Immune properties of cry1Ab-transgenic MON810 maize studied in mouse models of airway and food allergy

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Abbreviations

APC  Antigen-presenting cell
BALF Bronchoalveolar lavage fluid
Bt  Bacillus thuringiensis
Cry Crystal
CT Cholera toxin
E. coli Escherichia coli
ELISA Enzyme-linked immunosorbent assay
GM Genetically modified
HBSS Hanks balanced salt solution
HSP Heat-shock protein
i.g. Intragastric
i.n. Intranasal
i.p. Intraperitoneal
IFNγ Interferon gamma
Ig Immunoglobulin
IL Interleukin
MCP-1 Monocyte chemoattractant protein-1
MLN Mediastinal lymph node
MMCP-1 Mouse mast cell protease-1
NALT Nasal-associated lymphoid tissue
OVA Ovalbumin
SGF Simulated gastric fluid
TGFβ Transforming growth factor-beta
Th T helper cells
TLR Toll-like receptor
TNFα Tumour necrosis factor alpha
Treg Regulatory T cells
TrypCry1Ab Trypsin activated Cry1Ab
List of included papers


Cry1Ab protein from *Bacillus thuringiensis* and MON810 *cry1Ab*-transgenic maize exert no adjuvant effect after airway exposure

[Submitted]


Humoral and cellular immune responses in mice after airway administration of *Bacillus thuringiensis* Cry1Ab and MON810 *cry1Ab*-transgenic maize

[Submitted]


Lack of immunogenic, allergenic and adjuvant properties of Cry1Ab protein after intragastric exposure in a food allergy model in mice

[Manuscript]
1 Introduction

1.1 Genetically modified crops

Traditionally, crops intended for food and feed were improved by the selective breeding of desirable traits. Since the first genetically modified (GM) plant was produced in the 1980’s (James and Krattiger, 1996), more rapid and targeted methods have been developed. GM crops are created by direct transfer, insertion and ligation into the plant genome of a transgene containing the genetic code for expression of a protein that may confer an advantageous trait to the recipient plant. Commonly, traits that aim to reduce yield loss from insect damage or weed interference or a combination of these traits (stacked genes) are implemented (James, 2010). The International service for the acquisition of agri-biotech applications (ISAAA, 2013) reports that among the 27 countries that allowed cultivation of GM crops in 2013, 19 were developing countries and eight were industrial countries. Soybean, cotton, maize and canola are the major GM crops. For the same year, GM maize embraced 32 % of the global planting of maize crops. The potential benefits of GM crops are accepted beyond doubt, but a number of inherent GM crop properties with potential impacts on ecosystems and the health of humans as well as domestic and wild animals, are still debated or under investigation (Traavik and Lim Li, 2007, Haslberger, 2006, de Vendomois et al., 2010, Buiatti et al., 2013, Kumar et al., 2008). Unintended negative effects on aquatic (Bohn et al., 2008, Bohn et al., 2010, Linn and Moore, 2014, Rosi-Marshall et al., 2007) and terrestrial ecosystems (Lovei et al., 2009) have been reported, and potential toxicity (Hammond et al., 2013, Domingo and Gine Bordonaba, 2011) and impaired nutrition value have been postulated. Because the introduction of “foreign” genetic material often leads to the expression of novel proteins, concerns have been raised that the use of GM crops may lead to immune responses against these new proteins, because they may be of immunogenic, allergenic and/or express immune
adjuvant properties (Bernstein et al., 2003). The focus of this thesis will be on the potential immune effects of a common insect resistant maize, MON810, and different forms of purified Cry1Ab proteins produced in bacterial systems. It will not include the many other aspects related to GM maize or other GM crops.

1.2 MON810 maize

Since the introduction of the GM MON810 maize (trade name Yieldgard™) by Monsanto in the United States in 1996, it has been approved for direct use as food and feed in several countries worldwide. The MON810 event is generated by particle acceleration technology, and includes the use of the CaMV35S (cauliflower mosaic virus) promoter, an intron from the Zea mays L. Heat shock protein (Hsp) 70, as well as a modified cry1Ab gene from the gram positive soil bacterium Bacillus thuringiensis (Bt) subspecies kurstaki. The cry1Ab transgene enables the MON810 plant to express a protein that is toxic to lepidopteran target pests including European corn borer (Ostrinia nubilalis büchner), Mediterranean corn borer (Sesamia nonagrioides Lefebvre) and Buseola fusca.

1.3 The insecticidal Cry1Ab protein

In nature, the Bt bacterium produces insecticidal Cry proteins, also called δ-endotoxins, during the sporulation phase. The Cry proteins comprise of at least 50 subgroups with more than 270 members. One large group of Cry proteins, the Cry1Ab, are globular molecules containing three structural domains connected by single linkers. The Cry1Ab is primarily produced as an inactive ~130 – 140 kDa protoxin (Hofte and Whiteley, 1989, Bravo, 1997). Once ingested by the susceptible insect, the alkaline environment in the midgut facilitates solubilisations of the protoxin. Trypsin-like gut proteases cleave off domains from both the carboxy- and the amino-terminal ends, leaving a ~60 – 70 kDa protease resistant active toxin.
that binds to specific glycoprotein receptors on the surface of insect midgut epithelial cells (Bravo et al., 2007, Bravo, 1997). The underlying mechanisms of the toxic effect of Cry1Ab proteins are not entirely resolved, however it is suggested that an aggregation of toxins subsequently creates pores through the cell membrane causing a disruption of the gut integrity and leading to the death of the insects (Vachon et al., 2012).

1.4 The immune system

A wide range of defence mechanisms have evolved in order to protect the body from harmful external and internal threats. The immune system consist of the innate (“unspecific”) and the adaptive (“specific”) immune systems playing important roles both independently and in symphony. The mucosal surfaces acts as physical barriers, as well as active immune components in interface between the internal milieu and the external environment (Knight and Holgate, 2003). Epithelial cells in the intestinal and airway mucosa represent a highly regulated communication network that can transmit vital signals to cells and serve as targets of mucosal mediators (Kagnoff, 2014, Ryu et al., 2010). Upon encounter with infectious agents, the innate immune system responds quickly involving a large number of immune cells that may attack the pathogens by release of microbicidal proteins, complement activation, phagocytosis (engulfment and degradation of the pathogen) and the induction of inflammation with recruitment of other leucocytes. The adaptive immune system can be divided into cell-mediated and humoral (antibody-mediated) immunity. Cell-mediated responses primarily comprise the T cells, that, driven by the cytokines and signals in the immediate environment, differentiate into subsets like cytotoxic T cells, T helper cells (like Th1, Th2 or Th17 cells) or regulatory T cells (Treg) (Zhang et al., 2014). These sub-groups of T cells play an important role in establishing and maximising the immune response, characterised by different cytokine production profiles. Th1 cytokines produce predominantly interferon gamma (IFNγ) and
interleukin (IL)-2, whereas Th2 cytokines is associated primarily with IL-4 and IL-5 production. Both Th2 and Th1 cells coordinate the activation of B cells to produce different classes of antibodies and are thus driving the humoral response (see below). Th17 cells produce IL-17, and promote inflammatory responses to infections by recruitment of neutrophils. Tregs are characterised by their inhibitory functions and production of suppressive cytokines such as IL-10 and transforming growth factor-beta (TGFβ).

