens, or, by activating intestinal epithelial responses and immune responses.

This project was conducted to assess the role of several barrier disruptors in food allergy development, with the aim to identify markers of early effects on the gut barrier for later use in developing methods for screening environmental contaminants for immunotoxic effects. The barrier disruptors cholera toxin (CT), deoxynivalenol (DON), house dust mite (HDM) and glyphosate (GLY) were included.

Early effects of exposure to barrier disruptors were assessed in two short-term mouse experiments. We investigated markers of intestinal epithelial responses (IL-33, ST2, IL-25, TSLP), altered gut barrier integrity and/or permeability (tight junction proteins, IL-22, FABP2, Ara h 2) and mucosal (total IgA) and lymphoid immune responses (proinflammatory responses TNF-α, IL-6, IFNγ, IL-1β, IL-17 and pro-allergy responses IL-13, IL-10, IL-5, IL-4, IL-2). Food allergy development was investigated in a food allergy mouse model, assessed by anaphylaxis (clinical score, temperature-drop and mMCP-1), allergic sensitization (Ara h 2 specific IgE and total IgE) and lymphoid immune responses (cytokine profile from allergen-stimulated spleen cells).

DON and CT, but not HDM and GLY, had adjuvant effects in the food allergy experiment, with CT giving the strongest response. We detected an activated intestinal epithelial response (increased levels of IL-33 and ST2) and inflammatory immune responses in lymph nodes (increased levels of TNF-α, IL-6, IFNγ and IL-1β) as early effects of exposure to DON. Our results suggest that DON and CT may have different modes of action regarding effects on barrier function and adjuvanticity, as both showed adjuvant effects in food allergy development, but effects on the present early markers were detected only for DON.
Forord


Jeg også vil også takke familien min, en stor heiagjeng som til stadighet lurer på hva jeg egentlig holder på med, min skjønne nevø på to år som har blåst liv i min til tider litt slitne studentsjel, og selvfølgelig, mine gode studievenner. Hva hadde årene på Blindern vært uten dere? Rett og slett en trist tilværelse. Takk for alle oppdagelsesferdene vi har hatt i biologibøkene og ute i den virkelige verden. Rike, thank you for your unconditional support, encouragement and help. Og ikke minst, takk til min kjære Tore som har stått ved min siste hver eneste dag gjennom mine hittil mest lærerike, spennende og utfordrende år.

Elena Klåpbakken Drønen

Oslo, september 2018
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1 Introduction

1.1 Food allergy

Food allergy is a major public health concern that can affect individuals from all age groups. Clinical symptoms of allergic reactions are versatile and range in severity from mild or moderate to life-threatening anaphylaxis. Symptoms of food allergic reactions may be expressed in the skin, respiratory tract and/or gastrointestinal tract (GI-tract) and include itching, skin rashes, swelling, edema, and respiratory problems. Food allergy can be a serious condition and often impairs quality of life of patients and their families (Greenhawt, 2016).

An allergic reaction to food is an overreaction by the immune system to a food antigen (the food allergen) that is non-harmful to most individuals, but for unknown reasons cause adverse reactions in some individuals (Janeway, Murphy, Travers, & Walport, 2008). In the sera of allergic patients, allergen-specific immunoglobulin type E antibodies (IgE) can be detected. The detection of food specific IgE is a useful tool for diagnosing allergy and for identifying the triggering food allergen. This type of allergic reaction is referred to as IgE-mediated food allergy and is the focus in this project.

The prevalence of food allergy appears to have increased in the western world over the past decades (Allen & Koplin, 2012; Gupta et al., 2011; Koplin, Mills, & Allen, 2015; Sicherer & Sampson, 2014). Although the reason for this increase is unknown, there is a possible link to environmental exposures and lifestyle factors (Yoo & Perzanowski, 2014). Environmental impacts on the gut, such as exposure to contaminants through the diet, may contribute to the development of food allergy. Some environmental contaminants may have adjuvant effects, meaning that they modify the immune response towards an antigen and subsequently enhance the magnitude of the response (Janeway et al., 2008). As exposure to adjuvants together with food allergens have been shown to promote food allergy development in animal models (Bol-Schoenmakers et al., 2016; Li et al., 2000; Vinje, Larsen, & Lovik, 2009), possible adjuvant effects of environmental contaminants should be investigated.
1.2 Food allergy development

There are two stages in the development of IgE-mediated food allergy: a sensitization phase and an elicitation phase. The sensitization phase is non-symptomatic. Here, the individual is introduced to food allergens. The food allergen is internalized, processed, and presented by antigen presented cells (APCs) to T cells that recognize a specific part of the food allergen (Figure 1 A-B). Subsequently, food allergen-specific T helper cells (Th cells) becomes activated by the APCs and upregulate costimulatory molecules on their cell surfaces, and production of cytokines (Figure 1 C), which are secretory proteins important in cell signaling. In turn, activated Th cells activates allergen-specific B cells via receptor-ligand interactions and cytokine secretion, in which B cells develop into plasma cells that produce allergen-specific IgE antibodies (Figure 1 D-E). IgE binds to receptors on cells, such as mast cells and basophils (Figure 1 F).

An allergic response can occur quickly after exposure to the allergen, which is due to the receptor-bound IgE antibodies. When a food allergen is encountered after the individual has become sensitized, the binding of the allergen to IgE results in an immediate response with release of effector molecules (histamine and cytokines), causing allergic symptoms (Figure 1 G-H). This phase is called the elicitation phase.

Figure 1. Sensitization and elicitation to a food allergen in the intestine. A) Food allergens are taken up across the gut barrier by a microfold (M) cell. B) An APC takes up, processes and presents allergen peptides to an allergen-specific T cell. C) The T cell is activated by the APC and upregulates costimulatory molecules and secretes cytokines. D) An allergen-specific B cell is activated by a T cell. E) A B cell differentiates into a plasma cell secreting IgE. F) IgE binds to high affinity receptors on the surface of effector cells (mast cells or basophils). G-H) Encounter with the allergen after sensitization results in the release of effector molecules such as histamine and cytokines, leading to allergic symptoms.
Th cells aid in the activation of a range of immune cells, such as B cells. There are several subsets of Th cells, and each subset can affect how an immune reaction evolves, depending on what cytokines are secreted. Key subsets are type 1 helper T cells (Th1 cells), which promote cellular responses towards intracellular pathogens such as bacteria and viruses, and type 2 helper T cells (Th2 cells), which promote humoral responses towards extracellular pathogens such as parasites (Janeway et al., 2008; Romagnani, 2000). That is, whilst cytokines produced by Th1 cells evoke cell-mediated immune responses, cytokines produced by Th2 cells promote antibody responses, in particular IgE responses. Hence, Th2 cells play a key role in allergy development. Th2 cells secrete specific cytokines such as interleukin 4 (IL-4), IL-5, IL-10 and IL-13. These cytokines are known to drive allergic immune responses by promoting B cells to produce IgE antibodies (Hammad & Lambrecht, 2015). Cytokines typically secreted by Th1 cells are IFNγ, which is known to promote inflammatory immune responses, and IL-2, which is important for the proliferation of T cells. Other inflammatory cytokines associated with the acute phase of an immune response are TNF-α, IL-6 and IL-1β (Akdis et al., 2016).

Other cells with roles in allergy development are Th17 cells, regulatory T cells (Treg cells), and type 2 innate lymphoid cells (ILC-2 cells), all of which demonstrate the complex interplay of immune cells in allergic disease. Th17 cells are characterized by their secretion of IL-17, a cytokine known to play a role in chronic, allergic inflammatory states (Nakae et al., 2002). Treg cells produce cytokines that may promote immunosuppression and tolerance, such as IL-10 (Akdis et al., 2016), thus preventing a detrimental immune response towards food antigens. ILC-2 cells are a group of cells which also play important roles in food allergy development (Noval Rivas, Burton, Oettgen, & Chatila, 2016), and their roles in were largely unknown until recently (Lund, Walford, & Doherty, 2013). ILC-2 cells are a potential source of Th2 cytokines associated with allergy (IL-4, IL-5, and IL-13) and respond to the epithelium-derived cytokines IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) (Halim et al., 2014; Lee et al., 2016; Lund et al., 2013; Salimi et al., 2013). These cytokines are further discussed in section 1.4.

It is not fully understood which mechanisms determine how an immune response evolves, but the early cytokine environment that is present at the activation site of a T cell seems to affect the overall outcome of the response. In addition, several studies in vivo show that
there is often not a clear polarization to a Th1-driven or Th2-driven response, but rather a mixture, or continuum, between the two (Nygaard et al., 2015; Samuelsen, Nygaard, & Lovik, 2008). As the different subsets of Th cells secrete different cytokines, the levels of expressed cytokines can give us an idea about the evolution of an immune response, and may function as markers of immune responses. However, the detected levels of expressed cytokines are momentary pictures and cannot reveal whether an immune response has just started, is ongoing or has finished, as illustrated in Figure 2. The interplay between immune cells and the cytokine environment is illustrated as a network, rather than a straight line from “start” to “finish”. Illustrating the cytokine environment as a network is a more accurate, but also more complicated picture of the nature of an immune response.

Figure 2. Illustration of a cytokine network by Zhang and An (2007). Various interactions between immune cells and cytokines is shown as a network, illustrating the complex nature of an immune response.

1.3 The gut epithelial barrier

The gut epithelial barrier plays a vital role in preventing disease and maintaining a healthy gut (gut homeostasis). Exposure to various substances is a frequent event in the small intestine as nutrient uptake is its main task. An appropriate response is therefore essential when encountering substances such as allergens, pathogens, toxins and toxic compounds to maintain the gut barrier function. The gut barrier functions as both a physical barrier preventing uncontrolled passage of compounds, and, as a tissue that can elicit immune responses to maintain homeostasis, such as immunosuppressive
responses. The immunosuppressive environment in the gut is largely accomplished by oral tolerance (Brandtzaeg, 2011). Oral tolerance is an active immune response that maintains tolerance to food proteins, i.e. encounters with food allergens does not elicit an allergic immune reaction in healthy individuals (Brandtzaeg, 2010; Pabst & Mowat, 2012). When a food allergen is encountered, APCs will normally present the allergen to Treg cells to maintain oral tolerance. The immunosuppressive mechanisms of Treg cells are driven by their secretion of IL-10, which is a cytokine known for its regulatory and immunosuppressive roles, as mentioned in section 1.2.

The epithelial layer of the small intestine consists of several specialized epithelial cell, in which the enterocytes and microfold (M) cells are particularly important for the gut barrier function. Both cells are absorptive cells, but they play slightly different roles. Enterocytes are the most abundant cells in the gut lining and mainly transport nutrients, while M cells sample content from the gut lumen into Peyer’s Patches (PPs), and aid in the presentation of antigens to immune cells. PPs are lymph nodules where immune cells reside and are similar in function to lymph nodes (LNs), which function as a “meeting point” for antigens and immune cells. PPs are important starting points for initiation of immune responses in the intestine, and the content and cells migrate to the mesenteric lymph nodes (MLNs) through lymphatic vessels. MLNs are the LNs draining the intestine, and the immune response initiated in the PPs (Figure 1, section 1.2) will continue to evolve in the MLNs. Thus, MLN play a critical role in the intestinal immune system (Macpherson & Smith, 2006), and are especially important during sensitization in food allergy.

M cells and enterocytes also secrete immunoglobulin type A (IgA) antibodies into the gut lumen (Hansen et al., 1999). IgA regulates interactions between the intestine and foreign compounds by binding to them and is thus neutralizing and anti-inflammatory (Boyaka, 2017; Pabst, 2012; Pabst, Cerovic, & Hornef, 2016). In addition, IgA aids in antigen presentation to immune cells by forming an antigen-IgA complex that is transported over the gut barrier (Asano & Komiyama, 2011; Phalipon & Corthesy, 2003; Turula & Wobus, 2018).

The integrity of the epithelial barrier is maintained by tight junctions (TJs). TJs are networks of integral membrane proteins that seal the apical sides of the epithelial cells
tightly together, thus preventing uncontrolled transcellular transport across the barrier (Figure 3 B). Hence, an intact network of TJ proteins in the enterocytes is crucial for the barrier function. TJ proteins with central roles in maintaining the gut barrier integrity are claudin and occludin proteins (Heiskala, Peterson, & Yang, 2001). A disturbed barrier integrity can lead to increased epithelial permeability (Clemente et al., 2003; Drago et al., 2006). In this thesis, an increased intestinal permeability is used as one measure of altered gut barrier function. The expression of claudin-2 has been reported to be upregulated when the gut barrier integrity is impaired (Luettig, Rosenthal, Barmeyer, & Schulzke, 2015), while for occludin, a downregulation has been reported (Al-Sadi & Ma, 2007; Capaldo & Nusrat, 2009; Jin, Pridgen, & Blikslager, 2015; Zhao et al., 2014). Thus, there is a link between the levels of expressed TJ proteins and increased gut barrier permeability, or, “gut leakiness”.

