DISSERTATION

Titel der Dissertation

„Short and long term effects of GM foods in mice"

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Magistra der Naturwissenschaften Daniela Domniteanu Reiner Mag. rer. nat

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. Nat.)

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Abbreviations list

AAI	Alpha amylase inhibitor
Ab	Antibody
Ag	Antigen
AHR	Airway hyperresponsiveness
BAL	Bronchoalveolar lavage
Bt	*Bacillus thuringiensis*
CSIRO	Commonwealth Scientific and Industrial Research organization
CMV	Cucumber mosaic virus
DTH	Delayed Type hypersensitivity
ELISA	Enzyme-linked Immunosorbent Assay
EPSPS-CP4	5-Enolpyruvylshikimate3-phosphatesynthase
FAO/WHO	United Nation Food and Agriculture Organization/World Health Organization
GM	Genetically modified
GMO	Genetically modified organism
HA	Hectares
H&E	Hematoxylin eosin
HPF	High power field
Ig	Immunoglobulin
i.g.	Intragastric
IL	Interleukin

i.n.	Intranasal
i.p.	Intraperitoneal
ISAAA	International service for acquisition of agri-biotech application
MALDI-TOF MS	Matrix-assisted laser desorption/ ionization time of flight mass spectroscopy
OVA	Ovalbumin, (chicken egg)
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
RBL	Rat basophil leukemia
RT	Room temperature
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SSA	Sunflower seed albumin
TBC	Total BAL cell counts
Th	T helper
TMB	Tetramethyl benzidine
UN	United Nations
Vit	Vitamin
WAO	World allergy organization

1 INTRODUCTION

1.1   Genetically modified crops

According to United Nations (UN), last year the global population exceeded 7 billion. Over the past few years, it has become clear that food production is challenged by loss of arable land, changing climate and evolving plant pest and diseases. Therefore, issues such as ensuring an adequate food supply becomes very important. Genetic selection has been successfully used for thousands of years to improve plant products valuable to livestock and humans and create new plants for enhanced crop performance, yield, and nutritional value [1]. Genetically modified (GM) crops were designed to solve some of these problems and are increasingly used for food and feed production [2] and at the same time they provide a novel alternative to the increase use of insecticides [3]. Genetic modification involves the introduction of distinct genetic traits from organisms into a new host and thus, providing a solution to specific limitations that constrain crop production and/or quality. ISAAA estimates that since 1994, when the first transgenic crop was developed until 2011, genetically engineered crop developed rapidly being grown on 160 million hectares in 29 countries and the most cultivated are GM soybean, maize, cotton, canola, potato, papaya [4]. Noteworthy is that approximately the half of the area cultivated with GMO crops (80 million ha) is found in 19 developing countries [4] where increasing yield and fighting insect pests is critical to avoid large economic loss and fight starvation.

The "first generation" of GMOs was developed mainly to induce resistance against viruses, insects and diseases and thus, improving productivity. The "second generation" of GMO crops with characteristics beneficial to consumers and was generated in the last decade. High nutritional value, and low allergenicity are the main traits of this generation of GMOs and includes the "golden rice" and soybean with high oleic acid and laurate content (Figure 1). Subsequent followed the development of a "third generation", where genetic engineering was employed to manufacture vaccines and therapeutic medicines in different plants [5]. To date genetically modified foods are classified into three categories according to their

usage: a) food is genetically modified (potato, tomato, soya, maize, sunflowers, rice, peas, etc.), b) food contains constituents of genetically modified crops (vitamins, oil, amino-acids, etc.) or c) food contains genetically modified organisms (yoghurt contains transgenic microorganisms) [6].

1994-first generation of GMO crops

✓ herbicide & disease tolerance
✓ insect & virus resistance

Figure 1. First, second and third generation of genetically modified crops (adapted from [5]).

1995-second generation of GMO crops

✓ high nutritional value
✓ low allergenicity

1998-third generation of GMO crops

✓ vaccine
✓ antibody
✓ pharmaceutical proteins
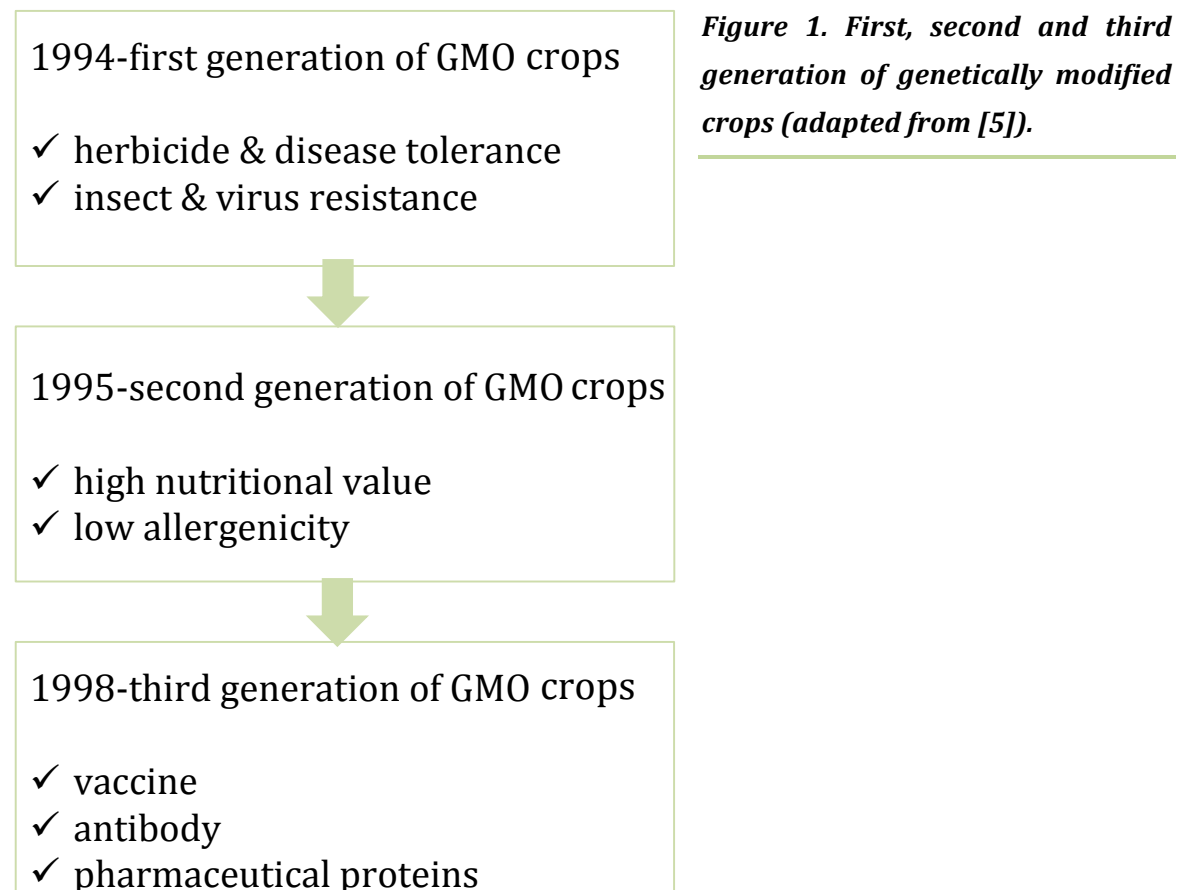
1.1.1 ADVANTAGES OF GM FOODS

Genetic modification of crop plants offers several advantages where other forms of plant breeding proved inappropriate to solve specific problems widespread in many developing countries.

a. Pest and disease resistance**:** to combat viruses, fungi, insects or diseases that damage the crops, farmers have to use pesticides. However pesticides might cause health problems to the consumers, poison water supplies or cause harm to other insects. Growing GM crops have great potential to combat pests and decrease dependency on chemical pesticides [7].

b. Cold tolerance**:** Tobacco and potato plants capable to tolerate cold temperatures were engineered with an antifreeze gene from cold water fish [8].

c. Drought tolerance**:** As the global population exceeded 7 billion more land is utilized for housing. Furthermore, changing in climate and increasing diseases and plant pests leads to the need to create plants that can tolerate long periods of drought.

d. Nutrition: In the third world countries people have to fight starvation and rely on a single crop, for example rice, as the main staple of their diet. However, a single crop cannot prevent malnutrition, as it does not contain sufficient nutrients. Genetically engineered rice or "golden rice" with high content of beta-carotene was created to improve the lack of nutrients and combat blindness which common found in third world countries [9].

e. Pharmaceuticals**:** Investigators are working to develop vaccines in tomatoes, potatoes or tobacco to overcome the high costs of medicines and vaccines. The aim of this research is to reduce costs and to improve storage and shipping [10].

f. Phytoremediation: Studies show that poplar trees have been genetically modified to purify heavy metal pollution from polluted soil [11].

1.1.2 RISKS ASSOCIATED WITH GENE MODIFICATION

a. *Risk to the environment*:

▪   Insects might develop resistance to GM crops: Bt toxin was introduced in GM maize to protect it against the corn borer. However, over the years, the insects have developed resistance against this toxin probably due to low levels of Bt toxin expressed in the transgenic maize. After some time the insects become resistant and pass this trait to the next generation.

- Harm to other organisms: The genes used to create transgenic plants are usually chosen because their products in the host plant will damage only the target insects and pests but not harm non-targeted insects or mammalian consumers. Yet, toxins produced in a GM plants/crops against a different pest may damage non-targeted species .

- Resistance to antibiotics: As part of genetic modification of organisms, marker genes, which provide resistance to antibiotics, are used to determine if the desired gene has been succesfully embedded. Even though marker genes are genetically scrambled before use to reduce the potencial for this danger their use could contribute to the growing problem of antibiotic resistance.

b. *Increased toxicity*:

- Genetic modification may enhance natural plant toxins in unexpected ways: when a gene is switched on, besides having desired effects it may also stimulate the production of natural toxins

c. *Allergenicity*:

- There is a possibility that introducing a gene into a plant may create a new allergen or cause an allergic reaction in susceptible individuals.

1.2   Unintended effects of genetic modification-allergenicity

The concerns of using GM material in foods and feed have focused mostly on possible unintended effects caused by the gene modification process. The safety issues of GM food need to be evaluated and are critical for their acceptance into the market. Although many studies have been conducted to evaluate the safety of GM crops, there is still a matter of debate about the risks induced by the GMO consumption. There is a need for additional evidence for GM food safety.

1.2.1 ALLERGY

The respiratory tract, skin and gut are confronted daily with a broad range of environmental antigens (Ags) varying from innocuous proteins to potentially harmful pathogens. These Ags are captured by Ag presenting cells (APCs) present in the tissues and may induce either immune tolerance or immune responses. Upon engagement of the T-cell receptor and peptide-MHC complex on APCs, helper T cells rapidly develop into distinct lineages with unique functional properties such as Th1 and Th2 and newly descovered Th9, Th17, Th22 and Tregs. Cytokines regulate the differentiation of naïve T cells in different effector CD4$^+$ T cells and the stimulatory microenvironment co-ordinate the actions of specific transcription factors (Figure 2) [12]. Th1 cells secrete IFN-γ and are mainly responsible for cell-mediated immunity, while Th2 cells, producing IL-4, Il-5 and IL-13, are involved in extra-cellular immunity [13].
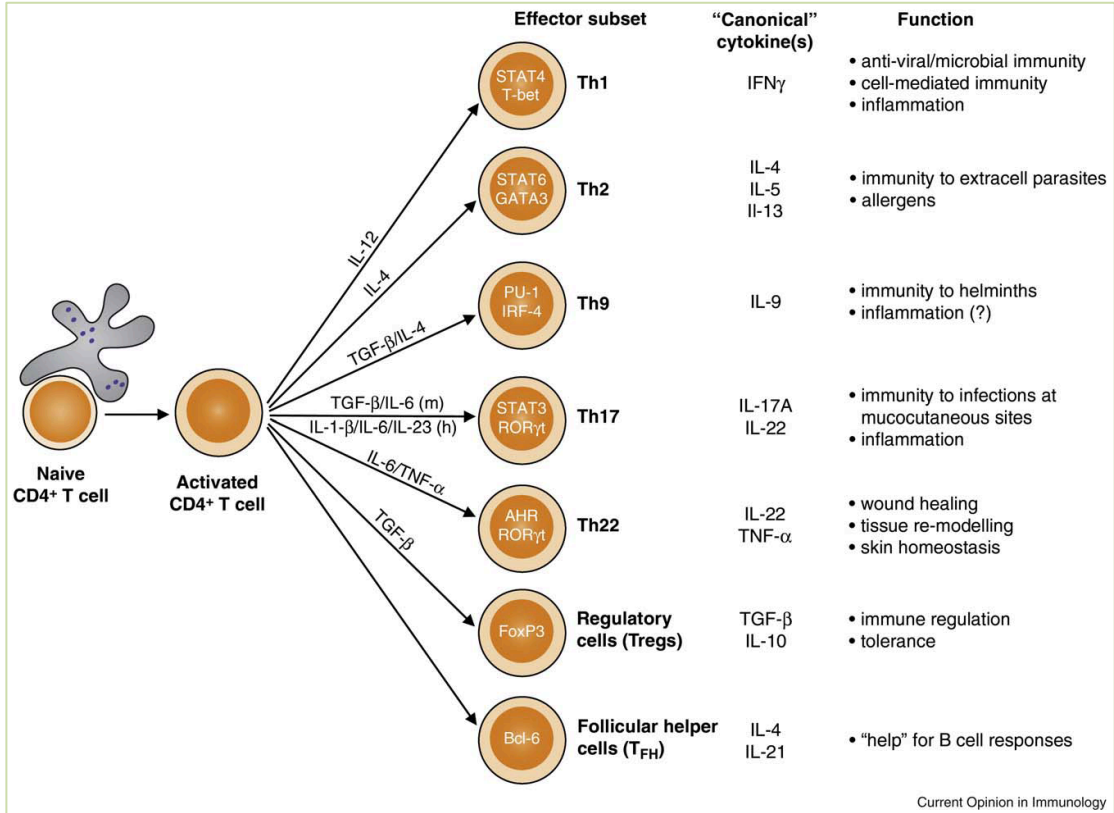
Figure 2. Naive CD4$^+$T cells differentiation into various subsets of effector T cells. *Specific cytokines activate transcription factors that regulate naive CD4$^+$T cell differentiation in distinct subsets of effector CD4$^+$ T cells. (according to Deenick et. al., 2011 [12]).*

Allergens such as proteins in pollen and foods, house dust mite, etc. are able to elicit Th2 responses and allergic reactions in atopic individuals [14]. Although the potential allergenicity of a protein cannot be predicted based on its structural characteristics, however some features are characteristic of many common allergens: low molecular weight, glycosylation and high solubility in body fluids [14]. An aberrant response to such proteins together with a genetic predisposition can generate allergic asthma [15]. Mouse models have been broadly used to investigate the recurrent inflammation, remodelling, airway hyper-responsiveness (AHR) and mucus overproduction main features characterizing disease [15, 16]. Ovalbumin (OVA) is one of the most common allergen, used to model asthma in mice. Allergic reactions can be divided into: *sensitization* and the *allergic reaction* [17]. *Sensitization* arises after single or repeated exposure to the allergen and results in the induction of allrgen specific IgE antibody, with the involvement of T cells, B cells and macrophages. The cascade of events that leads to *sensitization* are: Ag uptake, processing and presentation by APC, T cell and B cell activation with subsequent development of allergic hypersensitivity. Activation of T cells by APCs leads to the release of cytokines that control isotype switch of B cells and synthesis of Ag specific IgE antibodies. In this phase, IgE antibodies bind to FcεRI and II receptors expressed on the surface of mast cells and basophils present in the tissue and circulation [16, 17]. Once sensitized, re-exposure to the same allergen results in a marked and immediate reaction with cross-linking of IgE molecules on the mast cell, subsequent activation, degranulation and release of pharmacologically active mediators (eg. histamines, etc) into the tissue [16, 17].