Antibodies are divided into five classes with different functionality, immunoglobulins (Ig) IgM, IgD, IgG, IgA and IgE. Whereas IgM and IgD are mainly produced by B cells before activation and early in the immune response, IgG is the main Ig in serum, and is further divided into four subclasses with different functionality: IgG1, IgG2, IgG3 and IgG4 in humans and IgG1a, IgG2a, IgG2b and IgG3 in mice. Throughout the thesis, we refer to mouse Igs. IgA is particularly important on mucosal surfaces like the airways and the gastrointestinal tract as well as in secretions like milk, and is associated with neutralising characteristics (Macpherson et al., 2011). IgE has protective activity against parasitic worms, and plays an essential role in allergic responses (Fitzsimmons et al., 2014). The production of IgG, IgA and IgE is for all classes dependent on an antibody switch from IgM, an active process by recombination of Ig genes within the constant-region portion of the antibody heavy chain, driven to a large extent by the cytokine environment (Wu and Zarrin, 2014). To achieve an IgE switch and production, the ligation of the IL-4R on B cells by IL-4 cytokines is crucial, hence the close association between Th2 cells and allergic responses (Poulsen and Hummelshoj, 2007).
**Allergy**

The immune system is normally well-regulated and in a state of tolerance to most foreign proteins and aimed at elimination of harmful agents. In some cases, however, immune responses are initiated by antigens that are not associated with infectious agents, such as pollen and food proteins and drugs, leading to a break in tolerance, and induce harmful immune reactions generally referred to as hypersensitivity. Allergies, the most common type of hypersensitivity, are classified by Coombs and Gell (Murphy et al., 2012), as type I (IgE-mediated) and type IV (cell-mediated) hypersensitivity. Allergic diseases are an emerging public health burden (Sicherer and Leung, 2013), characterised by significant negative effects on personal life quality and often associated with severe morbidity (Bousquet et al., 2009). Common allergic conditions include allergic rhinitis, asthma, rhinoconjunctivitis, food allergy, urticaria and eczema (Asher et al., 2006, Berin and Sampson, 2013). Only IgE-mediated allergy will be discussed in this thesis.

The pathogenesis of the allergic reaction comprises two distinct stages, the sensitisation phase and the elicitation phase (Figure 1). During the sensitisation, the allergen in question is caught in the mucosa of the airways or the digestive system, processed by APCs like dendritic cells and presented to CD4 expressing (CD4+) Th cells. The presence of cytokine IL-4 will favour the differentiation of CD4+ T cells into Th2 cells, which typically release cytokines like IL-4, IL-5 and IL-13. An antigen-specific Th2 cell may then provide B cells with the necessary signals through co-stimulatory cell membrane molecules and cytokines leading to B cell activation and production of antigen-specific antibodies. Importantly, Th2 cells provide the cytokines (mainly IL-4) necessary for IgE isotype switch in B cells. Released IgE molecules from these B cells will bind the high affinity Fcε receptors on mast cells and basophils (Parham and Janeway, 2009). The cells (and the individual) are then sensitised. The elicitation
(triggering) phase involves the re-exposure or continued exposure to antigen and cross-linking of IgE molecules bound to the sensitised mast cell, causing the instant release of mediators such as histamines, proteases, leukotrienes and prostaglandins. These mediators typically initiate the clinical symptoms of allergy. A life threatening and sometimes fatal reaction occurs when mast cells and basophils evoke systemic responses, so called systemic anaphylaxis (Burks et al., 2008), characterised by marked vascular changes and loss of blood from vessels into the tissues (Fisher, 1986). Serum levels of mast cell proteases reflect an anaphylactic response originating in the intestines, and are used as markers for clinical anaphylaxis in murine models of food allergy (Vinje et al., 2009, Li et al., 2000, Vaali et al., 2006). In airways, the immediate allergic reaction can be followed by the late phase reaction, which includes Th2 associated recruitment of eosinophils, basophils and lymphocytes contributing to the immunopathology of airway allergy and asthma (Gould et al., 2003).

Figure 1. Schematic illustration of mechanisms involved in development of allergy.
1.5 Immune responses against novel proteins

Immunogenicity is defined as the capacity of proteins to induce an immune response, including both humoral and cellular reactions against novel proteins. In predisposed individuals, immunogenic proteins may stimulate type 1 hypersensitivity reaction through specific IgE responses. This may be result in allergy against the protein, via the mechanisms described in section 1.4. Furthermore, novel proteins may have the capacity, when co-administered with an antigen, to increase the immune response to that antigen (Brunner et al., 2010, EFSA, 2010), so-called adjuvant capacity. While adjuvants have been implemented to augment immune responses in experimental immunology as well as in practical vaccination for many years, several environmental compounds with allergic adjuvant effects in animals and associated with allergic disease in humans (i.e. ambient air particles, triclosan), has been suggested to have contributed to the increased prevalence of allergic diseases (Bertelsen et al., 2013, Alberg et al., 2014). The modes of action seem to vary between different compounds and are not entirely explained, but possible mechanisms are reviewed in Brunner et al. (2010).

In short, three types are described for clinical settings. The first type enhance antigen presentation by building a depot at the injection site, leading to a high local antigen concentration and an antigen release over time, therefore improving and prolonging uptake by APCs. The adjuvant may also accelerate the antigen recognition and uptake by direct stimulation of the APC or adjacent immune cells. The second type of adjuvants is more specific, and act as toll-like receptor (TLR) antagonists. They activate APC and trigger release or production of cytokines. The third type acts primarily via the up-regulation of co-stimulatory molecules on APCs. The specific mode of action of adjuvants will not be further discussed in this thesis. Nevertheless, the beneficial characteristics of adjuvants in vaccines are unwanted traits with respect to allergic diseases. Thus, introduction of novel proteins that are able to enhance immune responses against an allergen are undesirable.
1.6 Immunogenic, allergenic and adjuvant properties of novel proteins

Immunogenicity and allergenicity

The concerns that novel proteins in GM crops may confer risks of being allergenic or having adjuvant properties, have naturally evoked several risk assessment strategies, and both pre- and post-market surveillance. To date, the main focus of adverse immune responses related to novel proteins has been on allergenicity. An immunogenic property of proteins is not adverse per se, however, it is a prerequisite for the allergenicity. In general, allergenicity assessments of novel proteins expressed in GM foods are evaluated using several strategies, involving *in silico*, *in vitro* and *in vivo* approaches (EFSA, 2010, Ladics et al., 2014).

*In silico* approaches involve the search for similarity of amino acid sequences or structural features with known allergens to detect allergenicity and/or cross-reactivity (Ladics et al., 2011, EFSA, 2010). The approach requires comprehensive, updated databases of known allergens. Importantly, external factors such as post translational modifications cannot be accounted for (EFSA, 2010). Randhawa et al. (2011) aimed to identify potential sequence matches of Cry proteins to allergenic proteins using bioinformatic search tools. The authors concluded that the results indicated no significant alignment and similarity of Cry proteins at domain level with any of the known allergens suggesting that there is limited risk of allergenic cross-reactivity.

*In vitro* approaches involve the testing of protein degradation by the proteolytic enzyme pepsin or investigations in more physiologically relevant gastrointestinal digestion models. Because proteins that are able to escape the prototypical degradation in the digestive tract are more likely to reach the intestine mucosa and to sensitize the mucosal immune system, determination of stability to digestion is considered to be a relevant tool to assess the
prerequisite for allergenic potential of a protein (Moreno, 2007). However, such digestion tests have less relevance in the assessment of novel proteins with other relevant exposure routes, for instance via airway or skin exposures which may also induce food allergy. Guimaraes et al. (2010) used simulated gastric fluid (SGF) at different pHs and demonstrated that while the Cry1Ab protein was highly degraded at pH 1.2, it contained its immunoreactivity at pH 2, indicating that the simulated gastric conditions may influence the outcome of digestion tests. The results from such tests may therefore not be valid for infants with immature digestion, elderly with impaired stomach function and patients using medicines that interfere with the gastric pH (Untersmayr and Jensen-Jarolim, 2008).