Figure 3. Gut barrier functions. A) Sampling of gut content by an M cell into a Peyer's patch with immune cells. Content in the Peyer's patch is drained to LNs via lymphatic vessels. B) The intestinal epithelial layer with healthy enterocytes (green) is a mechanical barrier and prevents uncontrolled passage of compounds by the help of TJ proteins (yellow). Damaged enterocytes (red) with damaged TJ proteins has an altered barrier function with increased uptake of compounds, such as for instance allergens, over the gut barrier.
1.4 Consequences of a disrupted gut barrier function

For food allergy to develop, the oral tolerance to the food allergen must be broken. Why and how this occurs is not fully understood, but adjuvants may be a contributing factor during the sensitization process. As mentioned in section 1.1, adjuvants modulate and/or enhance the immune responses towards an antigen. A frequently used adjuvant in animal allergy models is cholera toxin (CT) (Bol-Schoenmakers et al., 2016; Bol-Schoenmakers et al., 2011; Capobianco et al., 2008; Li et al., 2000), a compound which also has barrier disruptor properties (Guichard et al., 2013; Lemichez & Stefani, 2013). Barrier disruptors are compounds that are capable of damaging or altering the intestinal epithelial barrier, which can lead to disrupted integrity and a leaky gut. Other compounds reported to have barrier disrupting capacities are the mycotoxin deoxynivalenol (DON) and house dust mite (HDM), in which both also have been reported to promote development of allergic disease in animal models (Bol-Schoenmakers et al., 2016; Hammad et al., 2009). Thus, the effects of barrier disruption and adjuvanticity may be linked. The barrier disruptors CT, HDM and DON will be further introduced in section 1.5.

Consequences of a leaky gut can be increased uptake of allergens and other compounds from the intestinal lumen that is normally prevented from crossing the gut barrier. A damaged or disrupted epithelial barrier will also elicit immune responses. The observed adjuvant effect of barrier disruptors may be a result of the production of endogenous danger signals, or “alarmins”, secreted by intestinal epithelial cells. Alarmins are danger signals released due to cell damage by infection or other cellular stress (Said-Sadier & Ojcius, 2012). The result can be an inflamed state, or, an upregulation of signals that promote immune responses, ultimately leading to break of oral tolerance, and promotion of ILC-2 and Th2 driven responses.

The cytokines IL-33, TSLP and IL-25 can act as alarmins (Hammad & Lambrecht, 2015; Moussion, Ortega, & Girard, 2008; Sy & Siracusa, 2016), and have several traits in common. They play important roles in maintaining gut homeostasis, and promote the production of cytokines typically produced by Th2 and ILC-2 cells (Ikeda et al., 2003; Lee et al., 2016; Noti et al., 2014; Schmitz et al., 2005). IL-33 is the ligand of the receptor ST2, which is found on ILC-2 cells, mast cells and Th2 cells. IL-33 is released from
epithelial cells that are damaged or infected, and binding of IL-33 to ST2 leads to production and release of cytokines (Kakkar & Lee, 2008).

To attain a more comprehensive picture of the underlying mechanisms of food allergy development there is a need for further research on how oral tolerance is broken and on the possible adjuvant capacity of barrier disruptors.

1.5 Properties and toxic effects of barrier disruptors

Cholera toxin

CT is used as a positive control and experimental model adjuvant for food allergy development, as it has strong adjuvant effects (Bogh et al., 2016). The mechanisms behind the adjuvant effect of CT are not fully understood, but the mechanisms behind its toxic effects are well established: in intestinal cells, CT leads to an efflux of electrolytes and fluid from the cell and into the gut lumen. The mode of action regarding adjuvant effects has been discussed by Boyaka (2017), suggesting that CT stimulates various immune cells.

Deoxynivalenol

DON is a mycotoxin belonging to the group of trichothecenes, which is known for their wide occurrence in food items and various toxic effects (P. Pinton & Oswald, 2014). DON is of the most common mycotoxin found in grain foods, making humans frequently exposed to DON (Organization & Cancer, 1993; P. Pinton et al., 2009). It has been demonstrated that DON has adjuvant effects in a food allergy model of whey allergens (Bol-Schoenmakers et al., 2016). Studies on intestinal integrity have reported that DON demonstrates barrier disruptor properties, as it affects the composition of TJ proteins and leads to alterations in intestinal permeability (Akbari et al., 2014; Philippe Pinton et al., 2010). It has been argued that one of the main targets for DON is the network of TJ proteins (P. Pinton & Oswald, 2014), but induced inflammation in the intestine by DON is also observed (Adesso et al., 2017). Whether the adjuvant capacities of DON is a result of its toxic effects leading to an inflammatory response with release of alarmins from epithelial cells, or that DON leads to degradation of TJ proteins by barrier disruption, is not clear.
**House dust mite**

Feces from HDM contain well-known allergens in allergic asthma (Hammad et al., 2009). Certain proteins from HDM feces, such as Der p 1, act as barrier disruptors in the lung by cleaving TJ proteins through proteolytic activity, and thereby facilitating trans-epithelial delivery of allergens in the lung (Wan et al., 2001; Wan et al., 1999). Although HDM proteins are mostly known for affecting the epithelial lining of the lungs, Der p 1 has been detected in the human GI-tract (Tulic et al., 2016). However, whether presence of HDM in the gut contribute to promoting sensitization and allergies to food items is not known, although it has been suggested that HDM may have an effect on the gut barrier integrity (Tulic et al., 2016; Wildenberg & van den Brink, 2016).

**Glyphosate**

GLY is a pesticide whose usage has increased substantially over the past decades (Benbrook, 2016) and its widespread use has caused concern on the possible health effects following exposure. Several studies concerning adverse effects of GLY exposure has been conducted, but effects on the GI-tract, especially regarding barrier-disruptor properties, has not been extensively investigated (Mesnage & Antoniou, 2017). A study on exposure to GLY-rich air and development of airway inflammation showed increased production of IL-33 and TSLP in mice, which suggests that GLY has an effect on the immune system (Kumar et al., 2014). It has been demonstrated that GLY exposure affects the gut microbiome (Aitbali et al., 2018), suggesting that GLY may have possible effects on the intestine.
1.6 Models for investigating allergy-promoting capacities

DON, HDM and GLY are environmental contaminants that humans are exposed to through various routes. As reviewed above, these compounds have possible immunotoxic properties that are linked to allergy development (adjuvant and barrier disruptor effects). Within toxicology, there is a lack of regulatory testing and a gap in knowledge on the possible immunotoxic effects of environmental contaminants. Thus, there is a need to investigate whether common environmental contaminants have these properties or not, and to find sensitive test systems.

In order to develop screening tests for detecting environmental adjuvants and barrier disruptors, suitable markers of key events (KE) in food allergy development needs to be identified and categorized. Screening tests using methods in vivo are costly, time-consuming, and often requires many animals. With focus on reducing animal testing, screening tests in vitro should be developed. The adverse outcome pathway (AOP) framework has been developed to identify the most efficient and predictive endpoints to use for tests in vitro. An AOP represents biological molecular initiating events (MIE) and KE that ultimately leads to adverse effects, which in this case would be clinical symptoms of food allergy. Recently, an AOP was suggested for food allergy development by van Bilsen et al. (2017). Here, the activation of epithelial cells is proposed as a KE, as it will lead to cytokine release and activation and migration of certain immune cells. For MIE, antigen uptake over the mucosal barrier by TJ disruption is proposed. Detecting early markers of these MIE and KE may be a promising way of assessing immunotoxicity of contaminants.

The immune system is a complex system of interactions between various cell types and tissues and is therefore impossible to fully mimic in vitro. Critical mechanisms may remain undetected and important markers of KE and their causal involvement in the pathway needs first to be identified or confirmed in vivo. Murine animal models are widely used in research on the immune system and underlying mechanisms for allergy development. Hopefully, through testing in vivo, suitable markers can be identified and further used in the development of screening methods in vitro for adjuvant and barrier disruptor properties of contaminants.
1.7 Aims and hypotheses

The overall aim of the present project was to identify early markers of altered gut barrier function or immune responses that could predict food allergy development. By conducting two types of experiments, we aimed to clarify the early effects of selected barrier disruptors on gut barrier function, and, the effect on food allergy development. The barrier disruptors CT, DON, HDM and GLY were chosen.

The main hypothesis was that damage to intestinal epithelial cells by barrier disruptors alters the barrier function, ultimately promoting allergy development. More specifically, we hypothesize that an affected barrier function will lead to impaired gut barrier integrity, i.e. increased barrier permeability and food allergen uptake, as well as an activated intestinal epithelial response. We further hypothesize that this in turn induce immune responses in lymphoid tissues that promote food allergy development. To test these hypotheses, we had the following sub-aims:

- To determine early effects of exposure to barrier disruptors
  - by quantifying epithelium-derived alarmins IL-33, TSLP and IL-25 in the intestine and ST2 in serum, as markers of an activated epithelial response
  - by quantifying IL-22, the peanut allergen Ara h 2, and intestinal fatty acid binding protein (FABP2) in serum, and the expression of the TJ proteins claudin-2 (upregulation) and occludin (downregulation) in the intestine, as markers of increased gut barrier permeability
  - by quantifying total IgA, as a measure of mucosal immune responses
  - by quantifying Th2 associated cytokines and/or production of inflammatory cytokines, as markers of activated immune responses in lymphoid tissues

- To determine possible adjuvant effects of the barrier disruptors on food allergy development, assessed as clinical symptoms of allergy (anaphylaxis) and allergic sensitization
2 Materials and Methods

2.1 Experimental design

We conducted two short-term experiments to investigate the effects of exposure to barrier disruptors on the gut barrier in mice. The allergy-promoting capacity of the barrier disruptors was assessed in one experiment in a food allergy mouse model, where mice were exposed to a barrier disruptor and a food allergen. Technicians provided support for animal care and handling for all experiments. Collection of tissues at the end of each experiment was a joint effort with supervisors. Method development, preparation and analysis of samples was the work of the candidate (with some assistance).

Choice of markers of gut barrier function and a mucosal and lymphoid immune response in short-term experiments

The alarmins IL-33, IL-25 and TSLP were chosen as markers based on their roles in gut homeostasis, and their ability to promote Th2 cytokine production, as reviewed in section 1.4. These alarmins are thus potential early markers of an altered gut barrier function and pro-allergic responses. Furthermore, as binding of IL-33 to its receptor ST2 leads to production and release of cytokines, ST2 was included as a possible marker of altered barrier function (Luthi et al., 2009; Schmitz et al., 2005). The chosen markers of an increased gut permeability were the TJ proteins claudin-2 and occludin and the cytokine IL-22 in intestine, and FABP2 and the peanut allergen Ara h 2 in serum. FABP2 and Ara h 2 has been used as markers of altered barrier integrity or barrier leakage (JanssenDuijghuijsen et al., 2017), while IL-22 has been reported to increase gut permeability (Wang, Mumm, Herbst, Kolbeck, & Wang, 2017). Total IgA in the intestine and serum was chosen as a marker of a mucosal immune response. The cytokines TNF-α, IL-6, IFNγ, IL-1β, IL-17, IL-13, IL-10, IL-5, IL-4 and IL-2 were chosen as markers of immune responses in MLNs (and PPs) and were assessed after stimulation with Concanavalin A (ConA) or lipopolysaccharides (LPS). ConA is a non-specific T cell activator promoting proliferation and differentiation of T cells (Dwyer & Johnson, 1981), while LPS primarily stimulates B cells and macrophages (MFs) (Janeway et al., 2008). An overview of the presented markers and endpoints for the short-term experiments is listed in Table 1.
### Table 1. Markers and endpoints reflecting effects on gut barrier permeability, function and immune response in exp. 1 and 2.

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Markers</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gut barrier permeability</strong></td>
<td>Composition of TJ proteins</td>
<td>Immunohistological analysis</td>
</tr>
<tr>
<td>(measure of barrier integrity)</td>
<td>Elevated levels of Ara h 2 (PE allergen) and FABP2 in serum, and IL-22 in supernatant of intestinal tissue</td>
<td>Ara h 2, FABP2, IL-22</td>
</tr>
<tr>
<td><strong>Intestinal epithelial response</strong></td>
<td>Levels of alarmins produced in supernatant of intestinal tissue</td>
<td>Enzyme linked immunosorbent assay (ELISA)</td>
</tr>
<tr>
<td></td>
<td>Levels of ST2 (IL-33 receptor) in serum and/or supernatant of intestinal tissue</td>
<td>ST2</td>
</tr>
<tr>
<td><strong>Mucosal immune response</strong></td>
<td>Levels of total IgA in supernatant of intestinal tissue and serum</td>
<td>Total IgA</td>
</tr>
<tr>
<td><strong>Lymphoid immune response</strong></td>
<td>Cytokine profile after <em>ex vivo in vitro</em> stimulation of cells from lymphoid tissue (MLNs, PPs, spleen) with ConA and LPS</td>
<td>Flow cytometric detection by multiplex Cytometric Bead Array (CBA)</td>
</tr>
</tbody>
</table>

#### Preparation of allergen and barrier disruptor solutions

The chosen food allergen for this project was peanut (*Arachis hypogaea*). Peanut (PE) is a widely used food ingredient, containing some of the most potent food allergens (Singh & Singh, 1991). PE consist of several well-characterized allergens of which some are very stable, as they are tolerant to heat treatment and the hostile environment of the human stomach (Sampson, 2004). The doses used for PE (Stallergenes Greer, London, UK), CT (EMD Biosciences Inc., San Diego, CA, USA), DON (Sigma Aldrich, St. Louis, MO, USA), and HDM (Stallergenes Greer) were based on literature where exposure to these doses gave effects (Table 2). Phosphate buffered saline (PBS, 10 mM 0.9% NaCl pH 7.4) was given to groups as negative control and was used as vehicle for the exposures.
Table 2. Doses and test solutions.