In the pathology of allergic asthma, a biased Th2 response is considered decisive. Additionally there are Th1, Th17 and possible Th9 cells involved in the pathophysiology of allergic asthma [15].

1.2.2 GENETICALLY MODIFIED CROPS AND FOOD ALLERGY

One major concern is that upon gene insertion, changes in gene expression of the existing genes and/or formation of new genes coding for new proteins (eg. allergens) may occur [18, 19]. According to world allergy organization (WAO), in the last decade the prevalence of allergic diseases worldwide has increased dramatically in both developed and developing countries. There is evidence that the incidence of food allergies around the world is also increasing [20, 21], affecting 2-4% adults and 4-8% children. The potential severity makes it critical to identify specific risk factors. The interplay between a very low number of proteins with the innate immune system may lead to this form of allergy. The reasons for the increase in sensitization are still not entirely understood nor is it clear whether the causes of food allergy are the same as those for inhaled or contact allergy.

Food allergies are induced by a broad range of foods or food constituents otherwise innocuous under healthy conditions. Under pathological conditions, they induce an abnormal reaction of the immune system. Upon feeding, the gut immune system encounters a large number of foreign proteins and potential allergens. The ability to prevent severe reactions to food resides mainly in the way they are presented to the immune system. Subsequent oral exposure to a food antigen may induce a T reg response that suppresses allergic sensitization called oral tolerance [22], initially defined in rodents and humans [23]. Failure in these mechanisms may lead to food allergy (Figure 3).

Most of the known plant food allergens belong to very few protein families on the basis of sequence homology, indicating that some preserved structures play a role in allergenicity [24]. Critical in the development of the allergenic potential is the level of exposure and the physico-chemical features of allergens [25]. Usually, common food allergens are stable to processing and digestion and are not easily degraded either by thermic processing, pH or by proteolytic digestion [25]. As a consequence, it is likely to be presented as conserved three dimensional protein structures to the immune system of the gut, thus, breaking the oral tolerance.
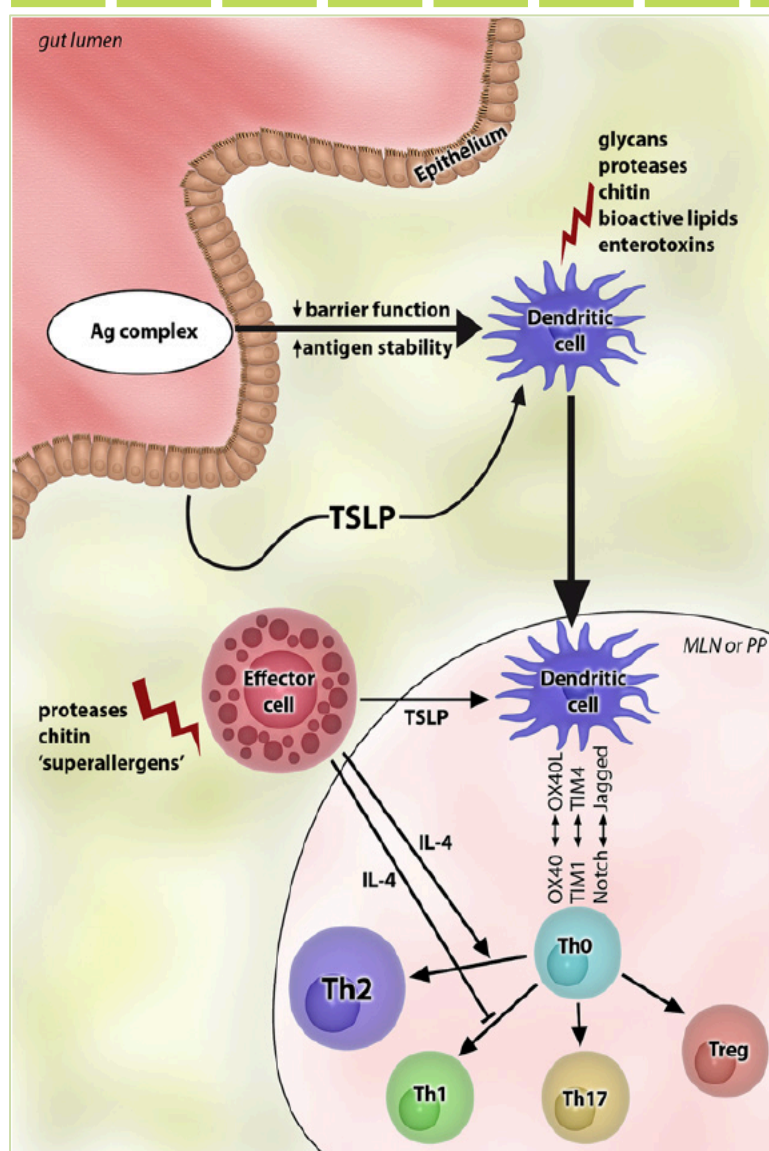
Figure 3. Potential mechanisms of allergic sensitization to antigens administered via gastrointestinal route. Ags are collected by M cells present in the gut and delivered to DCs. Intrinsic physico-chemical features of the Ags and the compromised epithelial barrier in the gut may lead to DC activation followed by Th2 differentiation [26] (according to Berin et. al., 2008).

There are increasing concerns about the introduction of GMOs into the human food chain because the insertion of novel genes into food might induce the expression of new proteins, which may cause allergic reactions in predisposed individuals. The risk assessment for newly created GM foods and their ability to sensitize the consumers is based on the knowledge of the source of new constituents and the possible cross-reactivity with known allergens [25]. Importing a new gene in a plant may produce a new protein able to trigger allergic reactions. For example, this occurred in 1996 when soybean was modified by the addition of a albumin gene from the brazil nut, to enhance the methionine content of animal feed [27]. However, the GM soybean expressed a new protein not found in soybean [7]. Tests with sera from brazil-nut allergic people revealed IgE binding reactions to the GM-soybean protein [7]. This incident emphasized one of the potential drawbacks of genetic

engineering, namely the transfer of an allergenic potential in the newly created GM. Another case related with potential allergenicity of GM foods was the Star Link maize [28]. This GM maize variety was engineered to express the Cry9C form of *Bacillus thuringiensis,* is resistant against the corn borer (*Ostrinia nubilalis*). Developers observed during the transformation process that Cry9c protein had potential allergenic properties, such as resistance to proteolysis in gastric-stimulated fluid. Moreover, there was an increase in both anti-Bt IgG and IgE response,s rash and erythema in farm workers [29, 30] and a significant anti-Bt IgG1 levels in rats fed transgenic Bt-rice spiked with purified Bt-toxin [31]. These results led to the hypothesis that consumers might develop allergenicity to Star Link maize. A third was the alpha amylase inhibitor (AAI) pea produced at CSIRO in Australia. The new AAI protein displays different structure when introduced in peas compared to AAI expressed in the native bean [50]. Upon feeding to BALB/c mice, the altered structure of AAI apparently induced Th2-type allergic inflammation.

1.2.3 GUIDELINES FOR ASSESSMENT OF ALLERGENICITY OF GM PLANTS

GMO crops are cultivated all over the world. The number of consumers have increased substantially since 1996. Due to the increase on GMO consumption, it was necessary to evaluate the potential allergenicity of novel GMOs. Experts expressed their concern that the introduction of genes into foods might lead to the development of food allergies to new proteins or to the whole plant and/or derived products [32]. The ability to predict new food allergies it is not well established [33]. Therefore, a decision-tree was implemented and approaches were developed with the aim to evaluate the allergenic potential of GM foods. The approach for evaluating potential allergenicity of GMOs is based on guidelines specified in Codex Alimentarius by the experts of FAO/WHO (Figure 4).
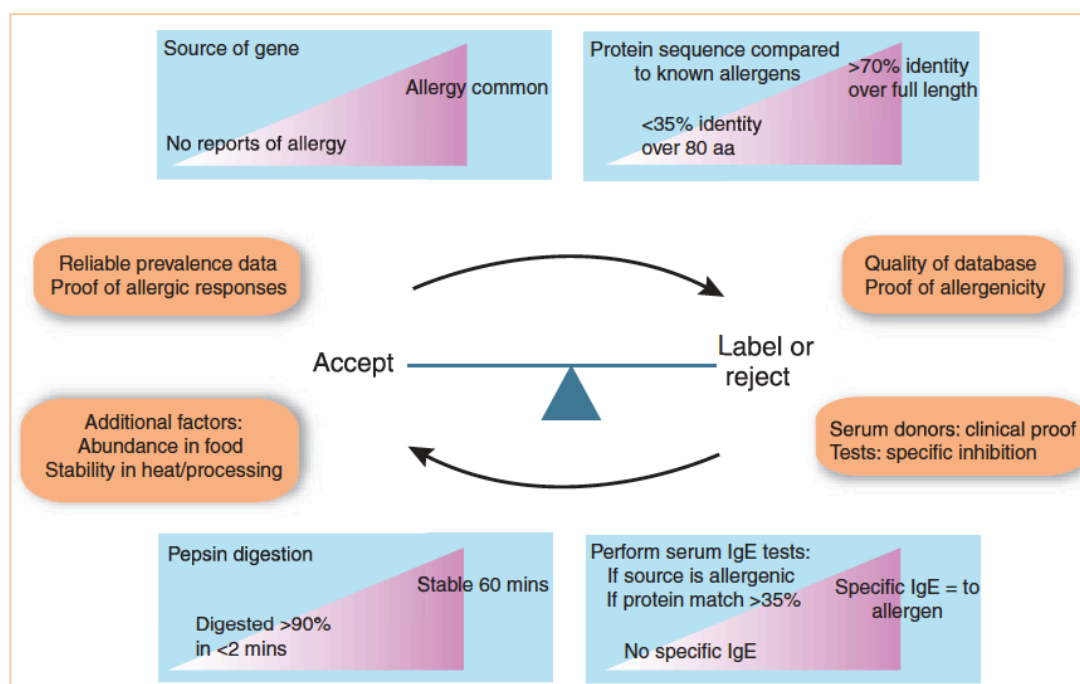
Figure 4. Graphic representation of the weight-of-evidence approach described by the Codex Alimentarius Commision Guidelines for Allergeniciy Assessment in 2003 (from Richard Goodman [7]).

These recommendations include gene source, comparison of newly introduced proteins with known allergens, *in vitro* testing of the reactivity with serum from allergic patients, and assessment of protein stability to proteolytic enzymes.

In the last decades, allergic disease has increased in prevalence with associated influence on life quality and economy. Thus, the WHO and other food safety authorities accept that allergy in general, and food allergies in particular are a major public health concern. Experts at the FAO/WHO are constantly reviewing this decision tree because it provides a suitable framework for the assessment of the allergenic potential of GM foods. These approaches provide possible evidence of the risk of induction of an allergic response by the transgenic protein in previously sensitized individuals. The evaluation of allergenicity potential of GMOs is based on a comparative approach and aims to determine if the allergenicity differ between the GM crop and its isogenic counterpart using scientifically relevant techniques. The allergenicity evaluation of GMOs under Codex Alimentarius/EFSA regulations concentrates on protecting already allergic consumers from unintended exposure to GMOs, but also *de novo* sensitization.

Evaluation of the source of the gene: The main issue to investigate in allergenicity assessment of GMOs is whether the source of the gene encoding the new protein is a known allergen or not. In both situations, a sequence homology search has to be conducted using FASTA or BLAST algorithms. If the sequence homology comparison of the transgene with known allergens gives a positive result or a positive serum screening, this might indicate the possible allergenic features of the transgene. In the case that the both sequence homology and serum screening are negative, then a combination of methods such as pepsin-resistance and animal/models are used to determine if the transgene has a high, medium or low likelihood of allergenicity [7, 34, 35].

Bioinformatics analysis: The guidelines in Codex Alimentarius specify that regardless of the source of all transferred proteins, homology analysis of the amino acid sequence should be performed. Comparisons with already known allergens should be done to establish whether the sequence similarity, if existing, is enough to ensure that the inserted protein would cause allergic cross-reactions in susceptible individuals. Based on the results obtained with FASTA or BLAST algorithms, the proteins with high amino acid sequence identity would further require testing for the evaluation of the potential IgE binding with serum from donors with allergies to the source of the sequence-matched allergen. If the amino acid structure identity of the transgene matches more than 70% with the allergen, the cross-reactivity potential is also high. A 50% to 70% sequence matching present a moderate risk of cross-reactivity. In both cases, serum screen for IgE binding should be performed. Less than 50% identity match indicate a lower cross-reactivity risk. However, a threshold value of 35% amino acid identity sharing might also maintain conformational epitope structure [7, 34, 35].