Furthermore, the Codex Alimentarius Commission (2003) suggests that IgE binding tests (reviewed in Goodman (2008)) should be conducted if the transferred gene was from an allergenic source or the sequence of the transferred protein has >35 % identity of 80 or more amino acids to a known allergen, or shares significant short amino acid identities. Commonly, however, these tests require available sera from several patients allergic to the allergen of interest, which are not readily available. A specific IgE response against plant Cry1Ab has to our knowledge not been detected in exposed food-allergic humans (Nakajima et al., 2007). However, greenhouse and farm workers exposed via the airways to the Cry1Ab protoxin biopesticide spray had detectable levels of specific IgE in serum (Dockes et al., 2004, Bernstein et al., 1999) indicating a sensitising potential of the protoxin in humans.

In vivo experiments using animal models, compared to in silico and in vitro experiments, allow better understanding of the interactions between cells and organs involved in allergic responses. Results obtained from animals can never be directly transferred to humans. The use of animal models is, however, still considered a useful strategy despite the obvious limitations, and is particularly regarded to be helpful in addressing the issue of
immunogenicity, allergenicity and adjuvant properties of novel proteins (EFSA, 2010, Ladics et al., 2010). Mouse models are widely used, because mice have a well characterised immune system (Aldemir et al., 2009). Some strains are susceptible for allergic disorders, and thus resemble individuals with a predisposed (atopic) genetic background. The BALB/c mouse shows propensity toward a Th2 phenotype and IgE production (Adel-Patient et al., 2005, Hilton et al., 1997) and are frequently used in models of airway allergy (Moreno-Fierros et al., 2000, Hilton et al., 1997). Animal models to investigate food allergy against novel proteins are usually dependent on adjuvants for overcoming or circumventing the mechanism of oral tolerance (Finkelman, 2007). While most previous studies focus on the sensitisation potential of novel proteins, there is a lack of knowledge concerning the allergic elicitation potential of such proteins. The C3H/HeJ mouse has however displayed responses related to elicitation of clinically relevant allergic responses (anaphylaxis) against well-known food allergens. The current knowledge from in vivo studies on Cry1Ab immunogenicity or allergenicity is described in section 1.8.

**Adjuvanticity**

While allergenicity has received some attention in risk assessments, opinions and evaluations, the possible adjuvant capacity of Cry1Ab proteins has so far largely been ignored. Adjuvanticity is, however, now included in the guidelines for testing of GM crops (EFSA, 2011a). A concern about the possible adjuvant capacity of Cry1Ab proteins has in part been based on the strong adjuvant effects reported for the structurally similar Cry1Ac protein. In mouse models, Cry1Ac proteins were found to promote immune responses to the same degree as the potent mucosal adjuvant cholera toxin (CT) (Vazquez et al., 1999). At the start of our project, to our knowledge, the capacity of Cry1Ab protein to act as an adjuvant had only been evaluated in one study. In a mouse model of peanut allergy, there were indications of an
allergy adjuvant capacity of orally administered Cry1Ab protein revealed in the elicitation phase in the lungs, as evidenced by early production of leukotrienes in bronchoalveolar lavage fluid (BALF) and late Th2 and Th17 production and eosinophil/neutrophil influx (Guimaraes et al., 2008). The study did not show indications of an adjuvant effect on the sensitisation phase, i.e. peanut-specific antibody responses. Taken together, the observations for Cry1Ac as well as for Cry1Ab in the Guimaraes study, indicated a need for further investigation of the potential adjuvant capacity of Cry1Ab proteins. A recent publication reported that feeding of mice with MON810 containing diets for 34 days did not promote the development of allergic asthma (Reiner et al., 2014).

1.7 Cry1Ab protein variants

Assessments of potential Cry1Ab immunogenicity and/or allergenicity have mostly been performed with purified protein isolated from the naturally occurring Bt, i.e. on the protoxin. It has been assumed that it is appropriate to extrapolate such results to Cry1Ab expressed in maize tissue (EFSA, 2012). The many important structure and context differences between bacterial and maize Cry1Ab may, however, make such extrapolations inadequate and lead to erroneous or irrelevant conclusions. In the Bt bacterium, Cry1Ab is expressed as a ~ 130 kDa protoxin that needs to be activated by enzymatic cleavage in the insect gut to obtain its toxicity. The MON810 transgene, on the other hand, encodes only the N-terminal half of the protoxin. Hence it expresses an already activated version of the Cry1Ab toxin of 91 kDa (Szekacs et al., 2010), which is structurally different from the intact protoxin (Guerrero et al., 2004, Guerrero et al., 2007, Kitami et al., 2011). Furthermore, transgenic insertions into a maize genome may cause a complex recombination event resulting in expression of “new” unanticipated proteins as well as modified molecular versions of the expected gene product. Such events may take place without interfering with the insecticidal activity of the Cry1Ab
protein or with the vigour and yield of the MON810 maize (Rosati et al., 2008).

The purification and extraction of Cry1Ab from GM plant material has been difficult to perform in sufficient amounts to be tested in animal trials, and for the purpose of obtaining active toxins that resembles the plant expressed toxins, a laboratory version has been produced with a bacterial host. Insertion of cry genes into *Escherichia coli* (*E. coli*) leading to the production of full length protoxin, followed by subsequent digestion by trypsin has given a bioactive toxin fragment of ~67 kDa to work with. Such trypsin cleaved toxin (trypCry1Ab) has been included in studies as a substitution for plant expressed Cry1Ab (Guerrero et al., 2004, EFSA, 2012).

Unanticipated posttranslational modification of the transgene-encoded protein is another basis for aberrant structural and immunogenic properties. Prescott et al. (2005) showed in a mouse model that transgenic expression of the bean alpha-amylase inhibitor-1 in peas led to the synthesis of a protein variant with altered immunogenicity, i.e. lung inflammation, due to divergent carbohydrate chain lengths at a glycosylation site that was common for both αAI versions. This finding indicates that subtle structural modifications of a protein may lead to significant changes in immunogenicity. However, it should be noted that these findings could not be reproduced in a study by Lee et al. (2013). Overall, it seems to be important to take into consideration both the source and version of the Cry1Ab used in experimental studies.

### 1.8 Exposure routes

Since Bt is a ubiquitous soil bacterium, mammalian (human, domestic and wildlife animal) immune systems have evolved in concert with the Cry protoxins, including Cry1Ab. Since 1996, however, human and animal populations, in areas of production or consumption, have
in addition been exposed to the GM plant-expressed versions of these proteins. The mucous membranes of the respiratory and gastrointestinal tracts may be the most important portals of exposure (Vazquez-Padron et al., 2000, Kitami et al., 2011) although it cannot be disregarded that also the skin is a potential route of exposure. The thesis will, however, only focus on exposures via the airways and the digestive tract.