<table>
<thead>
<tr>
<th>Doses (in 250 µL PBS)</th>
<th>Test solutions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 mg PE</td>
<td>4 mg/mL PE</td>
<td>Li et al. (2000)</td>
</tr>
<tr>
<td>15 µg CT, 1 mg PE</td>
<td>0.06 mg/mL CT</td>
<td>Bol-Schoenmakers et al. (2016)</td>
</tr>
<tr>
<td>100 µg DON, 1 mg PE</td>
<td>0.4 mg/mL DON</td>
<td>Bol-Schoenmakers et al. (2016)</td>
</tr>
<tr>
<td>20 µg HDM, 1 mg PE</td>
<td>0.08 mg/mL</td>
<td>Tulic et al. (2016)</td>
</tr>
<tr>
<td>20 µg¹ GLY</td>
<td>0.08 mg/mL</td>
<td>Ait Bali, Ba-Mhamed, and Bennis (2017); Jasper, Locatelli, Pilati, and Locatelli (2012); Zhao, Zhang, Wang, Han, and Xie (2016)</td>
</tr>
</tbody>
</table>

¹This concentration was based on one model for effects on behavior (Ait Bali et al., 2017), one for hepatic, hematological and oxidative effects of oral exposure to Roundup® (Jasper et al., 2012), and one model for effects on intestine of oral exposure to the pesticide chlorpyrifos (Zhao et al., 2016). Roundup® herbicide contains approximately 40% GLY (Monsanto Company, St. Louis, MO, USA).

Food allergy model

We conducted a food allergy mouse model (experiment 3) to investigate whether the compounds affecting the gut barrier function also had adjuvant effects and could break the oral tolerance when administered together with PE. The mouse model was based on a lupine food allergy model (Vinje et al., 2009). Endpoints and markers chosen for experiment 3 (exp.3) are listed in Table 3.
Table 3. Endpoints in the food allergy model.

<table>
<thead>
<tr>
<th></th>
<th>Endpoints</th>
<th>Measures of allergy response (assessed after i.g. challenge to allergen)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anaphylaxis</strong></td>
<td>Clinical symptoms of anaphylaxis</td>
<td>Score for severity of anaphylactic reaction</td>
<td>Scoring system (Table 12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drop in body temperature</td>
<td>Measuring rectal body temperature</td>
</tr>
<tr>
<td></td>
<td>mMCP-1 in intestinal tissue (Vaali et al., 2006)</td>
<td>mMCP-1</td>
<td>Immunohistology</td>
</tr>
<tr>
<td></td>
<td>mMCP-1 in serum</td>
<td>mMCP-1</td>
<td>ELISA</td>
</tr>
<tr>
<td><strong>Food allergy sensitization</strong></td>
<td>Ara h 2-specific IgE in serum</td>
<td>Ara h 2-specific IgE(^1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total IgE in serum</td>
<td>Total IgE</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphoid immune response</strong></td>
<td>Cytokine profile after \textit{ex vivo in vitro} stimulation of spleen cells with PE</td>
<td>TNF-(\alpha), IL-6, INF-(\gamma), IL-1(\beta), IL-17, IL-13, IL-10 and IL-2</td>
<td>CBA</td>
</tr>
</tbody>
</table>

\(^1\)Ara h 2 specific IgE was measured before challenge

2.2 Animals

Female C3H/HeOuJ mice were purchased from Charles River Laboratories (Sulzfeld, Germany) for exp.1 and 3 (Wilmington, MA, USA), and Jackson Laboratory (Bar Harbor, ME, USA) for exp.2. Mice were 4-5 weeks old at arrival and randomly allocated in groups. Mice were housed at room temperature (RT) of 21±2°C and 55±10 % humidity on Nestpack bedding (Datesand Ltd, Manchester, UK) and exposed to a 12 hours light 12 hours dark cycle. The mice were given pelleted feed (RM1, SDS, Essex, UK), tap water ad libitum, and acclimatized for at least one week before entering the experiment. The experiments were performed in conformity with the laws and regulations for experiments with live animals in Norway and were approved by the Experimental Animal Board under the Ministry of Agriculture in Norway.
2.3 Experiment 1: early effects of a single exposure (4, 24 and 48 hours)

Mice were exposed once by intragastric (i.g.) gavage to 250 µL of test solutions containing PE (1 mg), CT (15 µg) and PE (1 mg), DON (100 µg) and PE (1 mg), and HDM (20 µg) and PE (1 mg), see Table 4. The mice were observed for 30 minutes after the experiment in case of anaphylaxis.

Before the experiment started, blood from vena saphena was sampled as control samples (T0). At the site of blood sampling, hair was removed with a scalpel and mice were bled 100 µL using 100 µL Microvette tubes without heparin (Sarstedt, Nümbrecht, Germany). 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 with blood sampled in glass tubes before cervical dislocation.

Table 4. Test solutions, concentrations and number (n) of animals per group in exp. 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Doses (in 250 µL PBS)</th>
<th>Concentration</th>
<th>n (per time point 4, 24 and 48 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>250 µL PBS</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>1 mg PE</td>
<td>4 mg/mL PE</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>15 µg CT, 1 mg PE</td>
<td>0.06 mg/mL CT</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>100 µg DON, 1 mg PE</td>
<td>0.4 mg/mL DON</td>
<td>5</td>
</tr>
<tr>
<td>E</td>
<td>20 µg HDM, 1 mg PE</td>
<td>0.08 mg/mL</td>
<td>5</td>
</tr>
</tbody>
</table>

MLNs were localized by tilting the intestines to the side of the mouse using a cotton ball (David et al., 2017). The MLNs were excised using forceps 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% pencillin/streptomycin (10 000 units/mL pencillin, 10 mg/mL streptavidin, PAA Laboratories Inc, Etobicoke, ON, Canada), to ensure cell viability.
The small intestine was localized, excised and oriented so that the duodenum and ileum could be separated (Bol-Schoenmakers et al., 2016; David et al., 2017) as illustrated in Figure 4. We defined the duodenum as the top 7 cm of the small intestine and the ileum as the lower 7 cm. The intestinal segments were placed on metal plates kept on ice immediately after excision to minimize degradation of proteins. Duodenum and ileum were flushed thoroughly with cold PBS by using a syringe, and visceral adipose tissue was removed. 2 cm closest to the stomach and caecum of the 7 cm segments, were cut out (Figure 4). Further, the 2 cm segments were cut into two pieces of 1 cm, and snap-freezed in cryo tubes (Nunc Cryo Tube Vial 1.8 mL, Nalge Nunc International, Penfield, NY, USA) in liquid nitrogen and stored at -80 °C until homogenization. The remaining duodenum and ileum segments of 5 cm were prepared for immunohistological analysis.

![Figure 4. Illustration of excised intestine based on David et al. (2017). ST = stomach, CA = caecum, D = duodenum, J = jejunum, I = ileum. 7 cm of duodenum and ileum was removed, in which 2 cm of each segment were prepared for intestinal homogenization and 5 cm were prepared for immunohistological analysis.](image)

**Preparation of serum**

Blood was kept at RT for no longer than 3 hours after being sampled. Blood samples from vena saphena (T0) were centrifuged at 1000 x g for 10 minutes at RT and sera stored at -80 °C. Blood samples from exsanguination (TT) were collected in 5 mL meinecker tubes and centrifuged twice at 2000 x g for 10 minutes at RT. TT serum was transferred to 0.5 mL tubes (PCR® tubes 0.5 mL, Axygen Scientific INC, Union City, CA, USA) and stored at -80 °C until further use. T0 samples were not used for any further analysis.
**Lymph node cell preparation and stimulation**

For each mouse, MLNs were collected and pooled. Single-cell suspensions were prepared by forcing the nodes through a 70 µm strainer over a 50 mL centrifuge tube with subsequent washing with 10 mL cold HBSS buffer with 2% FCS and 1% penicillin/streptomycin. The tubes were centrifuged at 430 x g for 5 minutes at 12 °C. Supernatants were collected and cells were resuspended in 0.5 mL culture medium (RPMI 1640, Gibco ® by Thermo Fisher Scientific) containing 10% FBS and 1% penicillin/streptomycin. Single-cell suspensions were kept on ice, and all steps were performed under sterile conditions. Cell concentration was determined using a Coulter Counter Z1 (Beckman Coulter Incorporated, Indianapolis, IN, USA), and presented as the total cell number (10^6) per animal.

Cell-suspensions were adjusted to 3 x 10^6 cells/mL in culture medium. 180 µL cell-suspension and 20 µL stimulation solution (ConA, LPS or culture medium) were added to each well in Microtest plates (Microtest Plate 96 Well, C, Sarstedt) and incubated at 37 °C in 5% CO₂ for 48 hours (Galaxy S CO2 incubator, RS Biotech Laboratory Equipment Ltd., UK). Cells were stimulated with either ConA (Concanavalin A from Canavalia ensiformis, Sigma-Aldrich) in a final concentration of 5 µg/mL, LPS (lipopolysaccharides from Escherichia coli 026:B6, Sigma-Aldrich) in a final concentration of 10 µg/mL, or culture medium alone. After incubation, the plates were centrifuged at 180 x g for 5 min at RT and the supernatants were collected and stored at −80 °C until cytokine detection.

**Cytokine profile lymph node cells stimulated with ConA or LPS**

Cytokine release of TNF-α, IL-6, IFNγ, IL-1β, IL-17, IL-13, IL-10 and IL-5 and from ConA- and LPS-stimulated lymph node cells from MLNs was detected using the BD Cytometric bead array (CBA) Mouse Soluble Protein Flex Sets, measured on a BD LSR II flow cytometer and analyzed by the FCAP Array software (all from BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol.

**Development of method for homogenization**

Before conducting homogenization of intestines, we established a homogenization protocol based on methods by Bol-Schoenmakers et al. (2016) and David et al. (2017). We
conducted two tests to find the optimal speed, dilution, and amount of protease-inhibitor for an optimal homogenization and preservation of protein content. Intestinal tissue from three mice were used for developing the method. The optimal speed for homogenization was assessed by testing two speeds: 6000 rpm (recommended by manufacturer of Precellys 24 tissue homogenizer, Bertin Instruments, Montigny-le-Bretonneux, France) and 6800 rpm. For both speeds, the duration was 2x30 seconds with a 30 second pause. Default settings were followed as recommended by the manufacturer.

In the first test, 2 cm (approximately 50 mg) of ileum and duodenum segments from the three mice were pooled together and equally distributed into three samples. In two of the three samples, protease inhibitor was added (Table 5). The protein content of the intestinal supernatant was quantified by bicinchoninic acid assay (BCA) (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) according to the manufacturer’s protocol, and the optimal dilution of supernatant from intestinal tissue was found (see result section 3.3). The four dilutions chosen for the BCA-analysis are listed in Table 7. All samples were diluted with PBS.

In the second homogenization test, we tested if the presence of protease-inhibitor (Protease-inhibitor cocktail tablets, Sigma-Aldrich), and the amount of RIPA lysis buffer (Thermo Fisher Scientific) had an effect on the protein content in the samples, and to see if there was a different optimal dilution for ileum and duodenum. Changes in the second homogenization test were based on results obtained from the BCA analysis conducted for the first homogenization test. The chosen speed was 6000 rpm.

Three samples of 1 cm of ileum and three samples of 1 cm duodenum from the three mice were weighed to assess whether 1 cm was equal to approximately 25 mg. This time, ileum and duodenum were pooled separately (Table 6). Dilutions were chosen based on results from the previous BCA-analysis. We chose dilutions that gave the same ratio of buffer and tissue for our BCA-analysis, as we had different ratios of tissue and buffer (Table 7).
Table 5. Homogenization test 1. Duodenum and ileum pooled together, and equally distributed into three samples.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sample</th>
<th>Volume</th>
<th>Protease-inhibitor</th>
<th>Speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileum, mouse 1 (2 cm)</td>
<td>1</td>
<td>0.5 mL</td>
<td>Yes</td>
<td>6000 rpm</td>
</tr>
<tr>
<td>Ileum, mouse 2 (2 cm)</td>
<td>2</td>
<td>0.5 mL</td>
<td>No</td>
<td>6000 rpm</td>
</tr>
<tr>
<td>Ileum, mouse 3 (2 cm)</td>
<td>3</td>
<td>0.5 mL</td>
<td>Yes</td>
<td>6800 rpm</td>
</tr>
<tr>
<td>Duodenum, mouse 1 (2 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum, mouse 2 (2 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum, mouse 3 (2 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Homogenization test 2 (speed 6000 rpm). Duodenum and ileum pooled, and equally distributed into three samples.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sample</th>
<th>Volume</th>
<th>Protease-inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileum, mouse 1 (1 cm)</td>
<td>1</td>
<td>1.5 mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Ileum, mouse 2 (1 cm)</td>
<td>2</td>
<td>0.5 mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Ileum, mouse 3 (1 cm)</td>
<td>3</td>
<td>1.5 mL</td>
<td>No</td>
</tr>
<tr>
<td>Duodenum, mouse 1 (1 cm)</td>
<td>1</td>
<td>1.5 mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Duodenum, mouse 2 (1 cm)</td>
<td>2</td>
<td>0.5 mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Duodenum, mouse 3 (1 cm)</td>
<td>3</td>
<td>1.5 mL</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 7. Dilutions for BCA analysis.