Serum screen for IgE epitopes: Serum IgE-binding studies are needed to investigate whether proteins from an allergenic source or those that share a sequence identity higher than 35% over an 80 amino acid window are safe for the consumers. For this test, sera from patients with a history of allergenicity to the source are necessary to confirm that the transgenic protein encoded by the inserted gene does not bind IgE. Several factors as demographic factors are important for patients selection, since studies have shown that both age and habitat may influence the molecular recognition profiles of specific IgE. The outcomes of serum screening for IgE-binding

deliver critical informations for the possible incidence and intensity of sensitization to allergens. For these assays, sera from various, already sensitized individuals have to be tested due to the differences in specificity and affinity of IgE antibodies among the allergic patients. Also important to take in account are the use of appropriate negative control to prove assay validity. Moreover, IgE binding assays should be able to detect IgE binding to both linear and conformational epitopes [7, 34-36].

1.3   Mouse models to test allergenicity to GMO

The development and introduction of GMO food and feed on the market raised questions of the possible rise in allergenicity risk of the newly expressed proteins, but also of the whole GM food and feed. It is generally accepted that no single assay or feature categorically differentiates allergens from non-allergens. Currently, the allergenicity assessment for novel as well as for transgenic protein is performed using various methods. The existing approach is based on the ranked decision tree developed by experts in the area and recommends comparison of the inserted protein with known allergens [37]. Furthermore, experts also suggest, as a part of allergenicity assessment, to perform reactivity tests with sera from allergic patients and to assess the stability to digestive enzymes [37]. In different studies, it has been stated that resistance to enzymatic digestion is a common feature shared by various allergens and differentiate them from non-allergens [38, 39]. However, not all food allergens share this property. Therefore, association between increase allergenic potential and resistance to enzymatic digestion is not absolute [37]. According to the FAO/WHO prediction of sensitizing potential and evaluation of the possible allergenicity of newly expressed proteins in GMOs and the whole GM food should be done also using animal models. These animal models are considered to be advantageous when the protein expressed in the GMO has been not been on the human diet [40]. In various studies, the development of appropriate animal models was attempted [41, 42]. Preferably, the features of the disease developed by an animal model should resemble as closed as possible the human disease [37]. Besides, these models should ideally predict the allergenicity potential of novel proteins, and/or of the whole GM foods compared to the isogenic controls. Currently, the elaboration and validation of *in vivo* models to test the allergenicity of GM plants become an area of strong research and development. Several animal models

designed to identify potential hazards in GMO, and to test novel transgenic proteins have been proposed including the Brown Norway rat, BALB/c and C3H/HeJ mice, dogs and pigs [43].

Mice have been commonly used to assess allergenicity due to the fact that they share several essential immunological mechanisms with humans such as Th1, Th2, Th17 and regulatory responses [7]. In an oral sensitization model, Morafo *et al*. accentuated the importance of choosing the correct the mouse strain [44]. It is well known that IgE antibody isotype plays an important role in allergic reactions, and it is commonly associated and regularly used as a marker in food-allergic disease in both humans and animals [37]. Since BALB/c mice have a predisposition towards a Th2-type phenotype and are known as a high IgE responder strain, they are preferential used in different immunological investigations [45-47]. It has been also been shown in studies of food hypersensitivity, that BALB/c mice develop clinical symptoms of food allergy such as eosinophil and mast cells infiltration as well as antibody response to food proteins [48, 49].

Table 1: Mouse models for allergenic assessment of GMOs

GMO tested	Trait inserted	Evaluated parameters	Reference number
Pea	AAI insect resistant	Anti-AAI IgG1, mucus, airway and lung infiltrates, DTH, Th2-type cytokines	[50]
Tomato	CMV	Growth, body and organ weights, hematological and biochemical indices	[51]
Sweet paper	CMV	Growth, body and organ weights, hematological and biochemical indices	[51]
Maize	Bt endotoxin, insect-resistance Cry34Ab1 and Cry35Ab1 Bt	Hematology and serum chemistry values, organ weights, gross pathology and histopathology	[52]
Maize	2mEPSPS		

Potato	Cry 1, insect resistance	Microscopic structure of ileum	[53]
Soybean	CP4-EPSPS	Toxicity, food composition, body weight, gross pathology	[54]
Soybean	CP4-EPSPS, glyphosate-tolerant	Ultrastructural, morphometrical and immunocytochemical analyses of pancreas	[55], [56]
Soybean	CP4-EPSPS, glyphosate-tolerant	Structural and molecular modifications of nucleoplasmic and nucleolar constituents on pancreatic acinar cell nuclei	[57]
Soybean	CP4-EPSPS, glyphosate-tolerant	Ultrastructural analysis of testes	[58]
Soybean	CP4-EPSPS, glyphosate-tolerant	Fetal, postnatal, pubertal, and adult testicular development	[59]
Tomato	Coat protein resistance to cucumber mosaic virus	Growth, body weight, hematology and biochemical indices, acute toxicity, genotoxicity	[51]
Corn	Bt176, insect-resistant	Germ cell populations	[60]
Corn	NK603xMON810 insect resistant	Multigeneration study, performance and reproduction, organ weights, histological investigation of : intestine kidney, liver, pancreas, spleen, lungs	[61]

1.4   Future of biotechnology

Bioengineered foods were introduced on the food market silently and without obligatory labeling required in developed countries. Unintended exposure to allergens can follow through intentionally newly not labeled food components existing in GMOs. Genetically engineered crops contribute mostly in diminishing poverty in third world countries, through increasing crop productivity. The

advantages offered by the biotech crops such as improved resistance against insect and disease are unique and cannot be addressed with other form of plant breeding. According to ISAAA [62], the total area of cultivated GM crops between 1996-2008, surpassed 800 million hectares (2 billion acres). Interestingly, 10 years were needed to reach the first billion acres but only 3 years to reach the second billion acres of planted GMOs, yet only three years were required before the second billionth acre (800 million hectares) was cultivated in 2008 [62]. The high adoption rate of GM crops illustrated by the increase of the cultivated area in 25 countries all over the world, reveals the fact that biotech crops found universal approval and provided a substantial economic, environmental and social benefits to farmers in both in developing and industrial countries [62].

1.5   Genetically modified crops used in this study

1.5.1 AAI PEA, COWPEA AND CHICKPEA

Pea (*Pisum sativum*), cowpeas (*Vigna unguiculata*) and chickpeas (*Cicer arietinum*) gained worldwide economic importance as source of protein for animal as well as for human nutrition [63]. Peas are important rotation crop for Australian farmers, it improves the soil and reduces root disease among following crops (*personal communication T.J Higgins*). Furthermore, peas are well assimilated and have high energy content [64]. Cowpeas and chickpeas are major crops used as main food staple for human and animal nutrition in Africa (Nigeria, Niger and Burkino are the major producers for cowpeas) and on the Indian subcontinent (India and Pakistan are the major producers for chickpeas) providing a rich source of dietary proteins [65, 66]. However, bruchid beetles (*Brunchus pisorum*, *Callosobruchus maculatus* and *C. chinensis*) threaten and damage peas and chickpeas in storage and cowpeas in every developmental stage [67] leading to massive lost.

Figure 5. Field peas are susceptible to the pea weevil (Bruchus pisorum). The weevil lays eggs on the pea pod. In the non-protected isogenic (nGM) peas the eggs hatch and the larvae eat their way through the pod and into the seed, where they develop into adults, leaving a large hole when they emerge from the pea seed. GM peas engineered with a gene from Tendergreen bean are protected against the weevils (courtesy of T.J. Higgins, CSIRO).

To confer resistance against insects and to increase yield, peas, chickpeas and cowpeas containing AAI from the common bean (*Tendergreen bean*) were generated [65, 66] in Australia by Commonwealth Scientific and Industrial Research Organization (CSIRO). AAI is a member of a family of proteins known to play a role in plant defense [68] and occurs naturally in many food plants [69], including bean seeds (*Phaseolus vulgaris*) [68, 70]. Several studies revealed that AAI plays a role in natural defense mechanisms [67].

This protein family also includes phytohemagglutinin and arcelin, both with a diverse way of insecticidal action. AAI is absent in peas, cowpeas and chickpeas. Weevils can reduce pea yields by up to 30% and any sign of weevil damage downgrades the pea quality and value (*CSIRO interview*). Unlike peas, cowpeas and chickpeas, beans are protected against weevils as they express AAI protein that inhibits the activity of alpha amylase and causes the weevils feeding on the GM pea to starve before they cause any damage [71]. AAI from common bean has been introduced into peas [72], chickpeas [65] and cowpeas [66] using Agrobacterium-mediated transformation with the purpose of improving their resistance against bruchid pests. Genes encoding lectins and enzyme inhibitors seem to be valuable to use in genetic modification processes because their effects on the insect gut can be proved and their defending effect for the plant quantified [72]. Indeed, results show that using Agrobacterium-mediated genetic engineering, the AAI protein

accumulates in higher amounts in the pea, chickpea and cowpea seeds (up to 3%) compared to the AAI normally found in beans (1 to 2%) [72]. As a consequence of this gene insertion, field trials revealed that transgenic pea, chickpea and cowpea seeds expressing AAI are protected in the developing stages and also during storage. This particular AAI gene that encodes an insecticidal protein, was attractive for use in food crops mainly, because humans already consume AAI in beans without any known allergic effect [73] and secondly, because it is beneficial for the environment leading to decrease use of pesticides. α-amylase inhibitors occurs naturally in beans and have been extensively studied [74]. However several studies demonstrate that purified AAI inhibited starch digestion in the rat small intestine leading to losses of nitrogen, lipids and carbohydrates and thus, preventing optimal growth [75]. Furthermore, feeding studies performed by CSIRO and other groups in rats [64], pigs and broilers revealed reduced starch digestion in animals fed GM peas compared with the isogenic pea lines. Similar, *Prescott et. al* showed in a study published in 2005 that mice fed GM pea but not isogenic pea developed Th2 inflammatory immune response independent from IL-5 and eosinophils. Furthermore, they also showed that simultaneous gastrointestinal exposure to transgenic AAI and OVA cross-primes and increase the immune response to OVA [50]. Interesting, when the same group performed feeding experiments with GM chickpea, this did not lead to similar results. The authors suggested that the differences observed in the immune response between mice fed GM peas and beans reflect variations in posttranslational processing that occurs in beans and peas. AAI is a complex protein that undergoes complex posttranslational processing involving proteolytic cleavage of C-terminal amino acid residues and glycosylation [76].
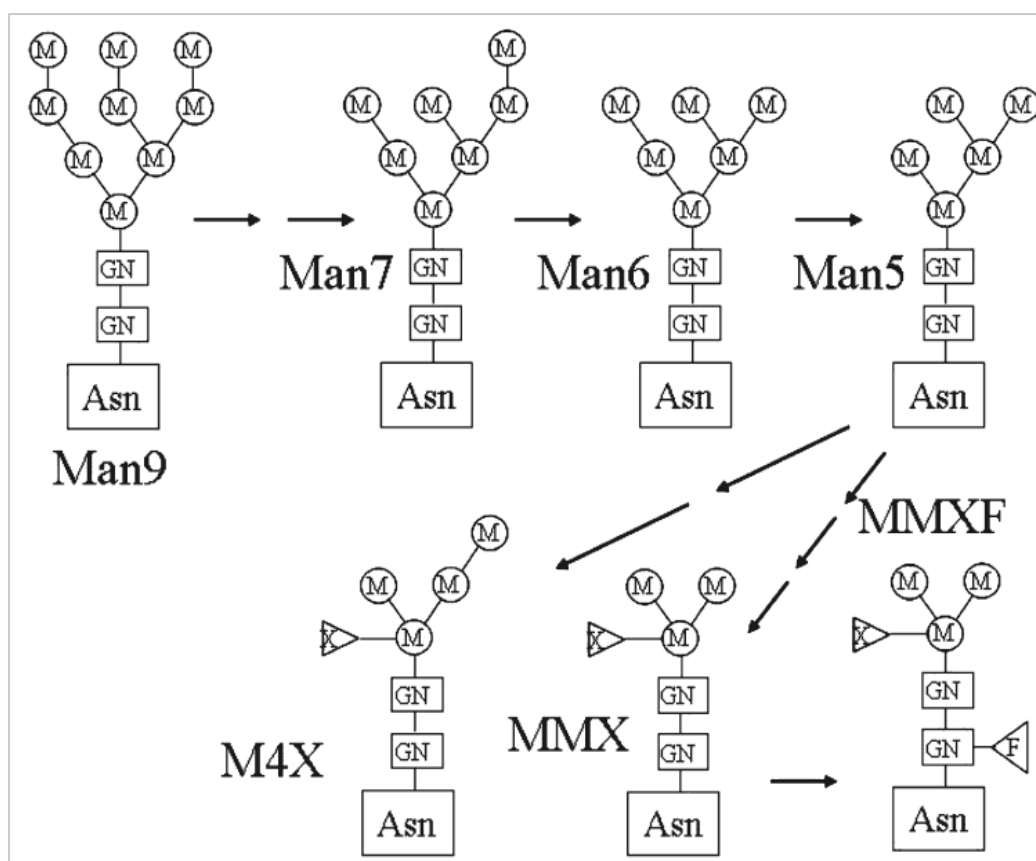
Figure 6. Simplified glycan processing in plants according to Campbell et al. [73]. N-glycosylation of proteins in plants and mammals starts in the endoplasmic reticulum by the transfer of Glc3Man9GlcNAc2 to an appropiate asparagine residue. With transport through the endoplasmic reticulum, the Golgi apparatus, and the vacuole, sugars are removed converting the precursor to high mannose-type N-glycans from Man9GlcNAc2 (Man9) to Man5GlcNAc2 (Man5).