Immune effects of Cry1Ab exposure via the airways

Inhalation of pollen and plant debris may be a realistic exposure route for humans as well as domestic and wild animals in the fields, and also of workers during processing of food and feed. To our knowledge, effects after airway exposure to adequate plant Cry1Ab proteins have not yet been investigated. Purified Cry1A protoxins and trypsin activated toxins have demonstrated specific humoral as well as cellular anti Cry1A immune responses after intranasal (i.n.) administration in mice (Guerrero et al., 2004, Guerrero et al., 2007). The same authors emphasised that the immune responses after i.n. exposure were unique, and suggested that binding of the lectin motifs of Cry proteins to the lectin binding receptors in nasal-associated lymphoid tissue (NALT) might contribute to that (Takata et al., 2000). The immunological potential of airway exposure to Cry1Ab-containing feed debris was directly illustrated, although unintended, when control fed and Cry1Ab-containing rice fed rats kept in the same room all developed Cry1Ab-specific IgM, IgG and IgA antibodies (Kroghsbo et al., 2008). In humans, Cry1Ab-specific antibodies have been detected after occupational inhalation of Bt-based insecticidal spray (Bernstein et al., 1999, Doekes et al., 2004). Studies like these indicate that specific immune responses may be induced after inhalation of Cry1Ab proteins, but so far no studies has investigated IgE sensitisation, or allergic airway responses, after airway exposure to Cry1Ab proteins.
Immune effects of Cry1Ab exposure via the digestive tract

According to (EFSA, 2010), the consumption of Cry1Ab containing food and feed is regarded safe. Also pollen from MON810 has been treated as a food product, and claimed to be safe (EFSA, 2011b). However, some feeding trials have revealed alterations in immune parameters. For example, it has been shown that MON810 diets potentiated oxidative cellular stress in the distal intestine of immune-sensitised fish, as indicated by increases in superoxide dismutase and Hsp 70 mRNA expression, suggesting that Cry1Ab protein or other antigens in Bt-maize had local immunogenic effects in salmon distal intestine (Gu et al., 2013). Finnamore and co-workers demonstrated MON810 maize induced alterations in the percentage of T and B cells and of CD4⁺, CD8⁺ T subpopulations of weaning and old mice fed for 30 or 90 days, respectively, at the gut and peripheral sites. An increase of serum IL-6, IL-13, IL-12p70, and macrophage inflammatory protein-1β after MON810 feeding was also found. On the other hand, in sows and offspring no Cry1Ab-specific antibodies could be detected after feeding transgenic maize to sows during gestation and lactation (Buzoianu et al., 2012). Blood monocyte count and percentage were higher, while granulocyte percentage was lower in Bt maize-fed sows on day 110 of gestation. Leukocyte count and granulocyte count and percentage were lower, while lymphocyte percentage was higher in offspring of Bt maize-fed sows. Bt maize-fed sows had a lower percentage of monocytes on day 28 of lactation and of CD4⁺CD8⁺ lymphocytes on day 110 of gestation, day 28 of lactation and overall. These results suggest that further investigations are needed for the gut and peripheral immune response to GM crop ingestion as well as the age of the consumer in the safety evaluations.
2 Aims of the study

The overall objective of the present study was to investigate the potential immunogenic, allergenic and adjuvant capacity of the Cry1Ab protein after i.n. and intragastric (i.g.) exposures in mice. Specifically, the aims of our study were to answer the following questions:

1. Can Cry1Ab proteins promote allergic responses (i.e. act as an adjuvant) to known allergens after airway or i.g. exposure of mice? (papers I and III)
2. Can Cry1Ab proteins induce specific humoral and cellular immune responses after airway or i.g. exposure of mice? (papers II and III)
3 Initial experiments

3.1 Cholera toxin as a positive control for an adjuvant effect

CT produced by *Vibrio cholerae*, is a potent adjuvant, consisting of the toxic subunit A and the nontoxic subunit B, where only the latter is normally used as an adjuvant. The subunit B binds epithelial cells and allows toxins to enter the cells. It has been shown to elicit a Th2 or a mixed Th1/Th2 response with a bias towards Th2 (Bharati and Ganguly, 2011). Because of these properties CT is often included as a positive control for adjuvant effect (Berin and Mayer, 2009). The airway model of ovalbumin (OVA)-allergy is described in paper I. Previous to the investigation of adjuvant capacity in paper I, a pilot study was conducted to establish a positive control for adjuvant effect by use of CT. Female BALB/c mice (n = 5), 5-6 weeks old at arrival, were sedated (Isofluran in 3.5 % O2) and i.n. immunised with 35 µL of the test solutions, containing 10 µg OVA or OVA together with 0.2 or 0.5 µg CT on days 0, 1, 2, 21, 22 and 23. Hanks balanced salt solution (HBSS) was used as vehicle. The mice were exsanguinated by heart puncture and blood was collected on day 26. OVA-specific IgE and IgG1 were detected in sera by enzyme-linked immunosorbent assay (ELISA). The results (Figure 2) demonstrated that both the 0.2 and 0.5 µg CT dose were able to induce OVA-specific IgE and IgG1 responses in mice compared to OVA alone. The 0.5 µg dose of CT was chosen for the following investigations, to provide a stable positive control for adjuvant effect.
3.2 Intranasal immunisations with maize pollen grains

Preceding the experiments of airway exposure to maize pollen grains (papers I and II), a pilot study was conducted to determine the amount of pollen possible to inhale without respiratory problems or discomfort for the mice. Pollen from both the MON810 and unmodified maize were kindly provided by Dr. Angelica Hilbeck, (Swiss Federal Institute of Technology, Switzerland. Pollen suspensions giving doses of 50, 200 and 600 µg pollen in 35µL HBSS were applied with a pipette on the nostrils of sedated female BALB/c mice (n = 4), 5-6 weeks old at arrival, on three consecutive days. The mice were terminated by an i.p. injection of ZRF-cocktail on day 4, and BALF were collected. We could not observe any respiratory problems after inhalation of the 50 and 200 µg pollen doses. The dose of 600 µg pollen, however, gave some discomfort in one mouse, evidenced as reduced activity during a 30 min observation period after the application. The dose of 500 µg pollen was therefore chosen for the following experiments. The levels of cytokines in BALF (figure 3) were determined using cytokine bead array analysed on a flow cytometer. In spite of very low levels of IL-5, 10, 17 and IFNγ at this time point, the results indicated that the highest dose of pollen increased the
BALF levels of IL-5, monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor alpha (TNFα) compared to control. Because this was a pilot study with a small sample size, and the highest dose (600 µg) of non-GM was not included, these results were inadequate to conclude on effects. However, the trends we observed supported that the BALF cytokines should be included as an endpoint in the main experiments.

![Figure 3](image_url)

Figure 3. Cytokine IL-5 (a), MCP-1 (b), TNFα (c), IL-10 (d), IFNγ (e) and IL-17 (f) levels in broncho-alveolar lavage fluid (BALF) collected in mice on day 4 (n = 4) after i.n. exposed to 50, 200 or 600 µg of MON810 maize pollen or 200 µg of non-GM maize pollen for three consecutive days. One group of mice received the vehicle (HBSS) only. Asterisk (*) denote groups significantly different from the HBSS group.