<table>
<thead>
<tr>
<th>Test 1</th>
<th>Homogenate</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mg tissue, 0.5 mL</td>
<td>1:1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test 2</th>
<th>Homogenate</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 mg tissue, 0.5 mL</td>
<td>0.25 mg tissue, 1.5 mL</td>
</tr>
</tbody>
</table>
Homogenization

Immediately after the intestinal samples were thawed, the two pieces of 1 cm intestinal segments were weighed in tared 1.5 mL tubes (Micro Tubes, Brand Scientific Equipment Pvt. Ltd., Wertheim, Germany), and weight was noted. 0.6 mL of RIPA lysis buffer with 0.04% protease-inhibitor was immediately added to the tubes to avoid tissue from drying and to reduce protein degradation. Protease-inhibitor stock solution was prepared according to manufacturers’ instructions.

The samples were kept on ice at all times except for during weighing. The tissue was cut into small pieces with clean scissors and transferred to 2 mL CK28-R tubes (Bertin Instruments) and homogenized using Precellys 24 tissue homogenizer (Bertin Instruments) at 6000 rpm (2x30 seconds, 30 seconds pause). Immediately after homogenization, the tubes were left to cool down on ice at RT for up to 30 minutes. Samples were centrifuged at 1970 x g for 5 minutes, and the supernatants were collected and stored at –80 °C until analysis. Because the method was time-consuming, batches of 19-20 samples were homogenized per day.

The concentration for each segment of duodenum and ileum per mouse were adjusted to their weight to account for variability (results for weights not included).

Markers of intestinal epithelial responses, gut barrier permeability, and a mucosal immune response

In the intestinal supernatant, levels of IL-33, ST2 (the IL-33 receptor), TSLP, IL-25, IL-22 and total IgA were analyzed by Enzyme-linked immunosorbent assay (ELISA). In serum, levels of total IL-33, ST2, total IgA, FABP2 and PE allergen Ara h 2 were analyzed by ELISA. All assays were performed according to the manufacturer’s protocols (Table 8). Where the manufacturer did not provide or recommend a specific diluent, PBS was use for sample dilutions. Optical density (OD) values were measured with a Microplate Reader (ELx808 Absorbance Reader, BioTek, Shoreline, WA, USA) connected to a PC using the software Gen5™ (BioTek). Concentrations were determined based on a standard curve generated on each microwell plate (plates were provided in the kits).
### Table 8. List of all ELISA kits used and manufacturer, tissue and dilution

<table>
<thead>
<tr>
<th>Marker</th>
<th>Tissue</th>
<th>Dilution</th>
<th>Kit</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST2</td>
<td>Ileum and duodenum</td>
<td>Undiluted</td>
<td>Uncoated Enzyme Linked Immunosorbent Assay</td>
<td>Invitrogen by Thermo Fisher Scientific</td>
</tr>
<tr>
<td>IL-33</td>
<td></td>
<td></td>
<td>Mouse IL-22 ELISA Ready-SET-GO!®</td>
<td></td>
</tr>
<tr>
<td>TSLP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-25</td>
<td>Ileum and duodenum</td>
<td>Undiluted</td>
<td></td>
<td>Invitrogen by Thermo Fisher Scientific</td>
</tr>
<tr>
<td>IL-22</td>
<td>Ileum and duodenum</td>
<td>Undiluted</td>
<td>Mouse IL-22 ELISA Ready-SET-GO!®</td>
<td>Abcam (Cambridge, UK)</td>
</tr>
<tr>
<td>IgA</td>
<td>Ileum</td>
<td>1:50</td>
<td>Precoated IgA Mouse ELISA kit</td>
<td>Abcam (Cambridge, UK)</td>
</tr>
<tr>
<td></td>
<td>Duodenum</td>
<td>1:5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>1:4000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ara h 2</td>
<td>Serum</td>
<td>1:2</td>
<td>Ara h 2 ELISA Kit¹</td>
<td>Indoor biotechnologies, (Charlottesville, VA, USA)</td>
</tr>
<tr>
<td>FABP2</td>
<td>Serum</td>
<td>1:2</td>
<td>Mouse IFABP/FABP2 ELISA Kit</td>
<td>Oourse (San Diego, CA, USA)</td>
</tr>
</tbody>
</table>

¹The antibody peroxidase-conjugated AffiniPure F(ab’) used for the Ara h 2 kit was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) and reconstituted following recommendations from the manufacturer of the antibody (Jackson ImmunoResearch Laboratories Inc.).

### Histology

5 cm of mice intestinal segments were oriented with the part closest to the stomach (duodenum) and caecum (ileum) upwards on filter paper wetted with PBS (Figure 5). The intestine was cut open using surgical scissors and folded outwards with forceps and the mucosa facing upwards. Residues of feces were carefully washed away with cold PBS. The intestine was rolled starting from the bottom of the petri dish. The finished intestine, ”swiss roll” (Moolenbeek & Ruitenberg, 1981), was held together with a hypodermic needle and fixed in 4% formalin (Sigma-Aldrich) for minimum 24 hours before paraffin embedding.

The samples were processed through a standard paraffin embedding protocol (Shandon Excelsior ES and HistoStar, Thermo Fisher Scientific). Histological sections of mouse intestine (swiss roll) were cut transversely at 5 µm thickness (Microm HM 355S, Thermo Fisher Scientific). Tissue samples were deparaffinized and stained with hematoxylin and hexamine (HE) according to standard protocols, as well as stained with antibodies specific...
to tight junction (TJ) protein claudin-2 (Claudin-2 polyclonal antibody, Labome, USA/Invitrogen by Thermo Fisher Scientific) and occludin (OCLN AA 480-520, Labome, USA/Bioss Antibodies, Woburn, Massachusetts, USA).

Prior to staining of TJ proteins, the sections were deparaffinized in xylene, following 100%, 96% and 70% ethanol (EtOH) and soaked in 3% hydrogen peroxide (H$_2$O$_2$) in EtOH for 15 minutes to reduce endogenous peroxidase. The epitopes were demasked in a pressure boiler for 20 min using decloaker buffer diluted 1:10 (Diva Decloaker 10X, Biocare Medical, Pacheco, CA, USA), and incubated with background punisher (BioCare Medical) to block unspecific background staining. Sections were stained with anti-claudin-2 diluted 1:4000 and anti-occludin diluted 1:500 in Renoir red (Biocare Medical) at +4°C overnight. Signal amplification was performed using donkey anti-rabbit-HRP secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 in Renoir red, and stained with Betazoid DAB (3,3’-diaminobenzidine) (Betazoid DAB Chromogen Kit, Biocare Medical). Following DAB-staining, the tissue sections were quickly

Figure 5. Preparation of intestine and swiss roll. Left: Duodenum (D) and ileum (I) oriented with the part closest to the stomach and caecum up, respectively, on wetted filter paper on a petri dish. The arrow indicates the direction of rolling of intestine. Intestines were cut open and folded so that mucosa faced upwards. Right: Finished swiss roll of duodenum. Arrow indicates the segment closest to the stomach, which is at the inner part of the roll (photo: Elena Klåpbakken Drønen).
counterstained with HE (Sigma-Aldrich). Sections were rehydrated in 70% EtOH, following 96% and 100% EtOH, and lastly xylene. Sections were mounted on Superfrost Plus glass slides (VWR, Radnor, PA, USA) using Pertex mounting solution (Histolab Products, AB, Västra Frölunda, Sweden).

The histological slides from each mouse were examined in a blinded fashion. Scoring of histological samples stained for the TJ proteins claudin-2 and occludin were done by scoring the color-intensity of each section compared to the control group, using a light microscope. The slides were screened at 20x and a representative area for the slide was chosen. The color-intensity of that area was then assessed at 40x.

2.4  **Experiment 2: early effects of a single exposure (4 and 24 hours)**

In exp.2, two time-points of 4 and 24 hours were chosen based on results from exp.1. In addition, one group was added and exposed to the pesticide GLY. Co-exposure to PE and immunohistological analysis was excluded from this experiment (Table 9).

Mice were exposed by (i.g.) gavage once to PBS (250 µL), or either PE (1 mg), CT (15 µg), DON (100 µg), HDM (20 µg) or GLY (20 µg) (Table 9). Mice were observed for 30 minutes in case of anaphylaxis. GLY was provided reconstituted in deionized water by the manufacturer (SUPELCO, Bellefonte, PA, USA).

Tissue preparation, ELISA, and CBA analyses were performed as described for exp.1 (section 2.3) with the following exceptions: PPs of the small intestine were added as an endpoint. The PPs were easily visible along the intestine (Figure 6 A) and cut out with scissors (Figure 6 B). PPs were processed and prepared as described for MLNs in exp. 1. Up to seven PPs, primarily sampled from the ileum, but if needed also from the jejunum and duodenum, were collected and prepared. PPs from each group were pooled, prepared, and counted.
Table 9. Test solutions and concentrations in exp. 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>Doses (all in 250 µL PBS)</th>
<th>n (per time-point, 4 and 24 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PBS</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>1 mg PE</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>15 µg CT</td>
<td>8</td>
</tr>
<tr>
<td>D</td>
<td>100 µg DON</td>
<td>8</td>
</tr>
<tr>
<td>E</td>
<td>20 µg HDM</td>
<td>8</td>
</tr>
<tr>
<td>F</td>
<td>20 µg GLY</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 6. PPs from small intestine. Left: PPs are indicated by arrows. Right: Excision of PPs (photo: Elena Klåpbakken Drønen).

The experiment was conducted over four days. We excised organs (intestine, MLNs and PPs) at the 4 hour time-point at day 1 and 3, and the 24 hour time-point at day 2 and 4. At day 1, time-point 4 hours, the PPs and not MLNs were collected from the mice. As the cell count in PPs was too low to expect detectable cytokine expression (cytokine pattern), the MLNs were collected in addition to the PPs for the following three days of the experiment to ensure high enough cell count.

In MLNs and PPs, cytokine release of TNF-α, IL-6, INF-γ, IL-1β, IL-13, IL-4 and IL-2 from ConA- and LPS-stimulated immune cells were determined by CBA. Analysis of IL-17, IL-10 and IL-5 were excluded in exp.2 based on results from exp.1. Levels of IL-4, a typical Th2 cytokine, and IL-2 was included in exp. 2 as they had not been analyzed in exp. 1.
For preparation of intestinal homogenate in exp.2, we increased the amount of tissue from 2 cm to 7 cm as swiss roll was not prepared due to limited usefulness of TJ endpoints. We also increased the volume of RIPA lysis buffer with protease-inhibitor from 0.6 mL to 1 mL and excluded the cutting-step of intestines with scissors. Because of this, we used different dilutions of samples for ELISA analysis in exp.2 than in exp.1, as listed in Table 10.

Due to low levels in exp.1, we excluded analysis of ST2 and IL-25 in ileum, and analysis of IL-22 in duodenum. We also conducted a titration of samples for IgA analysis, to find the optimal dilution of the tissue samples for ELISA analysis of IgA levels in exp.2. The titration was done by conducting a 1:2 dilution series of 9 samples (3 ileum, 3 duodenum and 3 serum), following the same procedure as described in exp.1 for the IgA kit. For all the ELISA analyses, apart from the IgA and FABP2, samples were incubated overnight on microwell plates at +4°C for maximum intensity.

Table 10. Dilutions of samples for ELISA analysis in exp. 2. Hyphens represent analyses not performed.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Ileum supernatant</th>
<th>Duodenum supernatant</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-33</td>
<td>1:2</td>
<td>1:2</td>
<td>-</td>
</tr>
<tr>
<td>IL-25</td>
<td>-</td>
<td>1:2</td>
<td>-</td>
</tr>
<tr>
<td>IL-22</td>
<td>1:2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ST2</td>
<td>-</td>
<td>1:2</td>
<td>1:2</td>
</tr>
<tr>
<td>IgA</td>
<td>1:100</td>
<td>1:400</td>
<td>1:200</td>
</tr>
<tr>
<td>FABP2</td>
<td>-</td>
<td>-</td>
<td>1:2</td>
</tr>
</tbody>
</table>
2.5 Experiment 3: Food Allergy Model

The food allergy model was based on the mouse model for lupine allergy developed by Vinje et al. (2009), and the mouse model for PE anaphylaxis developed by Li et al. (2000) with some modifications (unpublished data). The experiment included seven immunizations to PE together with the different barrier disruptors, and one high dose PE challenge at the end of the experiment. A high dose PE challenge was given to induce anaphylaxis to confirm clinically relevant signs of food allergy.

**Immunization, challenge and clinical anaphylaxis assessment**

The mice were immunized i.g. at day 0, 1, 2, 7, 21, 28 and 35 of experiment, with test substances as listed in Table 11. The CT+PE group was included as a positive control group for food allergy, since CT has previously been shown to have adjuvant effects in several models for food allergy (Andreassen et al., 2016; Bol-Schoenmakers et al., 2016; Capobianco et al., 2008; Li et al., 2000; Nygaard et al., 2015; Pablos-Tanarro, Lopez-Exposito, Lozano-Oj alvo, Lopez-Fandino, & Molina, 2016; Vinje et al., 2009). The groups given either PBS, PE or CT alone were negative control groups for non-exposed (PBS) and non-sensitized mice (PE, CT).