The synthesis of a mature, functional protein occurs in beans as the AAI precursor undergoes a complex posttranslational modification including glycosylation and proteolytic processing [77]. Mass spectra of the β subunit of AAI purified from different transgenic legumes were aligned (Figure 6) and compared to the corresponding AAI β chain extracted from the native *Tendergreen bean* [73]. The various transgenic β-chains differed from the *Tendergreen bean* first by showing less overall glycosylation and second by showing significant C-terminal truncations (Figure 6 and Table 2).
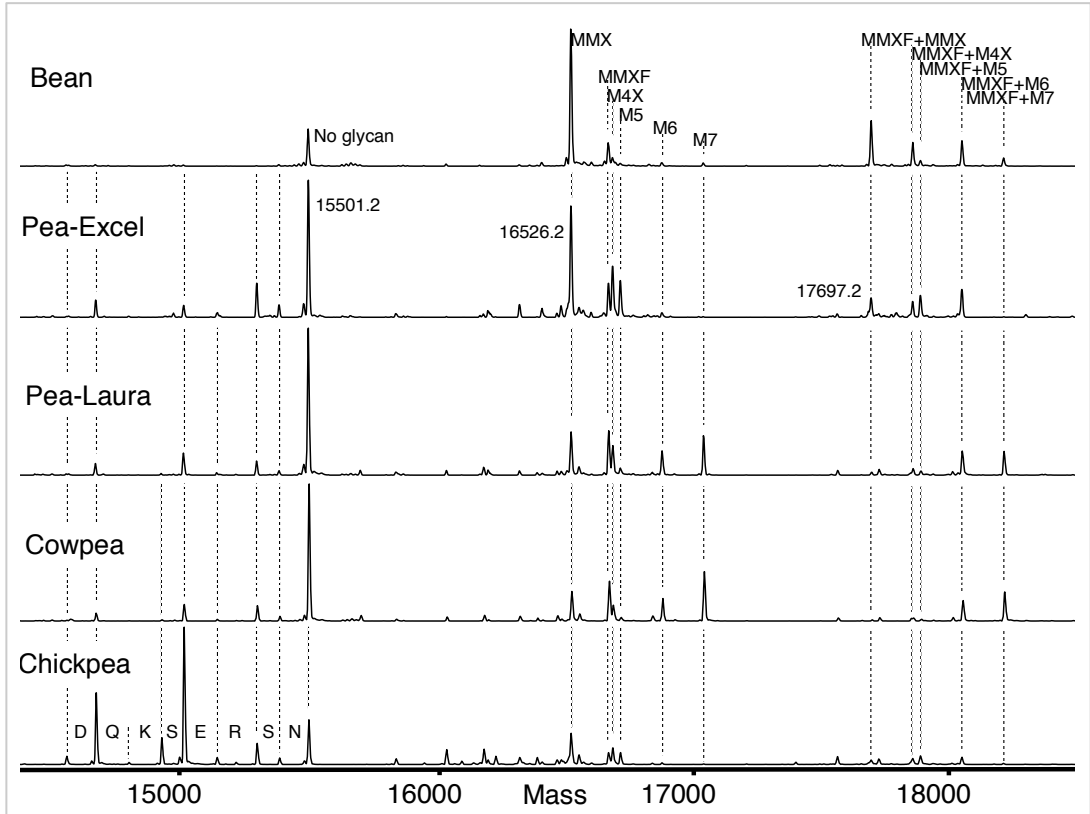
Figure 7. Deconvoluted mass spectra of the β-chain region of AAI *isolated from Phaseolus vulgaris cv. Tendergreen bean and 4 transgenic pea varieties, pea Excell, pea Laura, cowpea and chickpea (courtesy of T.J. Higgins-CSIRO).*

Table 2. Relative abundance of glycoforms and C terminally truncated forms of the α-amylase inhibitor β chain from varieties and various transgenic legumes [73].

	No glycam	One glycan total	Two glycans	MMX	% C-terminal truncation
T. bean	11	56	33	42	<1
P. bean	16	84	0	69	<1
Pea Excell	27	53	20	25	37
Pea Laura	34	49	17	11	30
Chickpea	29	52	19	24	87

Due to the findings of this study [50], CSIRO discontinued the development of the GMO peas.

1.5.2 MON810 (Bᴛ ᴄᴏʀɴ)

Progress in biotechnology led to an increasing number of GM containing foods, and among these GM corn is one of the most widespread. Following wheat and rice, corn is the third most cultivated crop worldwide and extensively used for food and feed production all over the world. According to ISAAA, in 2007 the area cultivated with corn worldwide was 158 million ha. and among this area 26% were genetically modified. In 1990, Monsanto developed the MON810 corn also known as *Bt* corn. Eight years later, it was one of the first GM crops approved for cultivation in the European Community. Maize MON810 expresses the transgenic insecticidal active Cry 1Ab protein, derived from *Bacillus thuringiensis,* which protects corn mainly against European corn borer (*Ostrinia nubilalis*) [78] responsible for almost 10% of damages in field. Cry proteins, produced by the gram-positive soil bacterium *Bacillus thuringiensis*, are insecticidal toxins extensively characterized. The receptors for Cry proteins exist only on the intestinal cells of certain insects [79, 80] but they lack in mammals, therefore they have been extensively introduced in the creation of new GM crops. The insecticidal traits of *B. thuringiensis* toxins have been used commercially, and preparations of insecticidal spray have been used to control lepidopterian and coleopterian insects. The crystalline (Cry) proteins are produced as inactive protoxins and upon activation in the insect´s midgut at a pH (>9.5) [81, 82] they release δ-endotoxins, which display a highly specific insecticidal activity. Cry proteins bind to receptors in the mid-gut cells of susceptible insects inducing pores in the membrane and cell lysis due to an influx of water. Ultimately the disruption of gut leads to death of the insect through starvation [83]. The use of Cry toxins has increased dramatically following the introduction of *cry* genes into rice, tomatoes, cotton, potatoes and maize with impressive results against insect pests. However safety concerns have been expressed about the effect Bt crops may have on the environment and on human health due to the frequent use of Cry genes in GMOs. Studies performed on BALB/c mice showed that Cry1Ac protein, with the similar structure as Cry1Ab, is immunogenic inducing the production of IgG, IgM and IgA antibodies upon i.p, i.g., i.n. administration [84]. Moreover, feeding MON810 to young and old mice leads to the alteration of intestinal and peripheral immune cell population and increase in Th2 cytokines [85]. Researchers also found that Cry1Ab induces a specific immune response in rats upon feeding GM rice containing or spiked with the cry protein [31]. Predicting potential allergenicity is a major issue in

safety assessments of GM crops. Taken together these studies show the intrinsic immunogenicity of Cry proteins in mice and rats. In recent studies, it was shown that genetically engineering of maize with a transgene from *Bacillus thuringiensis* corn induces the expression of one protein, γ-zein [85], previously identified as an allergen [86].

1.6 Aim of the study

The current study was performed to address two related questions. In light of the fact that AAI are differentially glycosylated in GM peas versus native beans, the purpose of this study was first to assess the intrinsic allergenic and immunogenic potential of the purified transgenic AAI proteins expressed in GMO chickpea and cowpea using two different routes of administration without using adjuvant. We also aimed to characterize the immune response obtained upon feeding GM chickpeas and cowpeas in a BALB/c mouse model compared to the near isogenic lines. Additionally, in a mouse model of initiation and relapse phase of OVA-induced allergic disease, we evaluated the allergenic potential of feeding MON810 and its isogenic counterpart.

2   ANIMALS AND REAGENTS

2.1   Mice

Four to six week old female BALB/c mice were purchased from Charles River Germany and maintained in the Veterinary Medicine University of Vienna animal facility on a 12-h light/dark cycle. Before starting the experiments mice were acclimated for at least 1 week to the animal facility conditions. Mice used in all experiments were provided OVA and corn-free chow but including soybean, (probably GM), from SSNIFF, Soest, Germany and water *ad libitum*. Charles River mice were also fed a standard diet probably containing GM-soybean. Thus, all experiments are supposed to be done in the presence of GM-soybean. All experimental protocols were performed in compliance with the appropriate Austrian laws and with the requirements of the Animal Care Committee of the Austrian Ministry of Science (68205/12-II/108/2009).

2.2   Chemicals

Ovalbumin (OVA, grade V) from Sigma Chemical Co. (St Louis, MO, US) was used for all experiments. Biotinylated anti-IgG1 and anti-IgG2a detection mAb and streptavidin horseradish peroxidase were from Southern biotechnology associates Inc. (Birmingham, AL, USA), while 3.3´, 5.5´-tetrametylbenzidine (TMB; BD OptEIATM) substrate and biotinylated anti-IgE detection mAbs were from Becton Dickinson Biosciences (Franklin Lakes, NJ USA).

2.3   GM materials

-AAI materials

Seed meal from GM cowpeas and chickpeas expressing AAI protein and their near isogenic controls (2 isogenic cowpea lines and one isogenic chickpea line) were provided by T.J. Higgins (CSIRO, Canberra, Australia) and generated as described in

[72] and grown side by side in green house. Seeds were grounded and sieved through a 40μm strainer and stored at 4ºC until administration. Shortly before intra-gastric administration to mice, the plant material was homogenized in phosphate-buffered saline (PBS).

-MON810 maize

Non-GM isogenic parent line of corn (Pioneer PR34N43) and GM corn (Pioneer PR34N44 event MON810) were grown simultaneously in Valtierra, Navarra, Spain and provided by Peadar Lawlor (Teagasc, Moorepark Research Centre, Fermoy, Co. Cork, Ireland). The GM and non-GM corn were tested for the presence of the cry1Ab gene by MON810 event-specific PCR. Mice were fed for 4 weeks *ad libitum* with corn-free chow, or mouse chow containing 33% Bt-maize (S0166-S040, SM M-Z, 10mm) or isogenic corn (S0166-S030, SM M-Z, 10mm) prepared by SSNIFF Soest Germany.

Table 3: Food formulation for MON810 maize experiments

Raw nutrients	Feed additives per kg		
Raw protein	Vit. A	[IE/IU]	15,000
Raw fat	Vit. D3	[IE/IU]	1,500
Raw fiber	Vit. E	[mg]	150
Raw ash	Vit. K3	[mg]	20
Starch	Vit. C	[mg]	---
Sugar	Copper	[mg]	5

2.4   Experimental design

2.4.1 INDUCTION OF IMMUNOGLOBULIN TO AAI

To establish whether differences in the structure between native and transgenic AAI proteins alter immunogenicity, we injected mice intraperitoneally (i.p.) with a solution of 10 μg of purified AAI proteins from transgenic peas, bean varieties compared to pea lectin protein and an well known allergen (OVA) on days 0 and 21

(in 200 μl PBS, without adjuvant). Seven days later, sera were collected and stored at -20C until further use.

2.4.2 INDUCTION OF AAI-INDUCED ALLERGIC ASTHMA

To determine whether pure AAI proteins from native and transgenic sources would induce allergic disease upon respiratory challenge, mice were instilled 50μg/50 μl of purified AAI protein dissolved in PBS i.n. on days 1, 3, 5, 15, 17, 19 and evaluated for allergic responses on day 22. As positive control we instilled OVA to mice according to the same experimental protocol.

2.4.3 INITIATION AND ONSET OF OVA-INDUCED ALLERGIC ASTHMA

To establish acute allergic asthma groups of mice were immunized intraperitoneally (i.p.) with 10μg of OVA (Sigma Chemical Co., St. Louis, MO) dissolved in 200μl phosphate buffered saline (PBS) on days 0 and 21. To induce acute phase of the disease, we nebulized 1% OVA in PBS (100 mL/nebulization period, Aerosol OVA) delivered by an ultrasonic nebulizer (Aerodyne, Kendall, Neustadt, Germany) for 60 minutes twice daily on 2 consecutive days (Figure 8).
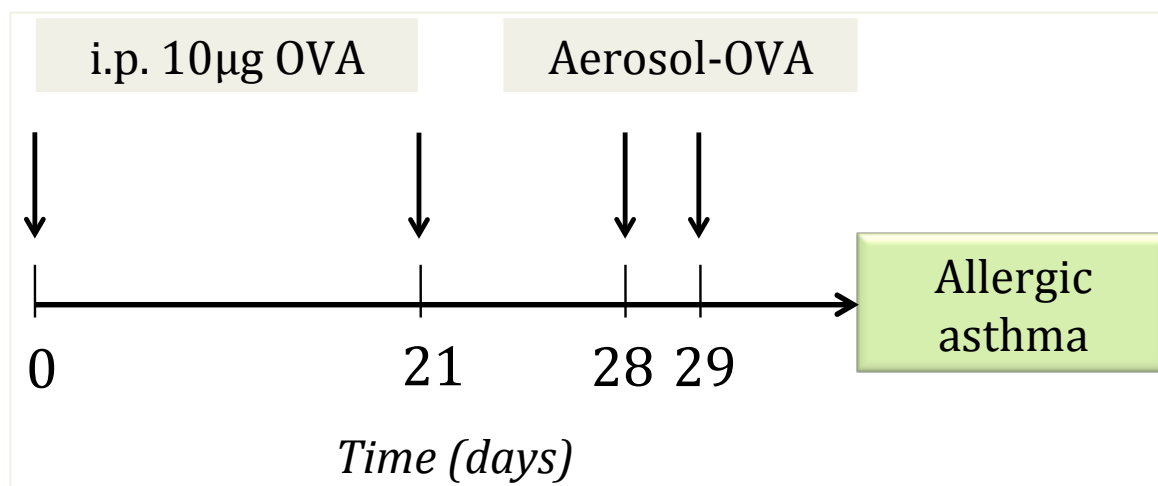
Figure 8. Experimental design of OVA-induced allergic disease