### 3.3 Cry1Ab positive sera

In order to develop the ELISA necessary to screen for the presence of anti-Cry1Ab IgE, IgG1 and IgG2a in sera of exposed mice (papers I, II and III), we needed a mouse anti-Cry1Ab antiserum to provide standard curves as well as a positive assay control. Positive and negative
controls are indeed essential for the standardisation of ELISA conditions (concentration of antigen, serum and secondary antibody as well as blocking conditions) to obtain the maximum sensitivity and the minimum background interference. Commercially, only mouse monoclonal Cry1Ab antibody was available. A monoclonal antibody was of low convenience for our study, since we needed to standardise an ELISA for the screening of animal antisera (polyclonal). The commercial monoclonal antibody is raised against an epitope contained in the full length version of Cry1Ab (protoxin) produced by the bacterium Bt, thus it was uncertainties whether the commercial monoclonal antibody could recognise the plant version of Cry1Ab. The mice in our study were in addition to the Cry1Ab protoxin, also exposed to the plant version of the protein (pollen and leaf extracts) as well as trypCry1Ab. Female BALB/c mice (n = 10) were i.p. immunised with 10 µg of trypCry1Ab protein (ca 67 kDa) together with 2 mg of the adjuvant Al(OH)₃ dissolved in HBSS (total volume 500 µL) on days 0, 20, 34 and 41. The mice were sacrificed by heart puncture and blood collection on day 48, and sera from all 10 mice were pooled. In addition, two mice were kept untreated and sacrificed on day 48, and the pooled sera served as negative assay control.

### 3.4 Development of Cry1Ab-specific ELISA

The ELISAs for detection of Cry1Ab-specific IgG1, IgG2a and IgE antibodies are described in the material and methods section in paper II. Previous Cry1Ab-specific antibody ELISAs reported (Nakajima et al., 2007, Takagi et al., 2006) have used indirect ELISAs, i.e. coating the plates with the protein. We hypothesised that ELISAs based on coating with Cry1Ab protein and incubation with test sera and then enzyme conjugated anti-mouse IgE were not appropriate for detection of IgE and IgG2a antibodies whose relative amounts are very small compared to IgG’s. The majority of Cry1Ab bound to the well will bind to specific antibodies of other more abundant sub-classes (e.g. IgG), making it difficult to detect the presence of
IgE/IgG2a. Instead we suggested to i) coat wells with anti-mouse IgE/IgG2a, ii) hybridise with the serum – the IgE/IgG2a present in the serum are captured on the well surface and the other classes of antibodies are washed away, iii) hybridise with trypCry1ab that will bind to the anti-Cry1ab IgE/IgG2a which, if present in the serum, are now trapped on the well, iv) hybridise with biotinylated anti-Cry1ab (detection antibody), v) hybridising with streptavidin–poly–horseradish peroxidase (HRP) for detection. Both procedures were tested using the antiserum from trypCry1Ab immunised mice. As expected, only specific anti-Cry1ab antibodies of class IgG1 and not IgE or IgG2a were detectable with the first approach. In contrast, the second approach detected the presence of both IgE and IgG2a in the sera, and this approach was used in the following determination of Cry1Ab specific IgE and IgG2a in sera (papers II and III).
4 Summary of included papers

Paper I

The aim of the study was to investigate whether plant material from the Cry1Ab-expressing maize variety MON810, or trypCry1Ab protein produced in recombinant bacteria, may act as adjuvants against the allergen OVA in a mouse model of airway allergy. Female BALB/c mice were i.n. exposed to OVA alone or together with i) trypCry1Ab, ii) MON810 pollen, or iii) MON810 leaf extract on three consecutive days. Furthermore, mice were i.n. immunised with OVA together with the known mucosal adjuvant CT, and the physiological buffer (vehicle) as positive and negative controls, respectively. Twenty days later, all mice received an i.n. booster with OVA on three consecutive days. OVA-specific antibodies were measured in serum, allergy associated cells and cytokines were measured in BALF, and cytokine release from OVA-stimulated mediastinal lymph node (MLN) cells was determined. A clear proallergic adjuvant effect of CT was demonstrated, determined as increased OVA-specific IgE and IgG1, eosinophils in BALF and Th2 cytokines in MLN cell supernates, while no elevation in OVA-specific antibodies, inflammatory cells in the BALF or cytokine release from MLN cells after stimulation with OVA were observed due to exposure to Cry1Ab-containing plant materials or the trypCry1Ab protein. Our data suggest that Cry1Ab proteins irrespective of source had no detectable systemic adjuvant effect in mice after airway exposure.
Paper II

We aimed to study the immunogenic and allergenic potential of Cry1Ab proteins from different sources after airway exposure. Thus, we exposed female BALB/c mice to i) purified Cry1Ab protoxin, ii) trypCry1Ab, iii) MON810 pollen, or iv) MON810 leaf extracts by i.n. application on day 0,1, 2 and booster immunised three weeks later. No anti-Cry1Ab specific antibodies were detected following exposure to the plant materials. Exposure to purified Cry1Ab resulted in specific anti-Cry1Ab IgG1 and IgE production, indicating inherent immunogenicity and allergenicity potential. Mice exposed to leaf extracts from MON810 and non-GM maize demonstrated significant influx of lymphocytes and eosinophils in the BALF, and also increased cytokine release (IL-10, IFNγ, IL-4, IL-13 and IL-17) in MLN cells. The results indicated that the cellular responses were related to maize plant material as such, and not to the Cry1Ab protein. These results suggest that airway exposure to Cry1Ab proteins may be a relevant route of exposure.
Paper III

I.n. and intraperitoneal (i.p.) immunisations with the purified Cry1Ab proteins have demonstrated immunogenic responses, and feeding trials have discovered changes in immune responses related to ingestion of Cry1Ab containing feed. While previous investigations primarily measured antibody responses to the protein, clinical symptoms associated with food allergy, or a potential promoted food allergy (adjuvant effect), associated with the Cry1Ab protein, have not been studied so far. Here we investigated the immunogenic, allergenic and adjuvant capacity of purified Cry1Ab toxin (trypCry1Ab) produced in E. coli after i.g. exposure, manifested as clinical relevant symptoms (anaphylaxis) as well as humoral and cellular responses. C3H/HeJ mice were orally gavaged with the food allergen lupin alone or together with CT or trypCry1Ab to evaluate adjuvant property of trypCry1Ab measured as anaphylactic responses (rectal temperature and clinical signs) as well as serum levels of total IgE, mouse mast cell protease-1 (MMCP-1) and lupin-specific IgG2a. Furthermore, C3H/HeJ mice were orally gavaged with trypCry1Ab alone or together with CT to evaluate immunogenic and/or allergenic properties, measured as Cry1Ab specific IgG1, IgE and IgG2a in sera. There was no indication of immunogenic, allergenic or adjuvant capacity of the trypCry1Ab protein after per oral exposure, within the limitations of the model and doses applied.
5 Discussion

5.1 Methodological considerations

Mouse models

Typically, laboratory mice are inbred for several generations and present with strongly reduced genetic variations compared with outbred mice. To work with genetically well characterised and nearly identical mice is by many considered necessary to reduce inter-individual variation and a prerequisite to obtain reproducible results. One could argue that reduced genetic variation could be both an advantage and a disadvantage. With inbred mice, the number of animals needed per experimental group can be reduced, and the results will be more easily interpreted with less variation between individuals. On the other hand, the conclusions are restricted to the specific genetic background of the animals used. The strains used in this thesis, BALB/c and C3H/HeJ, are specifically chosen to fit the research questions, since, like in humans, only mice of particular genetic backgrounds are able to develop IgE sensitisation and symptoms of allergic disease. The BALB/c strain is known to have an “allergic” phenotype and will more easily develop pathological immune parameters associated with allergic disease (Holt et al., 1981), in particular after i.n. installation (Hilton et al., 1997). C3H/HeJ mice, in addition to be an IgE-synthesis prone strain, also display clinically relevant responses to food allergens (Li et al., 2000, Vinje et al., 2009).