*Table 11. Immunization and challenge dose and number (n) of animals per group.*

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose per immunization (250 µL i.g.)</th>
<th>Challenge (5 mg PE in 250 µL i.p.)</th>
<th>Challenge (2.5 mg PE in 250 µL i.p.)</th>
<th>Total number of animals per group</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>250 µL PBS</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>1 mg PE in PBS</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>15 µg CT, 1 mg PE in PBS</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>D</td>
<td>100 µg DON, 1 mg PE in PBS</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>E</td>
<td>20 µg HDM, 1 mg PE in PBS</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>F</td>
<td>20 µg GLY, 1 mg in PBS</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>G</td>
<td>15 µg CT in PBS</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

Due to practical considerations, challenge was given over two days, at day 42 and 43 (about half of each groups per day). Prior to challenge, the mice were weighed, and blood sampled from vena saphena as described for exp. 1 (section 2.3), and serum stored at -
20°C. The mice were challenged with an intraperitoneal (i.p.) injection of 5 mg PE in 250 µL of PBS (Li et al., 2000). Due to dilution errors on day 42, 2.5 mg PE in 250 µL PBS was given to mice as listed in Table 11. Prior to challenge, and at 15 minutes and 30 minutes after challenge, rectal body temperature was measured using a BAT-12 microprobe thermometer with a RET-3 probe (Physitemp Instruments Inc, Clifton, NJ, USA). Mice were observed by the same investigator continually for 30 minutes after i.p. injection for clinical reactions and assessed by scoring system from 0 to 5 modified by Vinje et al. (2009) from Li et al. (2000) to assess the degree of anaphylaxis (Table 12). A score of 4 led to immediate euthanization followed by exsanguination and blood, spleen and intestines were excised and prepared as described for exp. 1 (section 2.3). After 30 minutes, all challenged mice were killed.

Table 12. Score scheme for anaphylaxis in the food allergy model.

<table>
<thead>
<tr>
<th>Score</th>
<th>Physiological response</th>
<th>Severity of reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No symptoms</td>
<td>No reaction. Normal activity</td>
</tr>
<tr>
<td>1</td>
<td>Itching and scratching around nose, eyes and head</td>
<td>Mild. Reduced activity, occasional scratching, muscle contractions, reversible symptoms within 5 minutes after challenge</td>
</tr>
<tr>
<td>2</td>
<td>Edema around eyes and mouth, diarrhea, erected body hair (pilar recti), reduced activity and/or impaired activity with increase in respiratory frequency</td>
<td>Moderate. Marked impaired or reduced activity, continuous scratching, abnormal respiration, symptoms reversible within 10 minutes after challenge</td>
</tr>
<tr>
<td>3</td>
<td>Wheezing, strained respiration and cyanosis around mouth and tail</td>
<td>Severe. Low or no activity, abnormal respiration, death, or irreversible 15 minutes after challenge</td>
</tr>
<tr>
<td>4</td>
<td>No activity after prodding and/or shivering and cramps</td>
<td>Severe.</td>
</tr>
<tr>
<td>5</td>
<td>Death</td>
<td>Severe.</td>
</tr>
</tbody>
</table>
Preparation of serum, intestinal tissue and spleen

Serum
Blood sampling was conducted the day before the start of experiment (T0) and the day before challenge (T41) and blood from exsanguination (TT) was collected and serum prepared as described in section 2.3. All analyses but one was done after i.p. challenge to either 2.5 mg or 5 mg of PE. Detecting the levels of Ara h 2 specific IgE levels in serum was carried out before challenge.

Spleen cell preparation and stimulation
We chose spleen cells to assess expressed cytokines (cytokine pattern) as these have been reported to be altered in previous food allergy models, reflecting systemic changes to the immune system (Li et al., 2000; Vinje et al., 2009). In addition, spleen is more easily accessible and provides a more abundant cell number compared to LNs. Preparation and stimulation of spleen cells was performed as described for MLNs (section 2.3), but to assess cytokine response of allergen-specific cells, cells were stimulated in vitro with PE at a final concentration of 1 mg/mL and incubated for 5 days.

Cytokine profile from PE-stimulated spleen cells
Cytokine release of TNF-α, IL-6, INF-γ, IL-1β, IL-17, IL-13, IL-10 and IL-2 from PE-stimulated spleen cells were determined using the same procedure as described for exp. 1 (section 2.3).

Histology
The procedure for intestinal tissue preparation for immunohistological analysis was followed as earlier described for exp.1 (section 2.3) with some exceptions. We only included ileum sections and increased the length of intestine for swiss rolls from 5 cm to 7 cm. As a measure of anaphylaxis (Vaali et al., 2006), we stained for mucosal mast cell protease-1 (mMCP-1). The mMCP-1 antibody (Moredun Scientific Limited, UK) was diluted 1:2000. For expression of mMCP-1 (only assessed in the food allergy model), a
representative area of the section was chosen in the same manner as described in exp. 1. At 40x, the number of cells stained for mMCP-1 in that area was counted.

**Measures of anaphylaxis and allergy sensitization in serum**

**Ara h 2 specific IgE**

In serum sampled at the last day of experiment before challenge, we analyzed the levels of Ara h 2 specific IgE, a measure of food allergy sensitization, by an ELISA assay previously 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 of plates (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. 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) in each well and incubated for 1 hour at 21°C. Plates were washed as previously described. For standard curve creation, 100 µL of diluted Ara h 2 specific IgE (mouse serum pool containing anti-Ara h 2 from PE-immunized mice prepared at the lab at The Norwegian Institute of Public Health) was added as doublets. The first dilution was 1:1, following a 2-fold titration to 1:64 in 4% BSA/PBS. Two wells (one doublet) was added 4% BSA/PBS only as blank samples. 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 added 100 µL of biotin-labeled Ara h 2 (3 µg/mL, Biotin Natural Ara h 2, B1-AH2-1, Indoor Biotechnologies) in 4% BSA in PBS. Plates were incubated 1 hour at 21 °C and then washed as previously described. Poly-HRP-strepatavidin (Poly-HRP-strepatavidin N200, Thermo Fisher Scientific) was diluted 1:40 000 in 4% BSA/PBS and 100 µL was added to each well. Plates were incubated for 1 hour at 21 °C, washed, and added 100 µL of TMB (TMB Stabilized Chromogen SB01, Life Technologies Europe B.V., Bleiswijk, Netherlands) solution and incubated at RT for 15 minutes. The reaction was stopped by adding 50 µL of 2N H$_2$SO$_4$.
stop solution (Sulfuric acid 95-97%, Merck Milipore, Burlington, MA, USA) in each well. Plates were read at 450 nm in a BioTek microplate-reader.

**Total IgE and mMCP-1**

In serum collected after challenge and exsanguination, levels of the allergic sensitization marker total IgE was determined using IgE Mouse Uncoated ELISA kit with Plates according to the manufacturer’s protocol (Invitrogen by Thermo Fisher Scientific). In the same blood samples, levels of the anaphylaxis marker mMCP-1 were determined using Mouse MCPT-1 (mMCP-1) ELISA Ready-SET-Go!® kit according to the manufacturer’s protocol (Invitrogen by Thermo Fisher Scientific).

**2.6 Statistical analysis**

To the majority of the endpoints, the assumptions of equal variance and a normality distribution in our data sets were violated, and we therefore chose to use non-parametric statistics. Statistical significance was determined by the non-parametric one-way analysis of variance Kruskal-Wallis test using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). If a p-value ≤ 0.05 was detected, we conducted a Dunn’s multiple comparison test to identify which groups were different. All bar graphs represent the group median, with a scatter plot where each mouse is denoted by a symbol. Correlation of score and body temperature drop in exp. 3 was done by Spearman rank-order correlation by using GrapPad Prism.
3 Results

3.1 Short-term experiments
Results from exp.1 and 2 showed that the most prominent effect was in the groups exposed to DON, with or without the allergen PE. The levels of the epithelium-derived alarmins TSLP and IL-33 in intestinal tissue, the IL-33 receptor ST2 in serum, and the lymphoid immune response markers TNF-α, IL-6 and IFNγ in LPS-stimulated lymph node cells from MLNs were all increased in the groups exposed to DON at 4 or 24 hours in both experiments. While CT affected some of the early markers, HDM and GLY did not clearly affect any of the markers in the short-term experiments.

Intestinal epithelial response: IL-33, ST2, TSLP and IL-25

IL-33 in ileum and duodenum
The DON+PE group had statistically significantly increased levels of IL-33 in ileum 4 hours after exposure compared to the PE group in exp.1 (Figure 7 A). As opposed to this, results from exp.2 at the same time-point showed statistically significantly increased levels in the CT group compared to the control group (Figure 7 B). IL-33 for 24 and 48 hours after exposure in exp.1 (Figure 20 E-F, appendix), and 24 hours after exposure in exp.2 (Figure 22 H, appendix) showed no statistically significant differences in ileum.

In duodenum, no statistically significant differences in levels of IL-33 were detected at any of the time-points in exp.1 (Figure 8 A and C for 4 and 24 hours and Figure 19 F for 48 hours, appendix). In exp.2, we detected statistically significant group differences between the DON group and groups exposed to PE, HDM and GLY, 24 hours after exposure (Figure 8 D). At 4 hours, we observed increased levels in the DON group and obtained a p-value of 0.0284 (Figure 8 C). This result suggests that DON increased the levels of IL-33 at 4 hours in exp.2, although Dunn's multiple comparison test did not show any statistically significant differences between any groups.
Figure 7. IL-33 in ileum 4 hours after exposure in exp. 1 (n=5) and exp. 2 (n=8). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a median value for each respective group and each symbol denotes one animal.

Figure 8. IL-33 in duodenum 4 and 24 hours after exposure in exp.1 (n=5) and exp.2 (n=8). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05, ** a p-value ≤ 0.01 and *** a p-value ≤ 0.001 Gray boxes denote a median value for each respective group and each symbol denotes one mouse. White box in C) shows the overall p-value obtained from the Kruskal-Wallis test.
**ST2 in serum**

In both experiments, exposure to DON resulted in statistically significantly increased levels of ST2 in serum after 4 (Figure 9 A and C). At 24 hours after exposure in exp.2, the DON group was also statistically significantly different from all other groups (Figure 9 D). This was not seen at the same time-point after exposure to DON+PE in exp. 1 (Figure 9 B). In addition, no statistically significant differences or effects were observed at 48 hours after exposure in exp. 1 (Figure 21 F, appendix).

**ST2 in ileum and duodenum**

ST2 in ileum was below the detection limit in both experiments. In duodenum, no clear effects of barrier disruptors on ST2 was observed, although we detected statistically significant differences in levels of ST2 at 4 hours in exp. 1 (Figure 19 G, appendix) and between the control group and the GLY group at 24 hours in exp. 2 (Figure 22 N, appendix). No statistically significant differences between any groups were detected at 24 or 48 hours in exp. 1 (Figure 19 H-I, appendix) or at 4 hours in exp. 2 (Figure 22 M, appendix).

**TSLP in ileum and duodenum**

In duodenum, the groups exposed to DON showed statistically significantly increased levels of TSLP at 4 hours after exposure compared to the HDM+PE group in exp. 1 (Figure 10 A), and the control group in exp.2 (Figure 10 B). We found no statistically significant differences between any groups at 24 and 48 hours after exposure in duodenum (Figure 19 B-C, appendix) or at any of the time-points in ileum in exp. 1 (Figure 20 A-C, appendix). We observed no changes in TSLP levels in duodenum after 24 hours in exp.2 or in ileum at 4 and 24 hours after exposure (Figure 22 B, C and D, appendix).

**IL-25**

No clear trend for IL-25 levels was observed, although statistically significant differences were detected between the DON+PE group and the HDM+PE group at 48 hours after exposure in exp.1 (Figure 19 L, appendix). There were no statistically significant differences at any of the other two time-points in exp.1 (Figure 19 J-K, appendix), and in exp.2, the levels of IL-25 in duodenum and ileum were all below the detection limit.
Figure 9. *ST2 in serum 4 and 24 hours after exposure in exp.1 (n=5) and exp.2 (n=8).* Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a median value for each respective group and each symbol denotes one mouse.

Figure 10. *TSLP in duodenum 4 hours after exposure in exp.1 (A, n=5) and exp.2 (B, n=8).* Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a median value for each respective group and each symbol denotes one mouse.
Gut barrier permeability: FABP2 and Ara h 2 in serum and IL-22, claudin-2 and occludin in intestine

FABP2 and Ara h 2 in serum and IL-22 in ileum and duodenum

For the serum levels of FABP2, there were statistically significantly lower levels only in the HDM+PE versus the PE group at 4 hours after exposure in exp.1 (Figure 21 A, appendix). No statistically significant differences were detected for FABP2 for 24 or 48 hours after exposure (Figure 21 B-C, appendix). The levels of the PE allergen Ara h 2 in serum samples and IL-22 in ileum and duodenum in exp.1 could not be detected as the levels were below the detection limit. Due to the low levels in exp. 1, levels of FABP2 and Arah2 and were not assessed in exp.2.

Claudin-2 and occludin in histological sections of intestine

We observed some differences in intensity of color in the claudin-2 and occludin stained sections, but the variation between each section was too large to make reliable conclusions. Therefore, we determined to not use the results to assess possible differences between groups for up- or downregulation of levels of the TJ proteins. HE stained sections showing the morphology of the intestine are illustrated in Figure 12 and sections stained for claudin-2 are illustrated in Figure 11.

Figure 11. Claudin-2 stained sections (swiss roll). Duodenum stained for claudin-2 (brown) in exp.1, 4 hours after exposure visualized in light microscope, magnified 10 times (PBS, PE, PE+CT, PE+DON, PE+HDM) and 40 times (PE+HDM 40X), respectively (photo: Ellen Namork).
Figure 12. HE stained sections (swiss roll) showing the morphology of the intestine. HE stained sections of ileum in exp.1, 24 hours after exposure to CT+PE. Magnified 10x (left panel) and 20x (right panel), in light microscope. Abbreviations: PP = Peyer’s patches, V = Villi with BB = brush border (microvilli of enterocytes), IC = immune cells (eosinophils, neutrophils, macrophages), C = crypt with P = Paneth cells, LP = lamina propria, MM = muscular mucosa (photo: Ellen Namork).