To induce disease exacerbation, mice induced with acute disease were allowed to recover and re-challenged with aerosolized 1% OVA for 60 minutes twice daily on 2 consecutive days (Figure 9).
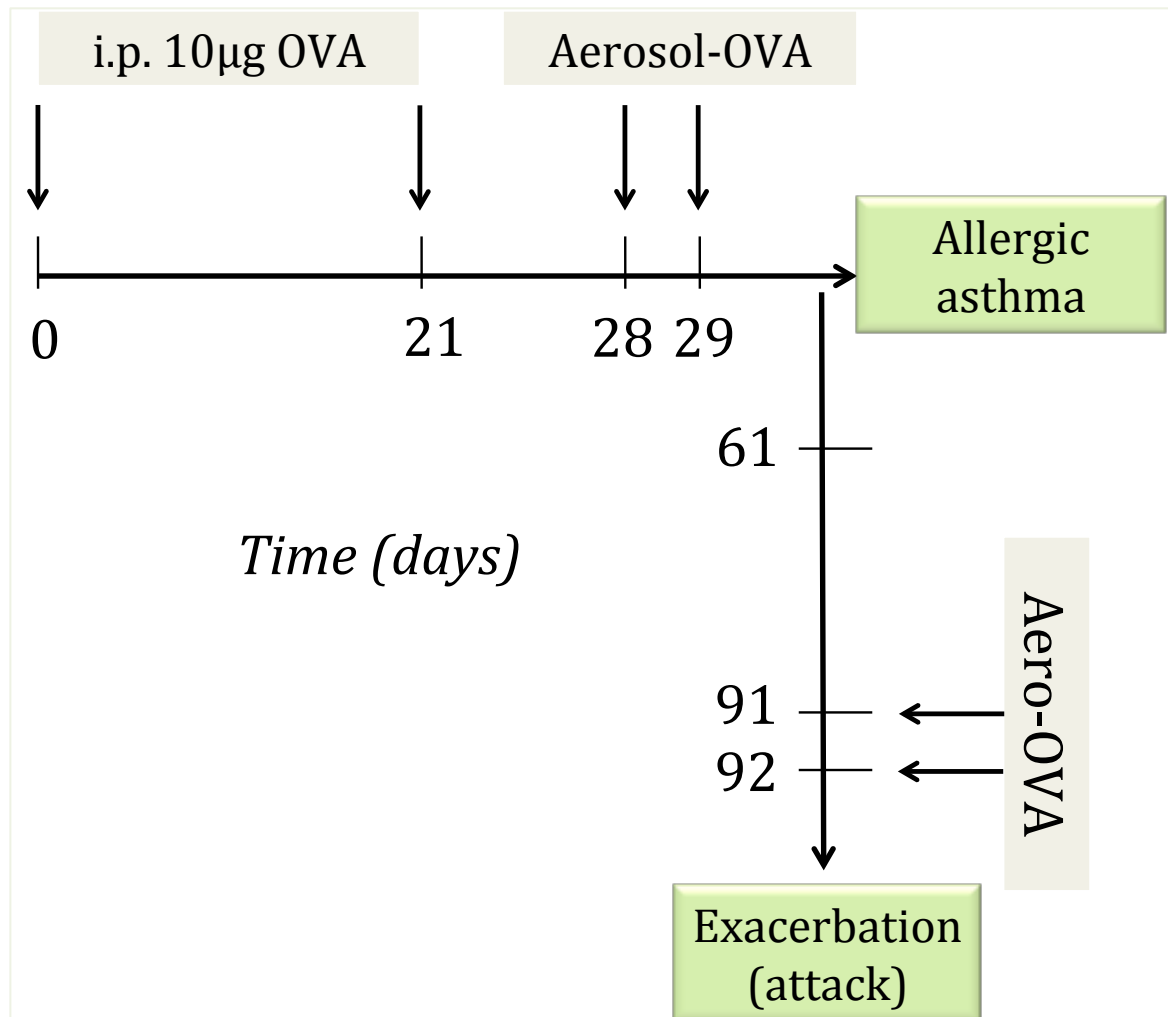
Figure 9. Experimental design of OVA-induced disease exacerbation

2.4.4 FEEDING PROTOCOLS

-Ad libitum feeding with corn

Animals were randomly assigned to 4 groups depending on their mean body weight and the experiments were performed at the same time in the same animal facility. Mice were fed before and during the onset or acute disease or before disease exacerbation with pellets containing 33% GM- or isogenic corn or corn-free normal mouse chow (Syngenta/SSNIFF) for 4 weeks. The diets were tested for the presence or absence of Cry1Ab DNA through PCR analysis. To evaluate the effects of the GM-feeding on allergic disease, we induced disease with OVA and then measured lung and airway inflammation, mucus hypersecretion and determined antigen-specific IgG1, IgE and IgG2a antibodies.

-AAI chickpeas experiments

Animals were randomly assigned to 5 groups depending on their mean body weight and the experiments were performed at the same time, in the same animal facility. Mice were force-fed (gavage) a seed meal suspension of either transgenic chickpea, isogenic chickpea, *Tendergreen bean* and Pinto bean seed meals at a dose of 100 mg of raw and heat-treated seed meal/ml in PBS (CSIRO, Canberra, Australia). The mice were fed twice a week for 4 consecutive weeks (250μl/mouse). In some experiments, we heated the suspensions for 30 minutes at 100°C in water bath. To determine whether GM chickpea feeding lead to AAI sensitization we challenged the mice i.n. with 50 μg of purified AAI protein purified from either AAI chickpea or native beans dissolved in 50 μl of PBS. Parameters associated with Th2 phenotype were measured 72h after the challenge.

-AAI cowpea experiments

Animals were randomly assigned to 6 groups depending on their mean body weight and the experiments were performed at the same time, in the same animal facility. Mice were force-fed (gavage) a seed meal suspension of transgenic cowpea, two isogenic cowpea varieties, Tendergreen bean and Pinto bean seed meals at a dose of 100 mg of raw and heat-treated seed meal/ml in PBS (*CSIRO, Canberra, Australia*). We fed mice twice a week for 4 consecutive weeks (250μl/mouse). We heated the suspensions for 30 min at 100°C in water bath. To determine whether seed meal

feeding lead to AAI sensitization, as result of feeding we challenged the mice i.n. with 50 µg of purified AAI protein purified from either AAI cowpea or native beans dissolved in 50 µl of PBS. Parameters associated with Th2 phenotype were measured 72h after the challenge.

2.5   Blood collection

At the beginning of the experimental approach, blood was collected from the tail vein. Seventy-two hours after the last aerosol challenge mice were anesthetized with a lethal dose of Rompun (Bayer AG, Leverkusen) Ketanest S (Pfizer) mixture. Whole blood was collected by cardiac puncture and allowed to clot overnight at 4^0C. On the next day sera were separated by centrifugation and stored at -20^0C until for further analysis.

2.6   Serum antigen-specific IgG1, IgE and IgG2a

Individual sera for the measurement of OVA and AAI-specific antibodies were tested by a standard sandwich ELISA. For the measurement of antigen-specific IgG1 and IgE, ELISA plates were coated with a solution of dissolved OVA or AAI at 10 µg/ml overnight at 4°C. The plates were washed and blocked with 2% BSA in PBS with for 2 hours at room temperature. Titrated sera were incubated for 24 h at 4°C. After washing, plates were incubated for 2 h at 4°C with biotinylated anti-IgG1, anti-IgG2a or anti-IgE detection mAb (Southern Biotech), followed by incubation with streptavidin horseradish peroxidase for one hour at room temperature. Plates were washed and incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (100µl) into each well. Following 10 min incubation in the dark at room temperature, dye development was stopped with addition of 100 µl of 0.18 M H_2SO_4 and the plates were measured at 450nm. Sera from naïve mice were used as negative controls. The antibody titers were expressed as the reciprocal of the dilution at which O.D. 450 nm > 0.1 readings were obtained.

2.7   Assessment of the airway inflammation

Seventy-two hours after last antigen challenge, mice were terminally anesthetized, tracheostomy was performed and a plastic catheter was clamped into the trachea. The lungs were washed 3 times with PBS in a total volume of 1 ml (0.4, 0.3 and 0.3 ml) to collect bronchoalveolar lavage fluid (BAL). The supernatant from the first lavage was frozen at -20°C for cytokine measurements. The total number of cells in BAL was enumerated in a Neubauer hemacytometer. The percentage of inflammatory cells was determined by morphological examination of at least 300 cells in cytocentrifuged preparations (Cytospin-4, Shandon Instruments, UK), stained with the Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) according to standard morphologic criteria.

2.8   Histopathological analyses of the organs

Kidney, spleen, and liver were harvested and weighted immediately. The ratio of organs weight to final body weight was calculated. The small intestine was collected, fixed by immersion in 4% paraformaldehyde. Fixed section were embedded in paraffin, 3 μm thick sections were cut and stained with hematoxylin and eosin (H&E) and analyzed on the light microscope. After BAL, lungs were flushed with PBS, removed and fixed by immersion in 4% paraformaldehyde and embedded in paraplast. Lung sections containing main stem bronchi from each lung specimen were cut (3 μm) and stained with hematoxylin and eosin for detection and scoring of inflammatory cell infiltration, LUNA for the detection and quantification of eosinophils and Periodic acid-Schiff reagent (PAS) for analysis of mucus hypersecretion. An expert respiratory pathologist blinded for the graded the extent of inflammation into the lungs according to semi-quantitative scoring system:

Grade 0 = no inflammation,

Grade 1 = inflammation present near central airways,

Grade 2 = moderate inflammation,

Grade 3 = severe inflammation and the extent

PAS stained sections were evaluated by an expert pathologist blinded, by grading the presence of mucus within the central and peripheral airway epithelia using the following grading scale:

Grade 0 = no mucus detected,

1= 0-20%,

2=21-40%,

3=41-60%,

4=61-80%,

5=81-100% of cells in airway wall positive for mucus.

For quantification of eosinophil infiltration, eosinophils were counted on ten random fields (400x magnification) containing alveoli and without major airways/vessels (which were selected from low power magnification) and averaged for each lung.

2.9   Staining protocols

i.   H&E staining was used for detection of cell infiltration in the lungs. Lung frozen tissue sections (5 μm), stored at -20°C, were thawed at RT for 10 min, fixed with 4% parafolmaldehyde (PFA) for 20 min, and then washed in 1xPBS for 5 min. Hematoxylin was added for 5 min and 1% HCl for 3 sec, three times, washed again with distilled water followed by staining with eosin for 30 sec. Slides were mount with EUKITT mounting medium for microscope preparation and visualized by light microscopy.

ii.   PAS of airway tissue sections allows clear visualization of mucus within goblet cells. Mucus cell development along the airway epithelium was visualized in paraffin embedded tissue sections (3 μm). Slides were deparaffined in xylol, then permeabilized in an ethanol gradient (100%, 96%, 80%) and stained with periodic acid-Shiff´s reagent.

iii.   Luna staining was performed for eosinophils detection in the lung parenchyma. Eosinophil accumulation in lungs was visualized in 3 μm paraffin embedded tissue sections. Slides were deparaffined in xylol for 10 min, followed by permeabilisation in ethanol gradient (100%, 96%, 80%). Staining with Weigerts iron hematoxylin as working solutions and lithium carbonate solution was performed. Sections were dehydrate in ethanol gradient (80%, 96%, 100%) then fixed with pertex mounting media and evaluated by light microscopy.

2.10 RBL cell mediator release assay

-in colaboration with Dr. Richard Weiss, University of Salzburg, Austria

We investigated the biological functionality of AAI-specific IgE. Approximately 4×10^4 RBL-2H3 cells/well were plated in 96-well tissue culture plates and incubated for 24h at 37°C in the presence of CO2. Subsequently, passive sensitization was performed by incubation with mouse sera from mice fed and challenged with native and transgenic meals containing AAI. The cell layer was washed twice in Tyrode's buffer to remove the unbound antibodies. Cross-linking of the Fc4ε-bound IgE and subsequent degranulation of RBL cells was induced by adding 100μl of AAI in Tyrode's buffer for 30 min in a humidified atmosphere at 37°C. The induced β-hexosaminidase release in supernatants was detected by incubation with 80μM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma) in citrate buffer (0.1 M, pH 4.5) for 1h at 37°C and fluorescence was measured at 360-465 nm using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are calculated by correlating measured values with total β-hexosaminidase released after addition of 1% Triton X-100, and reported as percentages.

2.11 DNA extraction from the quadriceps femoris muscle

-in collaboration with Dr. Éva Gelencsér, Central Food Research Institute – Budapest, Hungary

We extracted DNA form quadriceps femoris muscle from mice fed 33% GM corn, nGM corn or corn free diets were collected for DNA analysis. Tissues were collected after mice were terminally anesthetized, placed in individual eppy immediately frozen in liquid nitrogen and stored at -80° until further analysis. DNA was extracted from animal tissue as described by Meyer et al (1994). In brief, tissue samples (150 mg) were digested in 430 μL TRIS-EDTA-SDS extraction buffer (pH 8.0) containing 20 μL proteinase-K (20 mg/mL) and 50 μL 5M guanidine-hydrochloride. After 3h of incubation at 55°C the suspension was centrifuged for 10 min at 12,400 rpm. The supernatant (500 μL) was purified using the Wizard® DNA Clean-up system according to the manufacturer's instructions. Total DNA was quantified using a spectrophotometer at $O.D._{260nm}$ and purity was assessed by determining the $O.D._{260nm}:O.D._{280nm}$. The primer pairs targeting the *Cry1Ab* gene and a vertebrate mitochondrial gene fragment obtained from Invitrogen. Amplification was

performed in a reaction volume of 25 μL, which contained PCR Master mix (DreamTaq Green PCR mix) 0.3/0.5 μM primers and 250 ng tissue template DNA sample. Each set of PCR assays included reaction contamination control without template DNA (blank) and an extraction control sample to check the possible contamination during the DNA isolation protocol. A GeneAmp 2400 thermal cycler (Applied Biosystems, Foster City, CA) was used for SCytb specific PCR reaction and a GeneAmp 2700 thermal cycler (Applied Biosystems) for *Cry1Ab* specific PCR. PCR products were separated by electrophoresis using 10% polyacrylamide gels and visualized by staining with SYBR Green. Gels were run at 200 V for 50 min and the DNA was visualized with UV light (Kodak EDAS 290 system). Appropriate positive and negative controls were included in all analyses to ensure sensitivity and reproducibility of the DNA extraction and PCR assays.

2.12 Statistical analysis

Groups were compared by Kruskal-Wallis test followed by Dunn's multiple comparison test (for cell number and cell percentages in BAL) and by Wilcoxon Signed Rank test and Mann-Whitney test (for histological scores) using GraphPad Instat v.5.0 (GraphPad Software Inc.). Student *t test* was used to determine significance between heat-treated and row feeding groups. *P* values were considered significant at *< 0.05*.