The BALB/c mouse was used in both papers I and II, in the airway allergy model. The immunisation regime is regarded as sub-optimal because the OVA doses employed did not elicit an allergic response. With the presence of an adjuvant, however, OVA-specific IgG1 and IgE responses were demonstrated in our study (paper I). The advantage of using this sub-optimal immunisation in adjuvant investigations, in particular, is the possibility to detect also
weak adjuvants, because an elevation from no response will be easier to detect than a weak increase in an already elevated response. The model has previously been employed to study sensitisation to antigens (Hansen et al., 2011) and adjuvant effects (Nygaard et al., 2009, Alberg et al., 2011, Alberg et al., 2014) and the number of days between the last immunisations and the termination of experiments has been optimised in order to detect the peak of the IgE-response (Lovik et al., 1997).

The mouse model of food allergy has previously been described by Vinje et al. (2009) and Li et al. (2000). The model includes the use of the allergy prone strain C3H/HeJ mouse that display clinically relevant symptoms after oral immunisations and i.p. challenge with an allergen. However, it has been demonstrated that a clinical response is more clearly displayed after the i.p. challenge (Vinje et al., 2011). For the purpose of this study we therefore decided to use the i.p. challenge which enabled us to detect a significant and robust response in mice exposed to CT.

**Negative control animals**

As previously suggested (Kroghsbo et al., 2008), animals kept in the same room may get sensitised to the protein in question by inhalation of feed debris or airborne supplemental proteins. To avoid unintended airway exposures, the immunisations were given as a liquid, and also under controlled conditions with a pipette to the nostrils. We therefore did not anticipate that animals in the control groups were exposed to the Cry1Ab protein, which indeed is supported by our results.
Experimental conditions

For practical reasons, the two experiments of airway exposure with Cry1Ab (papers I and II) were conducted at two different locations. Because of that, the final results could not be directly compared. Nevertheless, the differences in experimental parameters were kept at a minimum. Mice were obtained from the same supplier (Charles River, Germany), and although the transportation time was longer from Germany to South Africa than from Germany to Norway, mice for both labs were transported by airplane and car. The same feed and bedding was used in both experiments (purchased from the same supplier), and the same investigator (MA) performed both experiments. Due to difference in availability of anaesthetic devices in the two animal facilities, the mice were sedated using different anaesthetic reagents in the two experiments, described in detail in paper I and II. We cannot exclude the possibility that these differences affected the parameters studied. However, the results from these two experiments supported each other and increased the reliability of the model to conclude on the absence of an adjuvant effect of Cry1Ab regardless of source (Figures 1 and 2 in paper I). Furthermore, results from both experiments confirmed the immunogenic and allergenic potential of the purified Cry1Ab protein (Figure 2 in paper II).

Immunising doses of Cry1Ab

The expression levels, and hence immunising “doses”, of Cry1Ab in different MON810 tissues may vary considerably within the plant (e.g. pollen versus leaves) and between plants grown in different ecosystems and locations (Agapito-Tenfen et al., 2013, Zolla et al., 2008). For the immunisation with fresh maize pollen grains as described in papers I and II, a perquisite was flowering maize plants of both the GM MON810 event as well as the conventional counterpart. The two maize events were grown under the same conditions, in adjacent pots in the same greenhouse. To assure that the plants were not mixed up under the
harvest of pollen; the Agdia Quick kit confirmed the presence of Cry1Ab proteins expressed in the MON810 plants. Also the content of Cry1Ab was quantified in fractions of collected samples from each of the days of pollen collection/exposure, confirming that plant material from the conventional non-GM maize did not contain detectable levels of Cry1Ab proteins. By using fresh pollen we utilised the possibility also to detect responses relevant in real life situations. It could be speculated that ligands on the pollen grain from GM maize could bind to receptors in the airways, and thus activate the pollen to express Cry-proteins (Hickling et al., 2004).

We experienced that the trypCry1Ab protein was difficult to dissolve, which therefore laid restrictions on the highest concentration that could be given as an i.n. instillation of 35 µL solution. For the exposure of plant material, also a maximum dose of pollen was given (based on the pilot study presented in section 3.2). Moreover, during the preparation of leaf extracts, the lowest possible volume of buffer was added to attain the highest possible Cry1Ab concentration. This restriction was a limitation of our study.

5.2 **Adjuvant capacity of Cry1Ab proteins**

The capability of Cry1Ab protein to act as an adjuvant was investigated in two independent models. In a mouse model for airway allergy, neither the Cry1Ab containing plant material (pollen suspension and leaf extract from MON810 maize) nor the purified Cry1Ab protein preparation (protoxin and trypCry1Ab) given i.n. were able to elicit humoral or cellular responses against the model allergen OVA (paper I). Further, there was no indication of an adjuvant effect after i.g. exposure to the trypCry1Ab protein solution on anaphylactic, humoral or cellular responses against the food allergen lupin (paper III). In both models, the well-known mucosal adjuvant CT was included as a positive control for adjuvant effects,
because of CT’s capacity to disrupt mucosal tolerance (as discussed in section 3.1). OVA co-administered with CT induced humoral and cellular responses after airway exposure (paper I). Similarly, humoral, cellular and anaphylactic responses were demonstrated after i.g. exposure to lupin co-administered with CT (paper III). Because the positive control with CT was as expected in two independent models, this strengthens our ability to conclude on the negative findings regarding adjuvant capacity of Cry1Ab proteins, irrespective of source and exposure route.

Our conclusion on lack of adjuvant capacity is in accordance with the recently published study of Reiner et al. (2014), showing no adjuvant effect of GM maize consumption on the onset or severity of allergic responses in a mouse model of allergic asthma. While trypCry1Ab protein did not demonstrate adjuvant effects after peroral sensitisation to peanut in a mouse model, possible adjuvant properties of Cry1Ab on allergic reactions became evident in the lungs after airway challenge to peanut (Guimaraes et al., 2008). Because Guimaraes and co-workers did not observe an elevated Cry1Ab-specific IgE production, one could speculate that the immune effects observed were not related to an enhanced IgE-mediated allergy development, but rather to local effects at the cellular level (T cells and eosinophils); however, priming for IgE production cannot be excluded.

The lack of investigations of a dose range of Cry1Ab proteins is a limitation of our studies of adjuvant effects (papers I and paper III). As discussed in section 5.1, practical limitations prevented us from testing higher doses. Since our objective was to identify possible effects, the highest possible dose of plant material or purified protein were chosen for all experiments. However, in one smaller study (Fig 4, results not included in paper I) we included three doses of protoxin, and compared the specific antibody responses against OVA in mice. In an
experimental setup like the one reported in paper I, female BALB/c mice were i.n. exposed to OVA alone or together with 0.5 µg CT, or together with 0.4, 2 or 10 µg Cry1Ab protoxin. As judged by the serum levels of OVA-specific IgE and IgG1 detected by ELISA, there were no indications of an adjuvant capacity of Cry1Ab protoxin regardless of dose (Figure 4).

Thus, using both “high” and “low” doses of the purified proteins, we were not able to provide a “proof of principle” for an adjuvant effect of Cry1Ab per se, neither via the airway nor the i.g. exposure routes. The lack of adjuvant capacity of MON810 plant material by airway exposure further suggests within the limitations of our study design and the doses applied, that the Cry1Ab toxin expressed in MON810 maize does not act as an adjuvant after inhalation or ingestion.