**Mucosal immune responses: Total IgA in intestine and serum**

**Mucosal immune response: total IgA in ileum, duodenum and serum**

In exp.2, the PE group had higher levels of total IgA expressed at 24 hours after exposure (Figure 13 A-C). In duodenum, total IgA in the PE group was statistically significantly higher than the control group and the DON group (Figure 13 A). There were no statistically significant differences 4 hours after exposure in the intestine or serum (Figure 22 I, K and M, appendix). The levels of total IgA were not assessed in exp.1 as the levels were undetectable due to either too high or low dilutions of intestinal samples and serum samples.

A summary of all results for markers of intestinal epithelial responses, gut barrier permeability, and mucosal and lymphoid immune responses, including markers not detected in both experiments are listed in Table 16 and Table 17 (appendix).
Figure 13. Total IgA at 24 hours after exposure in exp.2 (n=8). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a median value for each respective group and each symbol denotes one mouse.

Lymphoid immune responses: cytokine release from immune cells stimulated with LPS or ConA ex vivo

TNF-α, IL-6, IFNy and IL-1β in LPS-stimulated MLN and PPs cells

In LPS-stimulated lymph node cells from MLNs, the groups exposed to DON at 4 hours consistently showed increased levels of a number of the cytokines assessed.

At 4 hours after exposure, TNF-α was increased in DON groups, statistically significantly higher than the control group and the CT+PE group in exp. 1 (Figure 14 A), and the PE group in exp.2 (Figure 14 B). In exp.1 at 48 hours after exposure, no clear effect on TNF-α levels were observed, although a p-value of 0.0159 was found using the Kruskal-Wallis test, but no statistically significant group differences were found by Dunn's multiple comparison test (Figure 26 C, appendix). No statistically significant differences in TNF-α...
levels were found for any groups at 24 after exposure in neither experiment (Figure 26 B for exp.1 and Figure 27 B for exp.2, appendix) or at 48 hours in exp. 1 (Figure 26 C, appendix).

Also, the levels of IL-6 were increased by DON at 4 hours after exposure (Figure 14 C and D). This did not reach statistical significance in exp. 1, but in exp.2, the levels of IL-6 were statistically significantly higher than in the PE group and the HDM group (Figure 14 D). Despite not detecting statistically significant differences in exp.1, the elevated levels in both experiments suggest an increase in levels of IL-6 in response to exposure to DON 4 hours after exposure. No statistically significant group differences were observed for IL-6 at 24 in both experiments (Figure 26 E for exp. 1 and Figure 27 D for exp. 2, appendix) or at 48 hours in exp. 1 (Figure 26 F).

In exp.2, the DON group also had statistically significantly higher levels of IL-1β at 4 hours after exposure than the control group and the PE and CT groups (Figure 14 E). No statistically significant differences were found at 24 hours after exposure (Figure 27 H, appendix). We could not detect the levels of IL-1β in exp.1 as the levels were below the detection limit.

The levels of IFNγ were increased in the DON+PE group at 4 hours after exposure, statistically significantly higher compared to the HDM+PE group in exp.1 (Figure 15 A). For the same time-point in exp. 2, no statistically significant differences were obtained, although the levels apparently were increased in the DON group (Figure 15 C). 24 hours after exposure, IFNγ in the DON group was statistically significantly higher than all other groups (Figure 15 D). No effects of exposure to any of the compounds were found in exp.1 at the same time-point (Figure 15 B), or at 48 hours after exposure (Figure 26 I, appendix). The consistent observations over the experiments and time points support a finding of elevated levels of IFNγ in response to DON.

The levels of TNF-α, IL-6, IFNγ and IL-1β in MLNs were also assessed in PPs in exp. 2 at 24 hours after exposure. No statistically significant differences in levels of any of the cytokines were found in the PPs (Figure 27 right panel B, D, F, and H, appendix). Except for IL-1β, the cytokine levels in the PPs were lower than the levels in the MLNs.
Figure 14. TNF-α, IL-6 and IL-1β in MLNs stimulated with LPS ex vivo 4 hours after exposure in exp.1 (n=5) and exp.2 (n=4). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05. Gray boxes denote a median value for each respective group and each symbol denotes one mouse.
Figure 15. IFNγ in MLNs stimulated with LPS ex vivo 4 hour and 24 hours after exposure in exp. 1 (n=5) and exp. 2 (n=8). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a median value for each respective group and each symbol denotes one mouse.

**IFNγ, IL-17, IL-13, IL-10, IL-5 and IL-4 in ConA-stimulated immune cells**

We found no clear effects of the barrier disruptors on ConA-induced cytokine secretion in lymph node cells from MLNs. No statistically significant differences between the groups for the cytokines IFNγ, IL-13 and IL-10 and in ConA-stimulated lymph node cells at any time-points in exp. 1 (Figure 24, appendix). For the levels of IL-17 at 48 hours after exposure (Figure 24 I, appendix), a p-value of 0.0374 was obtained from the Kruskal-Wallis test but no statistically significant group differences were found by Dunn's multiple comparison test. For the levels of IL-5 at 24 hours after exposure (Figure 24 E, appendix), a p-value of 0.0245 was obtained but also here no statistically significant group differences were found using the same statistical methods. At all other time-points, we detected no clear effects of exposure to any compound.
In exp. 2, we found no statistically significant differences for any of the cytokines IL-6, IL-13, IL-5 and IL-2 from the ConA-stimulated lymph node cells (Figure 25, appendix). The levels of IL-4 and IL-1β were all below the detection limit (IL-4, IL-2 and IL-1β were only measured in exp. 2).

A summary of the results from ConA- and LPS-stimulated cells from both experiments are listed in Table 18 and Table 19, respectively (appendix).

### 3.2 Food allergy model

Overall, the results from exp.3 confirmed CT as a positive control for allergy adjuvant effect, as the CT+PE group was statistically significantly increased for all analyses (Figure 16 A-E). Additionally, several of the mice treated with DON+PE showed adverse anaphylactic score values, indicating that DON also promoted allergy development in our model.

**Food allergy sensitization: Ara h 2 specific IgE and total IgE in serum**

The levels of Ara h 2 specific IgE in serum before PE challenge were below the assay detection limit (results not included). For the levels of total IgE, there were statistically significantly higher levels in the CT+PE group compared to the control, PE, DON+PE, and GLY+PE groups (Figure 16 A). In addition, the CT group had statistically significantly higher levels compared to the control, PE, HDM+PE, and GLY+PE groups. The data also show a trend (statistically non-significant) of increased levels of total IgE levels in serum for the DON+PE group (Figure 16 A).

**Anaphylaxis: score of anaphylaxis, body temperature drop and mMCP-1 in serum and intestine**

When including only the mice challenged to 5 mg, the anaphylactic scores for the CT+PE and DON+PE groups were clearly increased, confirmed by statistically significantly higher anaphylactic scores compared to one or more groups (Figure 16 D). However, when all mice were included (challenged with either 2.5 mg or 5 mg, see Table 11), only the score for the CT+PE group was statistically significantly increased (to one or more groups; Figure 16 E). A drop in rectal body temperature was also measured to assess anaphylaxis.
Here, the CT+PE group was statistically significantly different from the control, PE, DON+PE and GLY+PE groups. For the levels of mMCP-1 in serum, the CT+PE group had statistically significantly higher levels than all other groups, and the CT group was statistically significantly different from the PE group (Figure 16 B). There was one strong responder one or more groups. in the HDM+PE group for levels of mMCP-1 in serum (Figure 16 B), and the same mouse had also a dramatic drop in body temperature (Figure 16 C) and an anaphylactic score of 3 (Figure 16 D). The strong responder is colored red (Figure 16 A-D, the mouse had been challenged to 2.5 mg PE and was excluded in E). Expressed levels of mMCP-1 in histological sections of intestine were not detected as the staining method was not successfully developed or tested due to lack of time. A summary of all results from exp. 3 for markers of anaphylaxis and allergy is listed in Table 20 (appendix).

**Lymphoid immune response: cytokine release from spleen cells stimulated with PE ex vivo**

Except for IL-1β (Figure 17 F), the CT+PE group had statistically significantly higher levels of all cytokines (Figure 17 A-E and G-H). These results show a clear pattern of increased levels of cytokines associated with an activated immune response and allergy. Despite no effects on group level, there was one strong responder in the HDM+PE group demonstrating elevated levels of inflammatory cytokines IFNγ, IL-6 and TNF-α (Figure 17 B, D and E). This was not the same mouse as the strong responder for markers of anaphylaxis and is colored blue. DON+PE or GLY + PE did not affect the PE-induced cytokine release. A summary of all results from exp. 3 for levels of cytokines from spleen cells stimulated with PE ex vivo are listed in Table 20 (appendix).
Figure 16. Total IgE, mMCP-1, temperature change and score in exp.3. A-D) One strong responder in the HDM+PE group is colored red. B) The upper detection limit for mMCP-1 was 139204.400 pg/mL and is marked with a dotted line. C) Temperature-change shows the change in rectal body temperature. D-E) Anaphylactic score after i.p. challenge shows the severity of anaphylaxis. D) Score of mice challenged to 2.5 mg or 5 mg PE only, E) score of all challenged mice. For all analyses, n=12 per group with the following exceptions: A and B), CT+PE (n=8) and GLY+PE (n=10) due to lack of serum. D) All mice challenged with 2.5 mg is excluded (PBS (n=8), PE (n=8), CT+PE (n=4), PE+DON (n=8), PE+HDM (n=4), PE+GLY (n=8), CT (n=8)). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05, ** a p-value ≤ 0.01, *** a p-value ≤ 0.001, and **** a p-value ≤ 0.0001. Gray boxes denote a median value for each respective group and each symbol denotes one animal.
Figure 17. Cytokine release from PE-stimulated spleen cells in exp. 3 (n=12). One strong responder in the HDM+PE group is colored in blue. Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05, ** a p-value ≤ 0.01, *** a p-value ≤ 0.001, and **** a p-value ≤ 0.0001. Gray boxes denote a median value for each respective group and each symbol denotes one animal.
3.3 Developed methods

Quantification of protein content in intestinal homogenate supernatant by BCA

Our results suggest that, despite absence of protease-inhibitor and increased speed, the dilution was the most important factor for optimal protein content. The optimal dilution in test 1 was 1:100 (Table 13), and the optimal dilution in test 2 was 1:50 and 1:15 (Table 14). The chosen speed for the homogenization protocol was 6000 rpm, as recommended by the manufacturer, and protease-inhibitor was added, in accordance with method developed by Bol-Schoenmakers et al. (2016).

Table 13. Absorbance values (OD-values) at 570 nm for test 1.

<table>
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<th>BCA test 1</th>
<th>OD-values at 570 nm (doublets)</th>
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</tr>
<tr>
<td>1:10 000</td>
<td>0,068</td>
</tr>
</tbody>
</table>

Table 14. Absorbance values (OD-values) at 570 nm for test 2.

<table>
<thead>
<tr>
<th>BCA test 2</th>
<th>OD-values at 570 nm (doublets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>0.25 mg tissue, 0,5 mL volume</td>
</tr>
<tr>
<td>1:10</td>
<td>Sample 1</td>
</tr>
<tr>
<td>1:10</td>
<td>0,8055</td>
</tr>
<tr>
<td>1:50</td>
<td>0,3035</td>
</tr>
<tr>
<td>1:100</td>
<td>0,218</td>
</tr>
</tbody>
</table>
Weighting of intestinal tissue samples

The weight of the samples showed that 1 cm of duodenum weighed approximately 30 mg (weight average 33.5 mg), while 1 cm of ileum weighed approximately 20 mg (weight average 24.1 mg), as listed in Table 15.

Table 15. Weight of 1 cm samples of duodenum and ileum.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: duodenum</td>
<td>3.29 mg</td>
</tr>
<tr>
<td>2: duodenum</td>
<td>3.32 mg</td>
</tr>
<tr>
<td>3: duodenum</td>
<td>3.45 mg</td>
</tr>
<tr>
<td>1: ileum</td>
<td>2.35 mg</td>
</tr>
<tr>
<td>2: ileum</td>
<td>2.41 mg</td>
</tr>
<tr>
<td>3: ileum</td>
<td>2.48 mg</td>
</tr>
</tbody>
</table>
4 Discussion

4.1 Allergy-promoting capacities of the barrier disruptors

CT and DON

The most consistent response detected in our food allergy model was anaphylaxis (score, temperature-drop and mMCP-1) and an allergic immune response (total IgE and cytokine profile) in the CT+PE group. We also detected indications of food allergy in the DON+PE group (clear increase in anaphylactic score and a trend for increased levels of total IgE), suggesting that DON did indeed have an adjuvant effect. However, score was the only statistically significant finding that was indicative of food allergy for the DON+PE group, which was lower than the score for the CT+PE group. In addition, the CT+PE group was statistically significantly different to one or more groups in all results for markers of food allergy, implying that the CT+PE group had a stronger allergic response compared to the DON+PE group, and that CT was a stronger adjuvant than DON even at a lower dose level, as we administered 15 µg CT and 100 µg of DON.