3   RESULTS

3.1   Effect of native and transgenic AAI administration on antibody response and airway inflammation in mice

3.1.1   BOTH NATIVE AND TRANSGENIC AAI PROTEINS ELICIT HIGH IgG1 AND LOW IgE ANTIBODY RESPONSE WHEN INJECTED I.P

Differences in the structure seen in the proteins extracted from transgenic peas compared to those extracted from native beans lead us to the hypothesis that the AAI proteins expressed in the genetically modified organisms (GMOs) are differentially glycosylated and this modification will probably lead to increased immunogenicity. In our experimental approach, the native and transgenic purified AAI proteins were systemically administered, without the use of adjuvants, which allowed for the evaluation of the allergenic potential of each protein. Mice (n=8) were exposed to 10 µg of either native AAI proteins expressed in the Tendergreen bean and Pinto bean, or to transgenic AAI proteins expressed in cowpea and chickpea. As positive control, we immunized mice with OVA, a major allergenic component of egg protein and as negative control, mice were systemic immunized with PBS (as depicted in Figure 10).
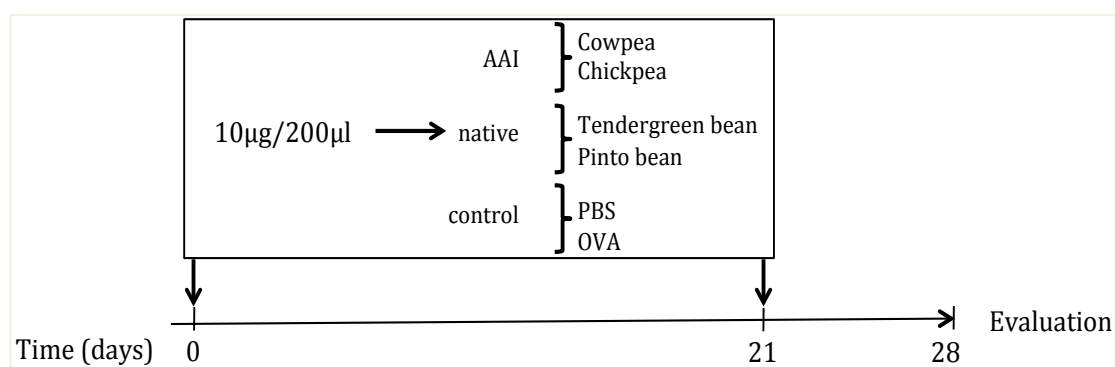
Figure 10: Experimental protocol to test whether transgenic proteins are more immunogenic upon systemic immunization. Groups of mice (n=8) were immunized twice, i.p. with 10 µg of native or transgenic AAIs on days 0 and 21. Seven days after the last immunization, blood was collected by cardiac puncture and individual serum samples were tested for the presence of anti-OVA and anti-AAI Th2 and Th1 antibodies by sandwich ELISA.

As expected, immunization with OVA stimulated the production of specific IgE antibody (1 in 225) and a vigorous IgG1 response (1 in 3513000), both markers for Th2 response (Figure 11 and Table 4). In contrast, PBS immunization failed to stimulate IgG1, IgE and IgG2a antibody production. Systemic exposure to both Tendergreen bean and Pinto bean AAI, the native proteins, resulted in a high level production of anti-AAI IgG1 antibody response as measured by ELISA. IgG1 antibody titers following treatment with Tendergreen bean AAI reached titers of 1 in 175500 whereas those derived subsequent exposure to Pinto bean AAI were more vigorous, 1 in 3200000, resembling the antibody titer obtained in response to OVA immunization (1 in 3513000) (Figure 11 and Table 4). When mice were immunized against transgenic cowpea and chickpea AAI, we measured a higher anti-AAI IgG1 titers when compared with Tendergreen bean AAI immunization, but lower if compared to the response obtained against Pinto bean immunization (1 in 462500 for cowpea and 1 in 1688000 chickpea). Despite Pinto bean inducing a strong IgG1 antibody response, both native and transgenic proteins were immunogenic under these conditions of exposure.

i.   Antibody response

Figure 11. Immunogenic response to the systemic administration of native and transgenic AAI, OVA and PBS in BALB/c mice. Serum OVA and AAI-specific IgG1, IgE and IgG2a antibody responses following systemic exposure in mice immunized with OVA Tendergreen bean AAI, Pinto bean AAI, cowpea AAI and chickpea AAI and PBS.
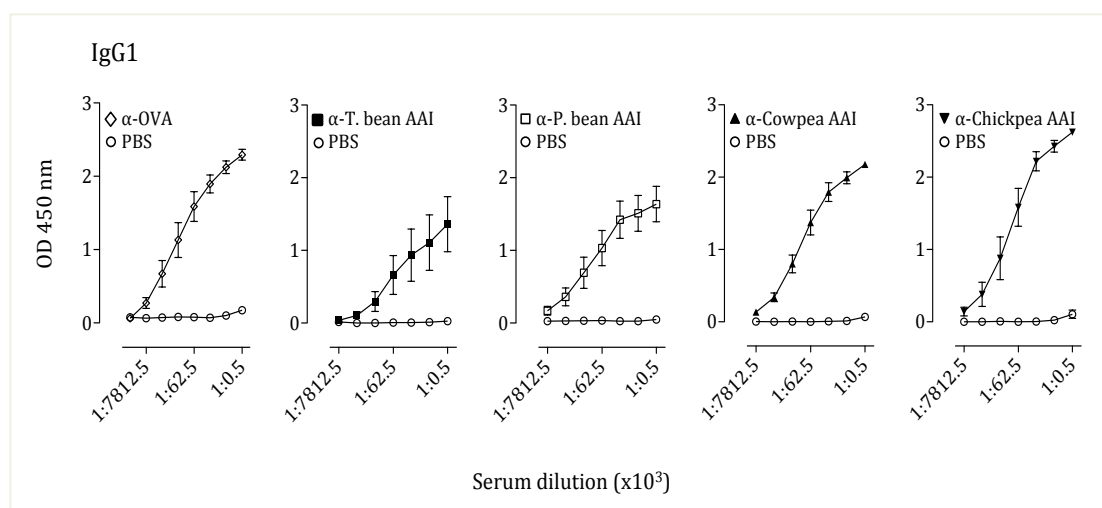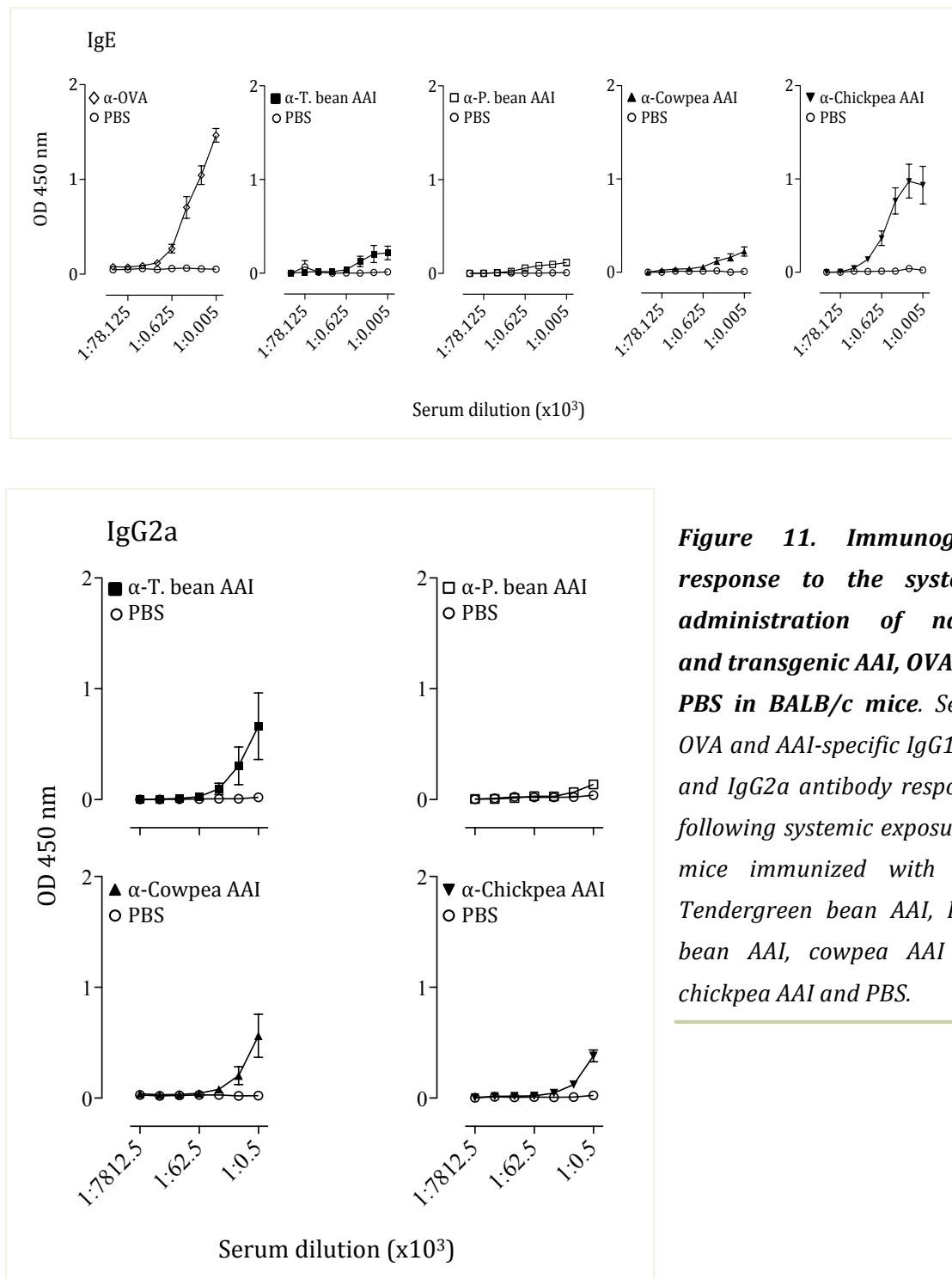
IgE antibody levels were also measured as an indicator for Th2 and allergy. As illustrated in Figure 10 and Table 4, despite the fact that i.p. administration of Tendergreen and Pinto bean AAI led to very low IgE antibody levels as the titer reached was only of 1/1 for both native proteins. Conversely, in the serum from cowpea-AAI immunized mice, the measured titers reached 1/30. Moreover,

sensitization with chickpea AAI induced higher IgE antibody levels with titers reaching 1/152, similar to those obtained in response to OVA immunization (1/225). Sensitization with all proteins induced comparable IgG2a levels, except that the titres for Pinto bean AAI were the titers were very low.

Table 4: Reciprocal titer values for IgG1, IgE and IgG2a production following systemic exposure to OVA, Tendergreen bean AAI (AAI-TB), Pinto bean AAI (AAI-PB), chickpea AAI (ChP-AAI), cowpea AAI (CoP-AAI) and PBS.

	OVA	AAI-TB	AAI-PB	ChP-AAI	CoP-AAI	PBS
IgG1	3513000± 1774000	175500± 114032	3200000± 1884000	1688000± 1533000	462500± 100000*	1±0
IgE	225±135	1.8±0.8	1±0	151.4± 120.8	30.6± 24.05	1±0
IgG2a	N.D	2501± 2500	1±0	600.6± 484.6	340.2± 102.8	1±0

*Statistical differences was determined using Man-Whitney test. Values are presented as mean titers ± SEM. *p<0.05, ** p<0.01, and ***p<0.001 were considered significant.*

3.1.2 IMMUNIZATION WITH BOTH NATIVE AND TRANSGENIC AAI PROTEINS INDUCE CROSS-REACTIVE ANTIBODIES TO PEA LECTIN

Seed lectins belong to a group that include concavalin A and PHA and may play a role in the seed defence [68]. In peas and beans, different lectins share a high degree of amino acid sequence identity with AAI [68]. To test whether the antibodies generated in response to AAI have epitopes that react with pea lectin, we measured the cross-reactivity between AAI antibodies generated to the immunization against the native Tendergeen and Pinto bean AAI, transgenic cowpea and chickpea AAI and pea lectin (Figure 12). Our results demonstrate that the immunization with all proteins induces antibodies that crossreact with pea lectin. However, Pinto bean and cowpea AAI immunization generate higher titers of cross-reactive antibodies compared to Tendergreen bean and chickpea AAI.
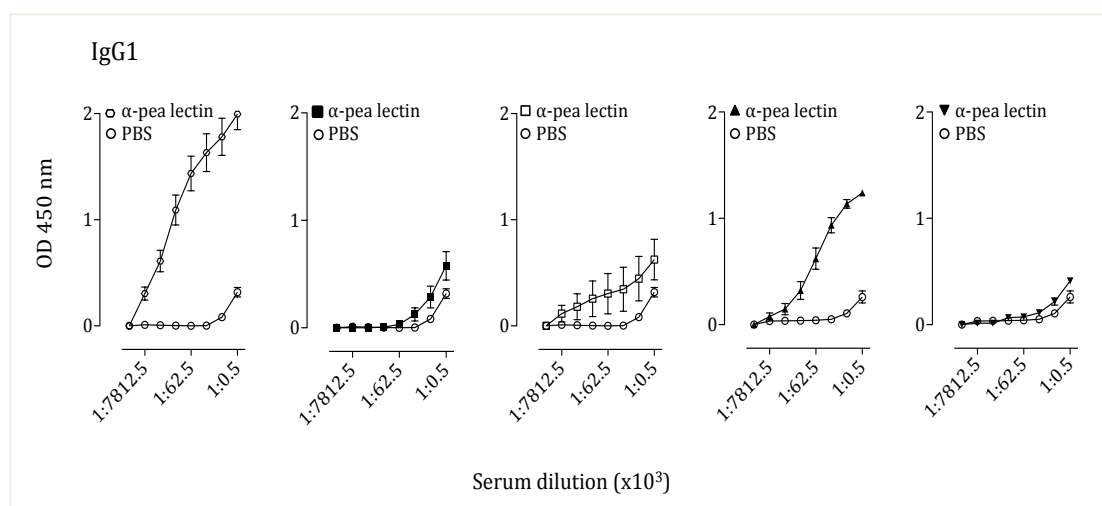
Figure 12. Cross-reactive antibodies to pea lectin. *Groups of mice (n=8) were immunized i.p. twice with 10 μg of pea lectin (open hexagon), native Tendergreen bean AAI (filled squares), Pinto bean AAI (open squares), transgenic cowpea AAI (filled triangles), chickpea AAI (filled triangles) and PBS (open circles) on days 0 and 21. Seven days after the last immunization, blood was collected by cardiac puncture and individual serum samples were tested for the presence of pea lectin cross-reactive antibodies by sandwich ELISA.*