Figure 4. Serum levels of ovalbumin (OVA)-specific IgG1 (a) and IgE (b) antibodies in mice intranasally (i.n.) immunised with OVA or OVA together with 0.5 µg cholera toxin (CT), or 0.4, 2 or 10 µg Cry1Ab protoxin, on days 0, 1, 2, 21, 22 and 23. Asterisk (*) denote groups significantly different from the OVA-group.
5.3 Immunogenicity and allergenicity of Cry1Ab proteins

The immunogenicity and allergenicity of Cry1Ab proteins were investigated after both i.n. (paper II) and i.g. (paper III) administration. In mice i.n. exposed to the purified Cry1Ab protein preparations (protoxin and trypCry1Ab; paper II), we detected significant Cry1Ab-specific IgG1 and IgE antibody responses, indicating that the purified proteins may have both immunogenic and allergenic properties. Notably, the antibody responses were induced without the use of an added adjuvant. We obtained comparable results in two independent experiments, although employing different versions of Cry1Ab (protoxin and trypCry1Ab), which strengthens the reliability of our observations.

Our data on IgG induction after airway exposure, implicating an immunogenic capacity of the purified Cry1Ab proteins, is in agreement with results obtained by others in previous animal experiments, employing i.p. immunisation with the protoxin (Adel-Patient et al., 2011) and i.p and i.n. immunisation with trypCry1Ab (Guerrero et al., 2004). Kroghsbo et al. (2008) suggested that the inhalation of Cry1Ab-containing feed debris or airborne purified proteins explained the specific antibody response (IgM, IgG and IgA) observed in both control fed rats and rats fed the Cry1Ab-containing rice diet. Our study demonstrating a humoral response after airway exposures thus supports the biological plausibility of the explanation by Kroghsbo and co-workers.

To our best knowledge, we are the first to report Cry1Ab-specific IgE production after airway immunisation. Our data suggest an allergenic potential of both the protoxin and trypCry1Ab proteins in the airways, which is in agreement with results from respiratory health studies of farm and greenhouse workers, revealing Bt-specific IgE in serum after inhalation of Bt spray products (Doekes et al., 2004, Bernstein et al., 1999).
The presence of IgE in serum reflects only the first phase, sensitisation, of the allergic response (Figure 1). While the presence of IgE on the mast or basophil cell surface is a prerequisite for the elicitation phase, the presence of IgE does not necessarily lead to an elicitation of clinical symptoms. This is a general challenge in the assessment of allergenicity, as IgE induction and binding studies by serological methods alone may lead to false positive results (EFSA, 2010). Although the observed humoral response suggested both an immunogenic and allergenic potential of purified Cry1Ab proteins (paper II), these findings could not be supported neither by an allergy-associated cellular influx nor a Th2 associated cytokine profile in BALFs and MLN cells, respectively. However, as discussed in paper I and III, our experimental design was not optimal for using a lack of BALF and spleen/MLN cell cytokine release to conclude on negative findings.

The immunogenic potential of Cry1A-proteins on cellular responses have previously been investigated by Guerrero et al. (2007) showing that in vitro stimulation of spleen cells from mice i.n. sensitised with Cry1A proteins demonstrated a Th1-associated cellular responses. In the present experiments, the lack of an inflammatory cell influx in BALF, however, which usually accompanies an IgE response in our models (Hansen et al., 2013, Alberg et al., 2014, Nygaard et al., 2009) does not support a notion that Cry1Ab induces a strong allergic response in mice.

Taken together, our cellular and cytokine data were not supportive regarding the immunogenic and/or allergenic potential of the purified Cry1Ab proteins (paper II). Nevertheless, the clear humoral response induced after airway exposures to these proteins, without an adjuvant, indicates that Cry1Ab has both immunogenic and allergenic properties. Further, our findings indicate that exposure via the airways may induce immune responses,
including allergy. The clinical relevance of Cry1Ab-specific IgE responses in mice, animals and humans needs further investigations.

Interestingly, while strong specific IgG1 and IgE responses were measured after the i.n. instillations of the purified Cry1Ab proteins, no such response could be detected after i.g. exposures to the purified trypCry1Ab protein as reported in paper III (Figure 4). Our findings are in agreement with previous studies that showed no indication of humoral antibody responses after i.g. immunisation of mice with the protoxin and MON810 protein extract (Adel-Patient et al., 2011) or after feeding salmon with Cry1Ab-containing diets (Gu et al., 2013).

The cytokine data presented in paper III, with concanavalin A (Figure 5, paper III), and Cry1Ab (not shown) stimulated splenocytes, and the gene expression data from the ileum (Figure 6 d, e, f, paper III) gives us no further evidence of the immunogenic or allergenic capacity of trypCry1Ab after i.g. exposure. Nevertheless, changes in immune responses related to the ingestion of Cry1Ab-containing feed by sensitised fish (Gu et al., 2013), weaning and old mice (Finamore et al., 2008), rats (Kroghsbo et al., 2008) and pigs (Walsh et al., 2011) have been reported, although the biological and clinical relevance of these alterations need further confirmation.

Our findings reflect the importance of investigating all relevant exposure routes, and not only the assumed primary route of exposure, which in the case of MON810 maize might be through consumption of food and feed. In mice, it has been shown that the inductive sites of immune responses in the nasal region and gastrointestinal tract, NALT and the Peyer's patches respectively, may have different helper T cell activity, which in turn may lead to
different responses to antigen exposures (Heritage et al., 1997). As discussed in paper III, the lack of immunogenic capacity of trypCry1Ab after i.g. instillation, could also be due to the degradation in the digestive tract. It has been demonstrated in vitro that Cry1Ab was exceedingly degraded in SGF at pH 1.2 and a high pepsin to substrate ratio, while the immunoreactivity was maintained at higher pH values (Guimaraes et al., 2010). These findings suggest that the relationship between the resistance of a protein to digestion and its allergenicity with respect to the physiological environment in the digestive tract should be further investigated.

Overall, our data do not provide evidence that i.g. exposure to trypCry1Ab induces immune, including allergic, responses in mice. Sensitised fish as well as mice at a vulnerable stage in life has been reported to display immune system alterations after feeding of Cry1Ab containing diets. Because our data is based on prime aged (young adult) individuals, they should not be extrapolated to more vulnerable stages of life or to immune compromised individuals.

Importantly, in contrast to the purified protein preparations which induced strong antibody responses after airway exposure, the MON810 plant material (pollen and leaf extracts) did not elicit humoral responses irrespective of airway or oral exposure (paper II). The MLN cell cytokine secretion levels were elevated, with a general immune stimulation rather than a skewing of the response in a particular Th1/Th2/Th17 direction, in mice exposed to extracts from both MON810 or non-GM maize leaves, suggesting that the plant material as such induced the immune response. Our data therefore do not suggest any immunogenicity or allergenicity of the Cry1Ab from MON810 plant material. The discrepancy in findings with purified Cry1Ab proteins and Cry1Ab containing plant material after airway exposure may be
explained by the structural and contextual (matrix) differences of the Cry1Ab protein mentioned previously (section 1.7). The trypCry1Ab protein is structurally similar to the plant expressed Cry1Ab, in that both versions consist of the N-terminal half of the Cry1Ab protoxin. However, to our knowledge, the assumption that these versions are functionally identical or fully comparable has never been confirmed. One can therefore speculate that subtle differences between the two versions, because of their different origin, processing and/or three-dimensional structure, could give a different outcome regarding immunogenicity and/or allergenicity capacity. The contextual differences, i.e. the pure proteins versus proteins within the plant matrix may also explain our findings. In paper II, it was discussed that the dose of Cry1Ab was significantly lower for mice exposed to plant material compared to those that received the purified protein solutions. These uncertainties limit the possibility to conclude on the lack of immunogenicity and allergenicity after exposure to Cry1Ab containing plant material. However, under our experimental conditions we did not find any indication of immunogenicity or allergenicity of MON810 plant material, irrespective of exposure route.