A possible explanation for the low score for the DON+PE group may have been the challenge dose. A miscalculation of the PE challenge dose concentration led to a substantial amount of mice being exposed to half the amount (2.5 mg PE) of the intended challenge dose (5 mg PE) (Table 11). For the DON+PE group, the anaphylactic response after a 2.5 mg PE challenge was in general low, while it was stronger for the mice challenged with 5 mg. This resulted in a group median that was high if including mice only administered 5 mg PE (a median score of 3), and considerably higher than the group median if all mice were included (a median score of approximately 1.5). This suggests that the PE challenge dose affected the overall result for anaphylaxis, affecting the DON+PE group more than the CT+PE group. Nevertheless, the challenge dose taken into account, a weaker allergic response to DON+PE compared to CT+PE was also reported by Bol-Schoenmakers et al. (2016) in response to exposure to DON or CT with whey allergen.
There was a trend for elevated levels of total IgE in the DON+PE group, and statistically significantly elevated levels in the CT+PE group and the CT group. The CT group was never exposed to PE proteins: the increased levels of total IgE in the CT group compared to levels of other groups suggest that these mice had become sensitized to proteins in their feed, as this was their only source of food proteins. We speculate that they had become sensitized to wheat, barley or soy, as this was the main components of their feed. In addition, the CT group also had statistically significantly elevated levels of mMCP-1 in serum and a trend in temperature-drop. This could be explained by a) the mice having a reaction towards proteins in their feed, which they were sensitized to, or, b) a cross-reaction to PE. Cross-reactivity between soy and PE can occur due to similarities in protein structures (Eigenmann, Burks, Bannon, & Sampson, 1996; Hurlburt et al., 2013).

HDM

HDM groups showed no change in levels for any of the early markers and no clear pattern of food allergy. The allergy-promoting capacities of HDM seems to be not as strong in the intestine as what has been reported for airway allergic disease (Chu et al., 2013; Hammad et al., 2009; Willart et al., 2012). However, some results from our food allergy model suggest a possible trend for developed food allergy by exposure to HDM+PE. Two mice had a strong response, in which one had levels of mMCP-1 in serum at the upper detection limit of the assay, a dramatic drop in body temperature, and an anaphylactic score of 3. However, the group median of the anaphylactic score was 1, suggesting that there was a weak group response mainly driven by two mice with a score of 3 (Figure 16 D). The other high responder had a strong response in levels of inflammatory cytokines TNF-α, IL-6 and IFNγ in PE-stimulated spleen cells. We can only speculate whether the strong responders are a source of natural variability, or indicators of possible effects not detected due to small number of mice per group. In addition, we do not know whether the number of responders in the HDM+PE group would have been higher if all mice had received the 5 mg PE challenge dose instead of 2.5 mg.

The adjuvant capacity of HDM in intestine and food allergy development remains speculative, but our results do indicate that HDM have adjuvant capacities in the intestine. Further investigation on the dose-relationship of HDM regarding adjuvant effects in the intestines will be required, also in light of the known effects in airways.
GLY

Exposure to GLY showed no adjuvant or barrier disruptor effects in our experimental system at the chosen dose. The dose for GLY was based on mice studies which assessed the effects of oral exposure to Roundup® regarding behavior (Ait Bali et al., 2017) and hepatic, hematological and oxidative effects (Jasper et al., 2012). As these studies had endpoints not suitable for our purpose, we also used one study that assessed intestinal inflammation and permeability of exposure to chlorpyrifos (Zhao et al., 2014). Although chlorpyrifos is a completely different pesticide from GLY, the endpoints of intestinal inflammation and permeability were more suitable for our study.

There is a lack in research on possible effects of exposure to GLY in the intestine and allergy-promoting effects. Although we cannot exclude adjuvant effects of GLY from the present study of one single dose, our lack of observed early effects on barrier function, immune responses or food allergy development do not suggest that GLY exert strong allergy-promoting effects. This is in agreement with an airway inflammation study in mice conducted by Kumar et al. (2014), where co-administration of GLY-rich air and OVA allergen did not substantially affect the immune response.

PE-stimulated spleen cells showed a mixed Th1/Th2/Th17 response to CT+PE

In agreement with the stronger allergic response in the CT+PE group relative to the DON+PE group, PE-induced cytokines from spleen cells were in general only increased in the CT+PE group. We detected a cytokine pattern in PE-stimulated spleens that showed a mixed Th1/Th2/Th17 immune response with elevated levels of TNF-α, IL-6, INF-γ, IL-1β, IL-17, IL-13, IL-10 and IL-2. These cytokines play different immunological roles, but the upregulation is in accordance with an ongoing, allergic inflammatory immune response. TNF-α, IL-6 and IFNγ are inflammatory cytokines, IL-17 is associated with ongoing allergic inflammation, IL-13 is a pro-allergic cytokine, IL-10 has regulatory roles and IL-2 plays a vital role in T cell proliferation (Akdis et al., 2016). While IL-10 is often referred to as a regulatory and immunosuppressive cytokine, an upregulation of IL-10 in allergic mice is in accordance with the literature (Morafo et al., 2003; Vinje, Namork, & Lovik, 2011). A possible explanation for the elevated levels is that the production of IL-10 is a counteractive response to regulate ongoing immune reactions (Kumar et al., 2014). The
upregulation of IL-2 and the inflammatory cytokines TNF-α, IL-6, IFN-γ and IL-17 illustrates an activation of a general immune response and an inflammatory state.

### 4.2 Early effects of the barrier disruptors

The most noticeable pattern of response detected in the short-term experiments were the rise in levels of the alarmin marker IL-33 and its receptor ST2, and the inflammatory cytokines TNF-α, IL-6, IFNγ and IL-1β from LPS-stimulated lymph node cells for the groups exposed to DON+PE (exp. 1) and DON only (exp. 2). Although varying for some of the markers, this was in general observed in both experiments. This suggest that DON exerts early, local effects on the intestine and promote inflammatory immune responses, and that the chosen early markers IL-33, ST2, TNF-α, IL-6 and IFNγ reflected the mode of action of DON better than for CT. No clear responses to CT, HDM or GLY were detected for markers of early effects on gut barrier function (intestinal epithelial responses, gut permeability, or mucosal and lymphoid immune responses).

**Elevated levels of IL-33 and ST2 imply an activated intestinal epithelial response to DON**

We hypothesized that the levels of IL-33, TSLP and IL-25 would rise early after exposure to barrier disruptors that promote allergy, because they a) are alarmins, which means levels rise in response to damaged epithelial cells, and hence an altered intestinal epithelial barrier, and b) drive Th2 responses, i.e., pro-allergic responses. Only IL-33 and its receptor ST2 showed a clear pattern of increased levels, which was mainly in the groups exposed to DON in both experiments. The only exception was for the CT group in exp. 2, where levels of IL-33 in ileum were increased only for the CT group.

After exposure to DON, increased levels of ST2 in serum and IL-33 in intestine, but not IL-25 and weakly TSLP, were detected. However, the consistency in elevated TSLP levels for groups exposed to DON in both experiments at 4 hours after exposure support that also TSLP levels increased as a response to exposure to DON. In agreement, Chu et al. (2013) demonstrated that IL-33 played a central role in PE sensitization, whilst IL-25 and TSLP did not. Further, the authors discussed how the sensitization pathway may affect the outcome of the cytokine response: studies showing a clear role of TSLP in allergy.
development had administered allergens by i.p. injection, as opposed to our model and Chu et al. (2013), which administered PE by i.g. gavage. It was reported by the authors that the PE food allergy was associated with production of TSLP, but that the development was independent of TSLP. This may explain why our study did not show effects on TSLP and IL-25, even though DON promoted allergy development. IL-33 showing up as the most consistent early marker of the allergy-promoting effect of DON, and CT in one case, is in accordance with Chu et al. (2013) reporting IL-33 as central in allergic sensitization to PE.

For both short-term experiments, the levels of ST2 in serum from groups exposed to DON corresponded to IL-33 in intestine, but the response was different from exp. 1 to exp. 2: In exp. 1, the group exposed to DON+PE had elevated levels in ileum, while in exp. 2, the group exposed to only DON had elevated levels in duodenum. Elevated levels of IL-33 in duodenum in response to exposure to DON without allergens were in accordance with Bol-Schoenmakers et al. (2016). However, Bol-Schoenmakers et al. (2016) also reported elevated levels of ST2 in intestine. We observed no such trend for levels of ST2 in duodenum, possibly due to a weaker response to DON in our study.

**Gut barrier permeability**

We did not detect any effects of increased barrier permeability in response to any of the barrier disruptors. Thus, we cannot conclude that the gut barrier integrity was altered, or that the permeability was increased. We see several possible explanations for this: firstly, our immunohistological method for assessing the TJ composition in intestinal epithelial cells was not suitable due to too large variation. Secondly, it is possible that the markers Ara h 2 and FABP2 in serum and IL-22 in intestine were not sensitive enough and in fact unsuitable as markers of barrier integrity and permeability in our experimental setup, although Ara h 2 and FABP2 has previously been used as markers of gut permeability (JanssenDuijghuijsen et al., 2017). Lastly, the doses of the barrier disruptors might not have been high enough to cause an effect on the gut barrier integrity and permeability after a single exposure.
ConA- and LPS-stimulated lymph node cells showed a pro-inflammatory response, but not a pro-allergic response to DON

No skewing towards Th2 (pro-allergic) cytokines was detected in the lymph node cells stimulated with ConA. As DON and CT promoted food allergy, we expected to detect Th2 cytokines from the ConA-stimulated lymph node cells. However, the lack of statistically significant findings at 24 and 48 hours could rather mean that the time-point was too early for detecting any Th2 response at all. It is well established that different LN responses and cytokines are expressed at different times and stages in an immune response, making it challenging to establish whether the immune response has started or finished, as illustrated in Figure 2 in the introduction (section 1.2). For instance, van Zijverden et al. (2000) showed that the MLN responses to allergen and adjuvants were not seen at 2 days, but at 5 days after exposure. Also, in a mouse allergy experiment with sensitization to OVA allergen and GLY with the earliest time-point being 8 days after exposure, an immune response with production of Th2 cytokines IL-13, IL-5 and IL-4 was detected (Kumar et al., 2014). Thus, these taken together with our results suggest that our latest time-point might in fact have been too early to detect Th2 response in the MLN and could therefore not serve to confirm the pro-allergic responses for CT and DON that was evident in exp. 3.

DON induced production of inflammatory cytokines TNF-α, IL-6, IFNγ, and IL-1β in lymph node cells stimulated with LPS. Inflammatory cytokines may promote sensitization and thus later allergic responses, as illustrated by reports of TNF-α acting as an allergy adjuvant in airway models (Lambert, Selgrade, Winsett, & Gilmour, 2001). Our results suggest that, at an early stage, exposure to DON elicits inflammatory responses, and since DON promoted food allergy in exp. 3, it is tempting to speculate that these responses were contributors to the adjuvant effects of DON.

Early markers of altered gut barrier function or lymphoid immune responses as predictors of food allergy development

The aim of our study was to detect early markers of effects of barrier disruptors that promote allergy development. The markers of interest were based on their known roles in allergy development. This was done in order to possibly link the markers to MIE and KE in an AOP for food allergy development. However, in our system, no general marker of early effects from exposure to CT and DON were detected in the short-term experiments,
although both had adjuvant effects in the food allergy experiment. No early effects of HDM or GLY were detected for the chosen markers reflecting an altered gut barrier function or immune responses either. We detected only clear responses for IL-33 in intestine, ST2 in serum and inflammatory cytokines TNF-α, IL-6, IFNγ, and IL-1β in LPS-stimulated lymph node cells as early markers of effects of DON exposure. Thus, as both CT and DON showed adjuvant effects in food allergy development, but early markers only were detected in DON groups, our results suggest that DON and CT may have different modes of action regarding effects on barrier function and adjuvanticity. None of the chosen markers reflecting early effects on the gut barrier function could be a general predictor of the adjuvant effects of CT and DON.

4.3 Methodological considerations

The first short-term experiment was a pilot study with few mice per group (n=5), serving as a basis for the design of the experimental setup for the second experiment with 8 mice per group. Thus, the two experiments were performed with similar methods and the results can therefore support each other, but they also had some differences in the experimental set-up and conditions.

a) The time-point of 48 hours was not included in exp. 2 as the overall responses detected in exp. 1 were at time-points 4 and 24 hours after exposure. The time-point of 48 hours might have been too early for detecting a Th2-skewed response in the LN cells (van Zijverden et al., 2000) while for detecting early effects, the time-point seemed to be too late as no effects were detected.

b) To detect possible effects of PE on the response to the barrier disruptor, we exposed the mice to a barrier disruptor with PE in exp. 1, and without in exp. 2. Hence, if any differences between exp. 1 and 2 were detected, we aimed to conclude that the presence of PE influenced the effect of the barrier disruptors. Unfortunately, the manufacturer of C3H/HeOuJ mice (Charles River) that were used in exp. 1 reported a positive finding for Streptococcus pneumoniae in the animal facilities, but that it was considered unlikely to have health consequences in immunocompetent mice. However, due to the positive finding, we chose to use C3H/HeOuJ mice from another provider in exp.2. We do not suspect the presence of S. pneumonia to have had any effect on the mice, as the mice were
declared healthy for research. The strain of mice was also similar in the two experiments. Nevertheless, we cannot exclude that any of the observed differences from exp. 1 to exp. 2 were due to these differences, and not the presence of PE.

4.3.1 Food allergy model

The food allergy model is well established in our lab with lupine (Vinje et al., 2009) and PE (unpublished data), and has the advantage of being able to reveal clinically relevant food allergic responses such as anaphylaxis. A disadvantage of using score of anaphylaxis as a marker of allergy is that the method is subjective, as it is based on one individuals’ assessment of the severity of an allergic reaction. Therefore, measuring rectal body temperature at the start and end of challenge, as well as measuring mMCP-1 in serum and in intestinal tissue (Vaali et al., 2006), is a way of assuring objective findings. Our measurements of score and drop in body temperature were correlated (Figure 18, appendix), which supports that score is applicable to assess anaphylaxis.