3.1.3 NATIVE AND TRANSGENIC AAI PROTEINS VARY IN THEIR ABILITY TO ELICIT TH2/TH1 ANTIBODY RESPONSES, BAL AND LUNG CELL INFLAMMATION UPON INTRANASAL ADMINISTRATION

To determine whether transgenic and native AAI proteins can induce allergic disease upon inhalation and the induced response would differ between the native and transgenic proteins, we chose a protocol previously established in the lab in which we apply 6 doses of OVA i.n which initiates an allergic response in mice. Using this experimental approach, illustrated in Figure 13, we tested the ability of transgenic and native AAI to induce allergic disease.
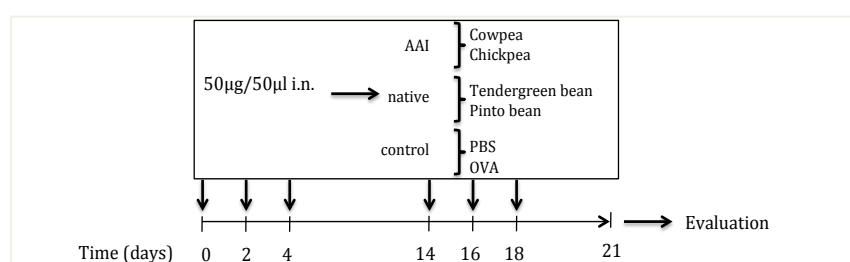
Figure 13: Experimental protocol to test whether transgenic proteins are more allergenic upon six i.n. challenges. *Groups of mice (n=8) were challenged i.n. 6 times with 50*

µg of native or transgenic AAI. Seventy-two hours after the last challenge, blood was collected by cardiac puncture and individual serum samples were tested for the presence of Th2 and Th1 antibodies by sandwich ELISA .

i.   Antibody response:

Table 5: Reciprocal titer values for IgG1, IgE and IgG2a production following six intranasal challenges to OVA, Tendergreen bean AAI (AAI-TB), Pinto bean AAI (AAI-PB), chickpea AAI (ChP-AAI), cowpea AAI (CoP-AAI) and PBS.

|	OVA	AAI-TB	AAI-PB	ChP-AAI	CoP-AAI	PBS
IgG1	4063000± 1531000	7813000±0	7031000± 781250	593750± 213587	7813000±0	1±0
One way ANOVA		*** vs. PBS, #vs.AAI-ChP	*** vs. PBS	#vs. AAI-PB	*** vs. PBS	
IgE	61±26.38	1±0	4±3	1±0	1.5±0.5	1±0
One way ANOVA						
IgG2a	1500± 577.4	10000± 1637	24438± 11148	875.3± 362.8	2477000± 1381000	1±0
One way ANOVA		*** vs. PBS	** vs. PBS		*** vs. PBS	

*Statistical differences was determined using the Man-Whitney test. Values are presented as mean titers ± SEM. *p<0.05, ** p<0.01, and ***p<0.001 were considered significant.*

As shown in Table 5 and Figure 14, i.n. challenge with OVA led to a very high increase in IgG1 (1/4063000), low IgE (1/61) and IgG2a antibody titers (1/ 1500). Intranasal challenges with a total of 300 µg of both pure native or transgenic AAI proteins induced a strong production of specific IgG1 and IgG2a antibodies but low IgE. A similar level of IgG1 and IgE antibody was produced following repeated challenges with Tendergreen, Pinto and cowpea AAI. The titers of IgG2a antibody in response to Tendergreen and Pinto bean challenges were modest (1/10000 and

1/24438), whereas repeated challenges with cowpea AAI led to a higher Th1 response, IgG2a reaching titers of 1/2477000. Systemic immunization with transgenic chickpea AAI, similar to OVA, induced high IgE titers (1/151), however, the response obtained subsequent the i.n. challenge failed to induce measurable IgE titers. Although the levels of IgG1 and IgG2a antibodies induced by repeated challenges were considerably higher than those stimulated by i.p. administration with the same proteins, this route of administration failed to stimulate measurable levels of IgE production. The presence of protein specific antibodies in serum samples of mice i.n. challenged with various AAIs are displayed in Figure 14 and recorded in Table 5.
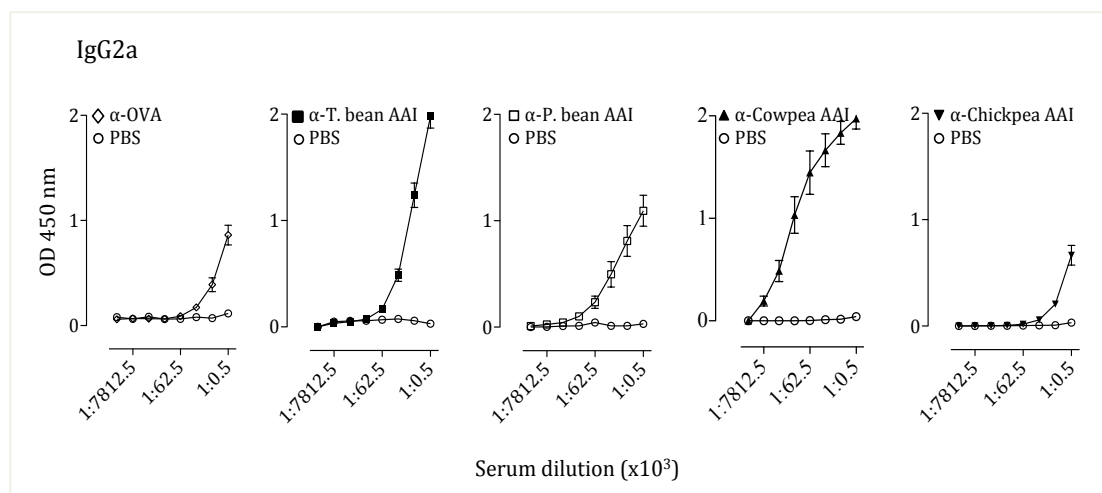
Figure 14. Allergic response to 6 i.n. challenges of native and transgenic AAI, OVA and PBS in BALB/c mice*. Groups of mice (n=8) were challenged i.n. 6 times with 50 μg of native or transgenic AAI. Seventy-two hours after the last challenge, blood was collected by cardiac puncture and individual serum samples were tested for the presence of Th2 and Th1 antibodies by sandwich ELISA. Serum OVA and AAI-spe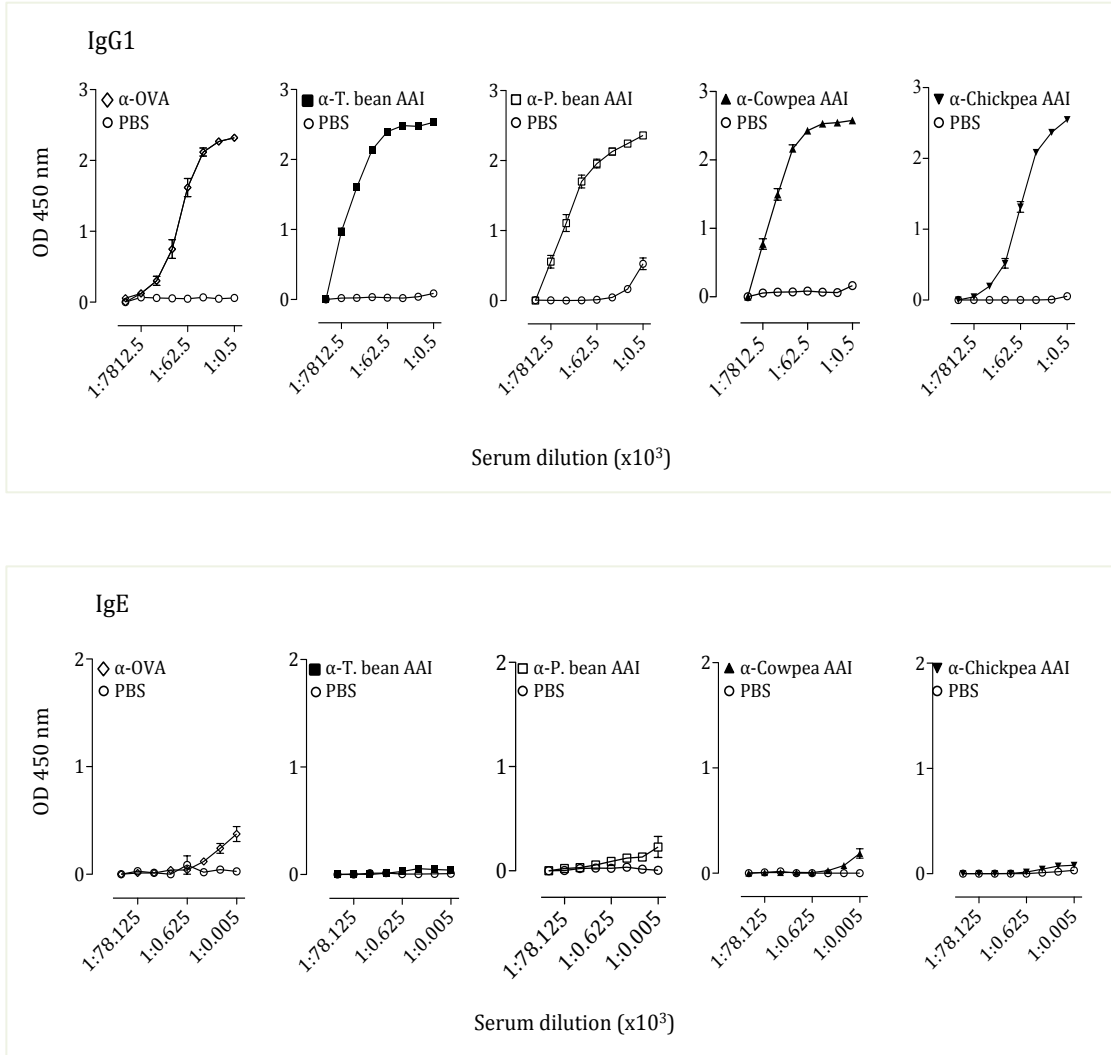cific IgG1, IgE and IgG2a antibody response following challenge in mice immunized with Tendergreen bean AAI, Pinto bean AAI, cowpea AAI and chickpea AAI or PBS.*

ii. Airway and lung cell inflammation

We next assessed the influence of AAI proteins on the airway and lung inflammatory response. Analysis of the cellular content of BAL fluid revealed that the total cell numbers in the airways were significantly increased in mice challenged with both native and transgenic AAI when compared to the PBS challenged group (Figure 15 and Table 6). The total airway cell numbers were higher in response to Tendergreen bean (523750±87105), Pinto bean *(*680000±102133) and cowpea AAI challenges (508125±92045) and lower for the chickpea AAI (261875±28125) and PBS challenges (154167±34531). Repeated i.n. challenges with Pinto and Tendergreen bean AAI, but also cowpea AAI, resulted in significantly elevated numbers of macrophages, neutrophils and lymphocytes in the airways. The lowest number of eosinophils was recruited in the airways of mice challenged with PBS (683±454.2) and transgenic chickpea AAI (7905±1455).

Table 6: Characterization of the BAL fluid in mice after six intranasal challenges with native and transgenic AAI proteins.

Cell counts	PBS	AAI-TB	AAI-PB	CoP-AAI	ChP-AAI
Eosinophils	683± 454.2	26812± 7390*	131198± 19829***,#	78178± 25970*	7905± 1455
Lymphocytes	1418± 755	31404± 7248**, #	35008± 9961**, #	14479± 4478	4727± 997.1
Macrophages	151805± 33429	435929± 77687*	505801± 78281*	478875± 40255*	245948± 26242
Neutrophils	261.3± 70.78	18217± 4750***	7994± 2538*	9184± 2042*	3296± 1131
Total	154167± 34531	523750± 87105*	680000± 102133*,#	508125± 92045*	261875± 28125

Statistical differences were determined using the Kruskal-Wallis test followed by Dunns comparison test for each cell type. Values are expressed as mean cell counts ± SEM. One-Way ANOVA, *p<0.05 vs. PBS, ** p<0.01 vs. PBS, ***p<0.001 vs. PBS, # p<0.05 vs. cickpea AAI.

Furthermore, differential cell counts on lavage fluid revealed that native Pinto bean AAI challenge recruited 1.6-fold more eosinophils compared to the mice challenged with transgenic cowpea AAI and 16-fold more eosinophils compared to mice challenged transgenic chickpea AAI. In contrast native Tendergreen AAI challenged mice only recruited 3-fold more eosinophils compared to mice challenged transgenic chickpea AAI. Interestingly, challenges with AAI extracted from Tendergreen bean recruited 2-fold more neutrophils into the airways (18217±4750) compared to Pinto bean AAI (7994±2538) and cowpea AAI (9184±2042) and even 5.5-fold more when compared to chickpea AAI.