5.4 Sources and versions of Cry1Ab proteins

In the experiments, we included four sources of the Cry1Ab protein i) protoxin (paper I), ii) the trypsinised version of *E. coli*-produced Cry1Ab (papers I, II and III), iii) Cry1Ab-containing pollen (papers I and II), and iv) Cry1Ab-containing leaves (papers I and II). We hypothesised that the Cry1Ab protein from different sources, because of their structural differences, might differ in their capacity to induce immune responses. The inclusion of purified protein preparations would provide a proof of principle, while the exposure to Cry1Ab containing plant materials would represent a more realistic exposure. In papers I, II and III, the absence of a detectable humoral response after exposure to the given doses of
plant material indicated no potential for immunogenicity, allergenicity and adjuvanticity of the Cry1Ab protein within plant material. The immune response after airway exposure to both versions of purified proteins (paper II) could therefore reflect i) a much higher concentration of Cry1Ab, and/or ii) that epitopes are masked within the plant matrix, and/or iii) plant expressed Cry1ab is structurally different. As discussed in paper II, the plant material contain only low levels of Cry1Ab, and the concentration is difficult to determine accurately (Szekacs et al., 2012). In comparison with the doses of purified Cry1Ab protein that were given during the i.n. and i.g. exposures, these levels could be too low to activate the immune system.
5.5 Ethical considerations

The use of laboratory animals

It is for obvious reasons unethical to experimentally sensitise human subjects with novel proteins to which they may be exposed again later to assess the potential of these proteins to elicit immune responses. As discussed (section 5.1), mice have been used for many years as models because of the well-characterised immune system that has extensive similarity to the human immune system. This has provided valuable new knowledge about the underlying mechanisms and causes of allergic diseases. The present animal experiments have been conducted in accordance with the 3R concept; replacement, refinement and reduction, as proposed by Russell and Burch (1959). Because of the intricate cooperation between cells and organs within the immune system, the replacement of animals with, for instance, *in vitro* or *in silico* models could in general not answer the research questions being asked. We implemented several steps to refine the experiments. Pilot studies with reduced group sizes were conducted to evaluate the use of CT as adjuvant in i.n. experiments, and for optimising the dose of pollen possible to inhale without affecting respiration and the general well-being of the animals. In addition, the model had previously been optimised to detect the second wave of specific IgE production after a booster dose of allergen (Alberg et al., 2011, Nygaard et al., 2009). A comprehensively tested and well-documented model was applied in the food allergy study (Vinje et al., 2009, Li et al., 2000). The number of animals needed per experimental group was determined using sample size calculations based on these previous experiments and the pilot studies (section 3.1 and 3.2). Corresponding human and mouse endpoints were clearly defined and followed in all experiments. Writing the papers, effort was made to meet the ARRIVE guidelines for reporting animal research and reporting *in vivo* experiments, as proposed by Kilkenny et al. (2012). High-quality reporting of animal
Experiments may lead to less need for replication of studies and thus a reduction in the number of animals needed for experiments.

**Ethical aspects of growing and use of the GM maize MON810**

With the introduction of insecticidal qualities, the cultivation of MON810 maize gives prospects of higher and more predictable yields due to reduced damage by pest insects (Areal et al., 2013, Shi et al., 2013). In view of the world’s growing population and the inevitably increasing demand for food, one could argue that it is unethical not to exploit the available technology and scientific potential of obtaining larger harvests of maize that could be utilised as food. However, one should first ask if these predictions are true, does insect resistant maize actually provide more food on the table? Further, if it does, are the benefits greater than the potential or impending adverse effects on ecosystems and the health of humans, domestic and wild animals? Also, the intellectual property rights including patents on GM seeds like MON810 maize may impact traditional ways of agriculture, e.g. by limiting seeds for recycling and sharing among farmers, which may have important consequences for biosafety issues of gene flow and co-existence of GM and non-GM crops as well as consequences for landrace diversity (van Heerwaarden et al., 2012, Pineyro-Nelson et al., 2009).

It has been postulated that the lack of research performed by unbiased scientists and the economic interests embedded in the GM industry complicates the search for answers to these questions (de Vendomois et al., 2010). Looking at the bigger picture, the investigation of possible adverse immunologic effects constitutes a small piece of the puzzle. Nevertheless, if MON810 maize was to increase the burden of allergic disease in terms of new allergies or augmenting the prevalence or severity of allergic diseases already present, the public health burden cannot be ignored. We have an ethical obligation to further address this issue as well.
as other aspects related to the “pros and cons” of growing MON810 maize and other GM crops.
6 Conclusions and further perspectives

The GM maize event MON810 has been inserted with a processed version of the transgene, cry1ab, derived from the soil bacterium Bt to express proteins with insecticidal properties. The introduced toxin may confer risks of immunogenicity, allergenicity and/or adjuvanticity to humans as well as domestic and wild animals, via inhalation of pollen and plant debris, or through consumption of MON810 containing food and feed. The lack of knowledge regarding immune effects after airway exposure and the possible adjuvant capacity encouraged us to investigate the immune properties of Cry1Ab protein, as purified bacterial versions and within plant material, in two mouse models of allergy.

Within the limits of our study design and dose range of Cry1Ab proteins, our data gave no indication of adjuvant capacity of Cry1Ab, irrespective of Cry1Ab version and exposure route. The clear humoral response induced after airway exposure to Cry1Ab protoxin and trypCry1Ab, without an added adjuvant, suggests that these proteins have both immunogenic and allergenic properties. The results confirm that airway exposure to Cry1Ab proteins may be a relevant route of exposure. Our data do not indicate that i.g. exposure to trypCry1Ab induces immunogenic or allergenic responses in mice. Furthermore, at the present doses we did not find any indication of immunogenicity or allergenicity of MON810 plant material irrespective of exposure route.

Based on our results, we see several suggestions for future studies. Well-designed experiments with purified plant proteins, relevant doses and exposure routes as well as long term exposure, need be conducted to further evaluate exposures experienced in real life situations. Studies in susceptible animals should be conducted. Future studies are needed to
investigate the clinical relevance of the observed Cry1Ab-specific IgE response after airway exposure. Firm conclusions regarding immunogenicity and/or allergenicity of Cry1Ab-modified crops must, unfortunately, await the large scale production of purified plant Cry1Ab.

In principle, post-market monitoring could be used to detect sensitising and allergenic properties of MON810 maize. However, to detect moderate changes in incidence and severity of a relatively rare and often diagnostically challenging condition like food allergy, the number of monitored individuals and the collection of sufficiently precise information to draw conclusions would be a formidable and perhaps impossible task.
7 Reference list


BUIATTI, M., CHRISTOU, P. & PASTORE, G. 2013. The application of GMOs in agriculture and in food production for a better nutrition: two different scientific points of view. Genes Nutr, 8, 255-70.


TAKATA, S., OHTANI, O. & WATANABE, Y. 2000. Lectin binding patterns in rat nasal-associated lymphoid tissue (NALT) and the influence of various types of lectin on particle uptake in NALT. Arch Histol Cytol, 63, 305-12.