A limitation of the present model is the lack of an assay detecting PE-specific IgE. It has been difficult to establish such an assay due to high amounts of background noise from the extract (unpublished data) or too high expenses if combining all recombinant Ara h allergens. Therefore, we chose to detect IgE specific to Ara h 2, which is one of the most important PE allergens (Koppelman, Wensing, Ertmann, Knulst, & Knol, 2004) and considered a good marker of severe PE allergy (Klemans et al., 2013). However, as the levels of Ara h 2 specific IgE were too low to be detected, we chose to include detection of total IgE levels. In comparison to PE specific IgE and Ara h 2 specific IgE, which are specific markers of PE sensitization, total IgE is a general marker for allergic sensitization to any antigen. However, in experimental settings with controlled exposures to one or a few allergens, like in for instance feed, increased total IgE levels have often been shown to reflect the allergen-specific IgE (Granum, Gaarder, & Lovik, 2001), thus, in our experiment reflects allergic sensitization.

In an anaphylactic reaction, release of histamine leads to vasodilation and an increased permeability of the blood vessels, resulting in an increased vascular leakage of fluid from blood to tissue with possible considerable leakage of IgE from the bloodstream. This may have affected the levels of total IgE in serum. Ideally, we would have measured total IgE
in blood sampled before challenge, but, the blood gave small volume and we therefore prioritized analysis of Ara h 2 specific IgE. As the levels of total IgE were measured in blood sampled after challenge, the levels were most likely decreased as a result of the anaphylactic reaction (Saenz de San Pedro et al., 2002; Vinje et al., 2011). Thus, this may have resulted in smaller group differences than expected and not reflecting the true level in sensitized mice.

Another limitation of our experimental design is that we were only able to administer one dose of our tested barrier disruptors. This was due to practical considerations like work load per experimental day and cost, as well as to reduce the number of animals used. Although the doses were based on dose levels previously reported to have effects (allergy adjuvant effects for DON and CT, and other less relevant effects for HDM and GLY), we cannot with certainty conclude that lack of effects reflect lack of adjuvant capacity. However, since the same doses were used in the short and long-term experiments, we could assess the possible links between expression of early markers and allergy adjuvant effects. Nevertheless, the barrier disruptors CT and DON showed adjuvant effects in our experiment.

4.4 Future perspectives

We detected only IL-33 in intestine, ST2 in serum and TNF-α, IL-6 and IFNγ as early markers in groups exposed to DON, thus, our chosen markers could not additionally identify the adjuvant capacity of CT. A suggestion for further studies is to use a screening approach, in which a wider range of possible markers can be tested so that the best subset of markers needed for prediction of adjuvant capacity could be identified.

For the short-time experiments, the chosen time-point of 4 hours was suitable for assessing early effects in the intestines of exposure. In order to link barrier disruption with increased risk of food allergy development, time-points longer than 48 hours should be included to be able to detect Th2 cytokines in LNs associated with later allergy development.

There is a possibility that the somewhat weak response detected in the short-term experiments was partly due to the single exposure to barrier disruptors. Several
exposures in one study with a longer duration might create a stronger and more easily detectable response, as well as represent a more relevant exposure scheme.

To assess the possible adjuvant effects of HDM in intestine and possible links to food allergy development, a dose-response experiment should be performed.

The current methods for the immunohistological analysis of the TJs need further development. Alternatively, other methods may be more suitable for assessing TJ composition, as histological methods are time-consuming, subjective methods that are difficult to generalize. A suggestion is measuring mRNA levels of TJ proteins, as was conducted by Bol-Schoenmakers et al. (2016).
5 Conclusions

The barrier disruptors CT and DON promoted development of food allergy in our model, while HDM and GLY did not at the present doses. Hence, CT and DON had adjuvant effects, with CT giving the strongest response. None of the chosen early markers reflecting intestinal epithelial response, altered gut barrier integrity and/or permeability and mucosal or lymphoid immune responses, could be a general predictor the adjuvant effects of both CT and DON. These early markers were also not affected by HDM and GLY.

We cannot conclude whether the barrier disruptors had early effects on the permeability, but we detected an activated intestinal epithelial response (IL-33 and ST2) and a pro-inflammatory response in LPS-stimulated lymph node cells to DON exposure. As both DON and CT had adjuvant effects, but the early markers were only affected by DON, our results suggest that DON and CT have different modes of action at the early stages and after a single exposure to the given doses. Our results indicate that a combination of a wider set of markers, reflecting different modes of action, is necessary to enable prediction of barrier disrupting compounds with food allergy adjuvant capacity.


Koppelman, S. J., Wensing, M., Ertmann, M., Knulst, A. C., & Knol, E. F. (2004). 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 & Experimental Allergy, 34*(4), 583-590.


Moussion, C., Ortega, N., & Girard, J. P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLOS One, 3*(10), e3331.


Appendix

Figures

Figure 18. Correlation between score and drop in body temperature in exp. 3. Drop in body temperature from time before i.p. challenge and at time of euthanasia and death, and the anaphylactic score is shown. White box shows the Spearman r-value (r) and p-value (p). Each circle denotes one animal.
Figure 19. TSLP, IL-33, ST2, IL-25 and IL-22 (not detected) in duodenum in exp. 1 (n=5). Results for time-points 4, 24 and 48 hours (hr.) after exposure are shown. Levels of IL-25 (J-L) are expressed as OD-values. Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05. Gray boxes denote a median value for each respective group and each symbol denotes one animal.
Figure 20. TSLP, IL-33 and ST2 (not detected) in ileum in exp. 1 (n=5). Results for time-points 4, 24 and 48 hours (hr.) after exposure are shown. Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a median value for the respective group and each symbol denotes one animal.
Figure 21. Levels of FABP2, ST2 and Ara h 2 (not detected) in serum in exp. 1. Results 4, 24 and 48 hours (hr.) after exposure are shown. Per group, n=5 with the following exceptions due to lack of serum: A) DON+PE group (n=4) and C) control group (n=4). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a median value for the respective group and each symbol denotes one animal.
Figure 22. TSLP, IL33, ST2, IL-22 (not detected) and IL-25 (not detected) in intestine in exp.2 (n=8). Results for time-points 4, and 24 hours (hr.) after exposure are shown. Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05, ** a p-value ≤ 0.01 and *** a p-value ≤ 0.001. Gray boxes denote a median value for the respective group and each symbol denotes one animal.
Figure 23. Total IgA and ST2 in serum in exp.2. Results for time-points 4 and 24 hours (hr.) after exposure are shown. Per group, n=8 with the following exception due to lack of serum: C) GLY group (n=7). Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** a p-value ≤ 0.01. Gray boxes denote a respective group, and each circle, square, triangle and sphere filled or open denotes one animal.
Figure 24. Cytokine release from MLN cells stimulated with ConA ex vivo in exp.1. Results for time-points 4, 24 and 48 hours (hr.) after exposure are shown. Per group, n=5 except for the CT+PE group (n=4) at 4 hours due to lack of LNs. Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05. Gray boxes denote median value for the respective group and each symbol denotes one animal. White boxes in E) and I) shows the p-values obtained from the Kruskal-Wallis test.
Figure 25. Cytokine release from MLN cells stimulated with ConA ex vivo in exp.2. Results for time-points 4 (n=4) and 24 hours (hr.) (n=8) after exposure are shown. At 24 hr., n=7 for the PE group due to lack of LNs. Gray boxes denote median value for the respective group and each symbol denotes one animal.

**IL-1β not detected**

**IL-4 not detected**
Figure 26. Cytokine release from MLN cells stimulated with LPS ex vivo in exp.1 (n=5). Results for time-points 4, 24 and 48 hours (hr.) after exposure are shown. Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05. Gray boxes denote median value for the respective group and each symbol denotes one animal. White box in C) shows the p-value obtained from the Kruskal-Wallis test.

**IL-1β not detected**
Figure 27. Cytokine release from MLN and PP cells stimulated with LPS ex vivo in exp.2. Results for time-points 4 (n=4) and 24 hours (hr.) (n=8) after exposure are shown. PPs and MLNs are separated by a stapled line. All PPs per group were pooled due to low cell count, giving 3 points per group except for the CT group with 2 points. Brackets symbolize statistically significant differences between two groups, where * symbolizes a p-value ≤ 0.05 and ** symbolizes a p-value ≤ 0.01. Gray boxes denote a median value for the respective group and each symbol denotes one animal.
### Tables

**Table 16.** Summary of results and observed trends (intestine only) from exp. 1 and 2 for markers of intestinal epithelial response (TSLP, IL-33, ST2 and IL-25), gut permeability (IL-22) and mucosal immune response (total IgA).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Exp. 1</th>
<th></th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duodenum</td>
<td>Ileum</td>
<td>Duodenum</td>
</tr>
<tr>
<td><strong>Time-point</strong></td>
<td>4h</td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>TSLP</td>
<td>↑DON</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-33</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑DON</td>
</tr>
<tr>
<td>ST2</td>
<td>S</td>
<td>N.S.</td>
<td>N.D.</td>
</tr>
<tr>
<td>IL-25</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-22</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Total IgA</strong></td>
<td>Not analyzed</td>
<td></td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S. = non-significant (no statistically significant differences), N.D. = not detected (under detection limit), S = statistically significant differences, but no trend. Red color = group with significant difference to other group(s). Yellow color = observed trends. ↑ denotes higher levels of a marker in the respective group compared to the levels in other groups.

**Table 17.** Summary of results from exp. 1 and 2 (serum only) for marker of intestinal epithelial response (ST2), gut barrier permeability (FABP2 and Ara h 2), and mucosal immune response (total IgA).

<table>
<thead>
<tr>
<th><strong>Time-point</strong></th>
<th>Exp. 1</th>
<th></th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4h</td>
<td>24h</td>
<td>48h</td>
</tr>
<tr>
<td>ST2</td>
<td>↑ DON</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>FABP2</td>
<td>S</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Ara h 2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Total IgA</strong></td>
<td>Not analyzed</td>
<td></td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S. = non-significant (no statistically significant differences), N.D. = not detected (under detection limit), S = statistically significant differences, but no trend. Red color = group with significant difference to other group(s). ↑ denotes higher levels of a marker in the respective group compared to the levels in other groups.
Table 18. Summary of results from exp. 1 and 2 for markers of lymphoid immune responses in ConA-stimulated MLN and PPs cells.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MLN</td>
<td>MLN</td>
</tr>
<tr>
<td>Time-point</td>
<td>4h</td>
<td>24h</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Not analyzed</td>
<td>N.D.</td>
</tr>
<tr>
<td>IL-4</td>
<td>Not analyzed</td>
<td>N.D.</td>
</tr>
<tr>
<td>IL-2</td>
<td>Not analyzed</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Not analyzed</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-5</td>
<td>N.S.</td>
<td>p = 0.0245</td>
</tr>
<tr>
<td>IL-10</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-13</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-17</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IFNγ</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S. = non-significant (no statistically significant differences). N.D. = not detected (below detection limit). S = statistically significant differences, but no trend. Red color = group with significant difference to other group(s). Yellow color = observed trends. P-values are obtained from the Kruskal-Wallis test.

Table 19. Summary of results from exp. 1 and 2 for markers of lymphoid immune responses in LPS-stimulated MLN and PPs cells.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MLN</td>
<td>MLN</td>
</tr>
<tr>
<td>Time-point</td>
<td>4h</td>
<td>24h</td>
</tr>
<tr>
<td>TNF-α</td>
<td>↑DON</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Trend ↑DON</td>
<td>N.S.</td>
</tr>
<tr>
<td>IFNγ</td>
<td>↑DON</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.S. = non-significant (no statistically significant differences). N.D. = not detected (below detection limit). S = statistically significant differences, but no trend. Red color = group with significant difference to other group(s). Yellow color = observed trends. P-values are obtained from the Kruskal-Wallis test. ↑ denotes higher levels of a marker in the respective group compared to the levels in other groups.
Table 20. Summary of results from exp. 3 for anaphylaxis (score, temperature-drop, levels of mMCP-1 in serum), allergic sensitization (total IgE in serum) and lymphoid immune responses (cytokine release from PE-stimulated spleen cells TNF-α, IL-6, INF-γ, IL-1β, IL-17, IL-13, IL-10 and IL-2).

<table>
<thead>
<tr>
<th>Group</th>
<th>PBS (ctrl)</th>
<th>PE</th>
<th>CT+PE</th>
<th>DON+PE</th>
<th>HDM+PE</th>
<th>GLY+PE</th>
<th>CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆Temperature</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↓</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↓ Trend</td>
</tr>
<tr>
<td>Score</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>↑¹</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Total IgE</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>↑ Trend</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
</tr>
<tr>
<td>mMCP-1</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
</tr>
<tr>
<td>TNF-α</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-6</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IFNγ</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-17</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-13</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-10</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-2</td>
<td>N.S.</td>
<td>N.S.</td>
<td>↑</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S. = non-significant (no statistically significant differences). Red color = group with significant difference to other group(s). Yellow color = observed trends. P-values are obtained from the Kruskal-Wallis test. ↑ denotes higher and ↓ denotes lower levels of a marker in the respective group compared to the levels in other groups.

¹Only statistically significant differences for mice challenged with 5 mg PE.