To examine whether repeated challenges with AAI will also increase the numbers of eosinophils in lungs, we quantified the numbers of inflammatory cells in tissue. Challenges with transgenic cowpea AAI (18.57±4.59), but also native Pinto (25.63±4.16) and Tendergreen bean AAI (13.75±3.09) significantly increased mean eosinophils per high power field (HPF) compared to PBS treated mice (Table 7), which corelates with the results observed for airway eosinophilia. Only a moderate increase in eosinophils was observed when mice were challenged with transgenic chickpea AAI (8.57±2.1). The numbers of eosinophils in BAL and lungs suggest that,

despite the differences in glycosylation and structure, both native and transgenic proteins induce a influx of eosinophils.
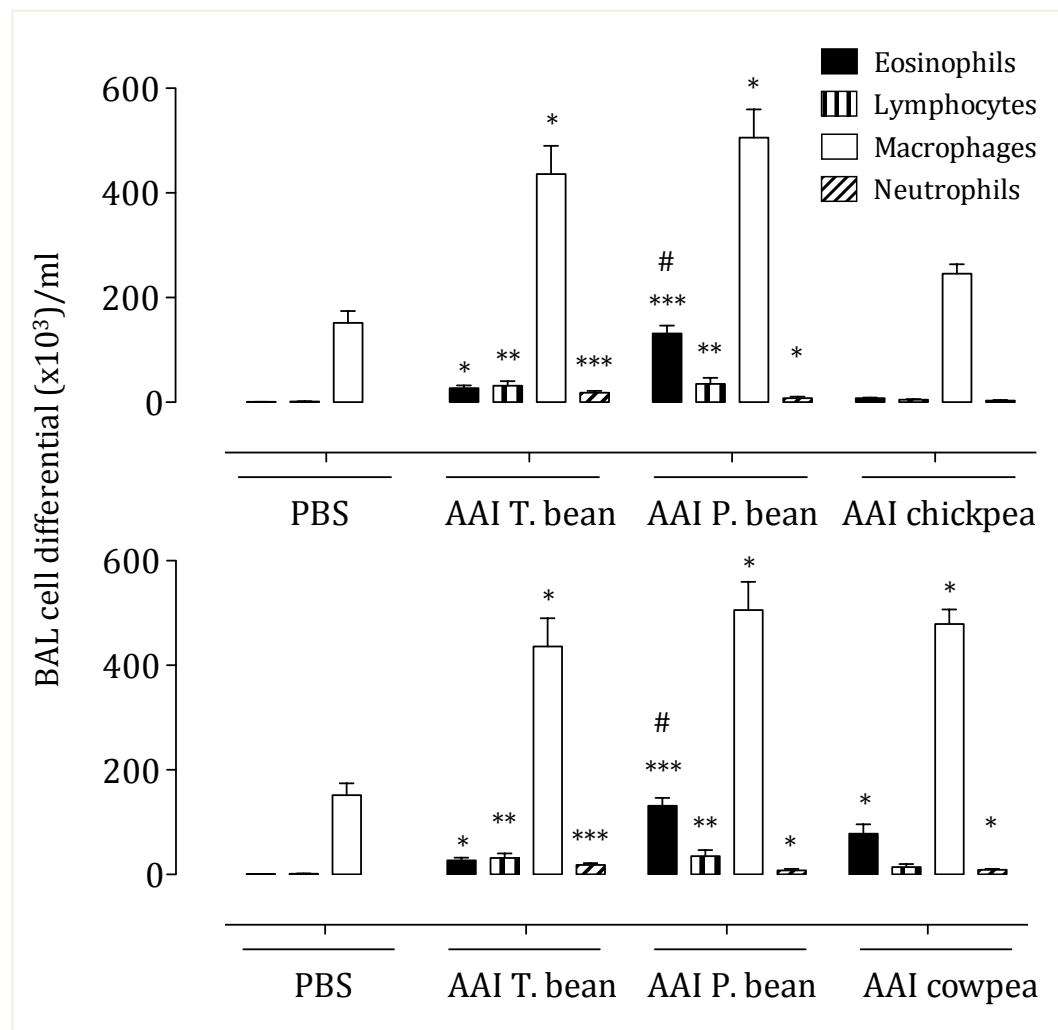
*Figure 15. Characterization of inflammatory cells in BAL fluid retrieved from BALB/c mice challenged native Tendergreen and Pinto bean, transgenic chickpea and cowpea AAI. Groups of mice (n=8) were challenged 6 times with 50 µg in 50 µl of purified native or transgenic AAI proteins. Seventy-two hours after the last i.n challenge BAL fluid was collected and differential cell counts were performed. Differential counts are derived from at least 300 cell counts and expressed as total cell numbers. Data represent total cell numbers ± SEM of counts; n= 8 mice. Statistical differences were determined using the Kruskal-Wallis test. *p<0.05 vs. PBS and #p<0.05 vs. sample.*

Table 7: AAI-induced overall inflammation in lung sections of mice challenged with native and transgenic AAI proteins.

	PBS	AAI-TB	AAI-PB	CoP-AAI	ChP-AAI
Grade of cell infiltrates	0±0	2.25±0.26	2.12±0.58	2.14±0.24	1.31±0.7
Wilcoxon test		*PBS	*PBS	*PBS	*PBS
Grade of mucus production	0.16±0.25	1.5±0.75	1.43±0.77	1.78±0.63	0.5±0.86
Wilcoxon test		*PBS	*PBS	*PBS	
Counts of eosinophils	0±0	13.75±3.09	25.63±4.16	18.57±4.59	8.57±2.1
Kruskal-Wallis			*PBS	*PBS	

*Values are expressed as grade of cell infiltrates ± SD, mucus production ± SD and mean of eosinophil counts ± SEM. Statistical differences between groups were determined using Wilcoxon test for the first two parameters, and Kruskal-Wallis test for the last parameter. *p<0.05 indicates statistical significance of groups compared to the PBS group.*

Because repeated challenges of native and transgenic AAI recruits inflammatory cells in the airways, we hypothesized that these results will be mirrored by inflammation and mucus hyperproduction in lungs, two important parameters characteristic of allergic asthma.
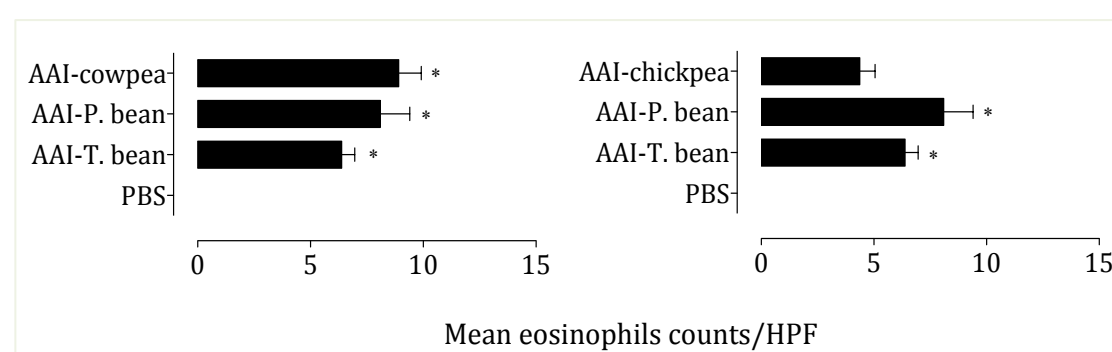
Figure 16. Mean of eosinophil counts per high power field (HPF). Quantification of eosinophils in lung parenchyma of mice i.n. challenged with pure AAI protein derived from GM cowpea and GM chickpea compared with mice challenged with native Tendergreen or Pinto bean AAI. Control mice were challenged with PBS. Data are presented as mean cell counts ± SEM; n=8 mice. Statistical differences were determined using the Kruskal-Wallis test and p values <0.05 were considered significant.

The density of inflammatory cell infiltrates around the central bronchi, alveoli and blood vessels was scored as described in Material and Methods. Lungs were processed for histological analysis 72h after the last i.n. challenge. The results show that in control mice challenged with PBS, lung tissue sections did not exhibit inflammatory cell infiltrates. Histological analysis of lung tissue stained with hematoxylin and eosin (Figure 18) revealed a significant increase in airway inflammation in mice challenged with both native and transgenic proteins when compared to PBS control group (Figure 17). We also observed a lower score for lung inflammation in mice challenged with AAI derived from GM chickpea, but no statistic significant differences were detected between groups of mice challenged transgenic and native AAI proteins. Mucus hypersecretion is another important feature of inflammation. To assess the extent of muco-polysaccharides within the goblet cells, lung sections were stained with periodic acid-Schiff reagent (PAS). Histological examination of lung revealed that PAS stained lung section on most conducting airways in mice challenged with native and transgenic AAI proteins stained positive for mucus production and the grades obtained for mucus mirror those seen for cell infiltrates. Similar to the results obtained for airway eosinophils and the grade of cell infiltrates, the grade of mucus production was lower for mice challenged with AAI derived from GM chickpea (Table 7 and Figure 19).
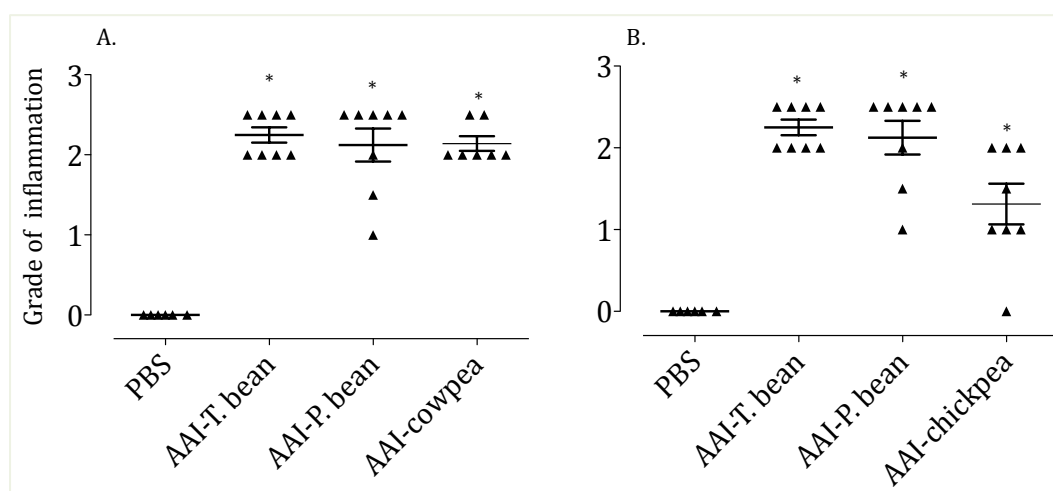
Figure 17. Grade of cell infiltrates in lung parenchyma of mice challenged with native and transgenic AAIs and PBS. Data are presented as the grade of cell infiltrates ± SD from n=8 mice. Statistical differences were determined using Wilcoxon test, p<0.05 was considered significant.
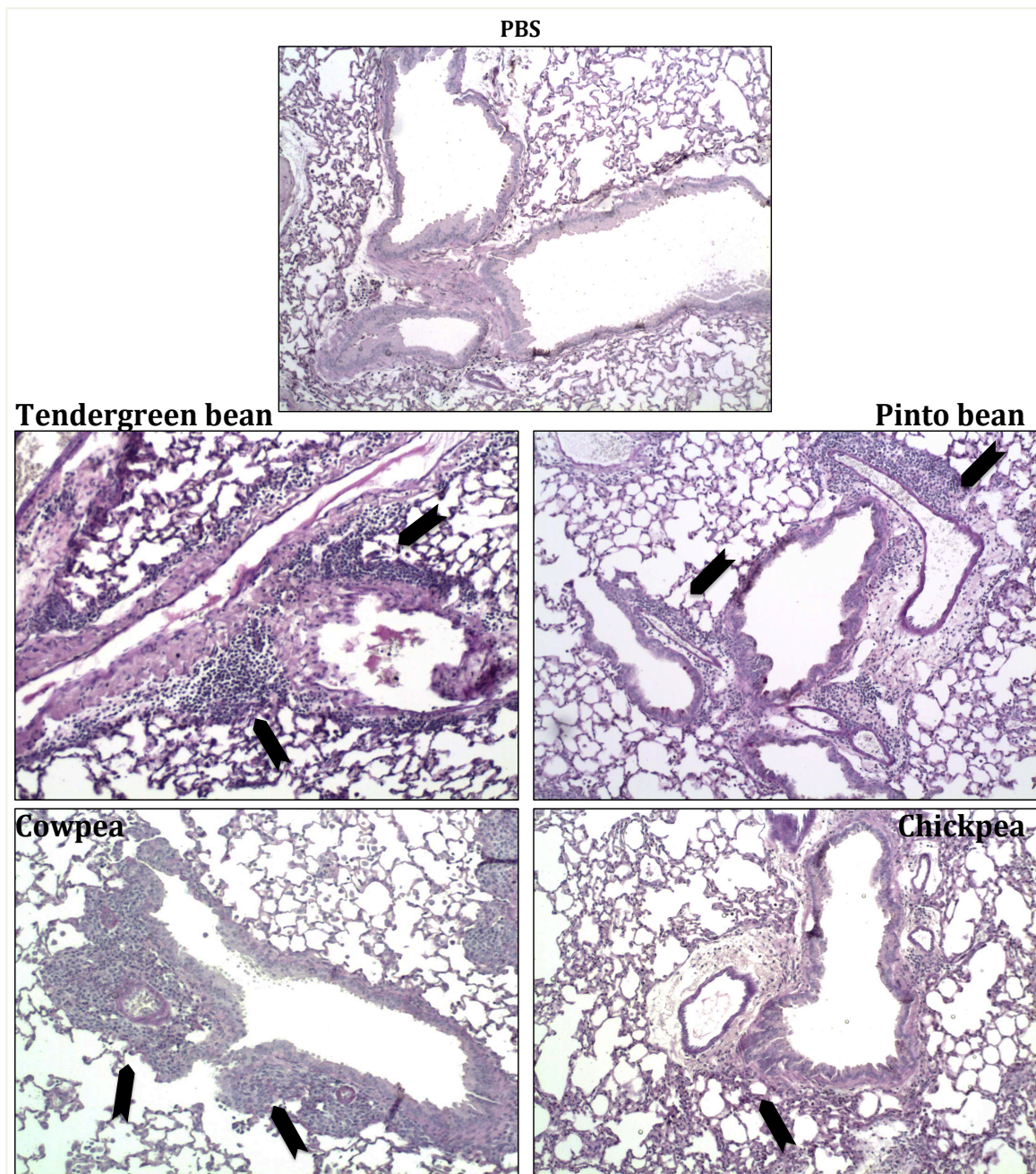
Figure 18. Histology of airways and lung parenchyma*. Representative photomicrographs of lung sections stained with H&E of mice challenged with PBS, Tendergreen AAI, Pinto bean AAI, cowpea AAI and chickpea AAI. Black arrows indicate cell infiltrates. Original magnification x 10.*
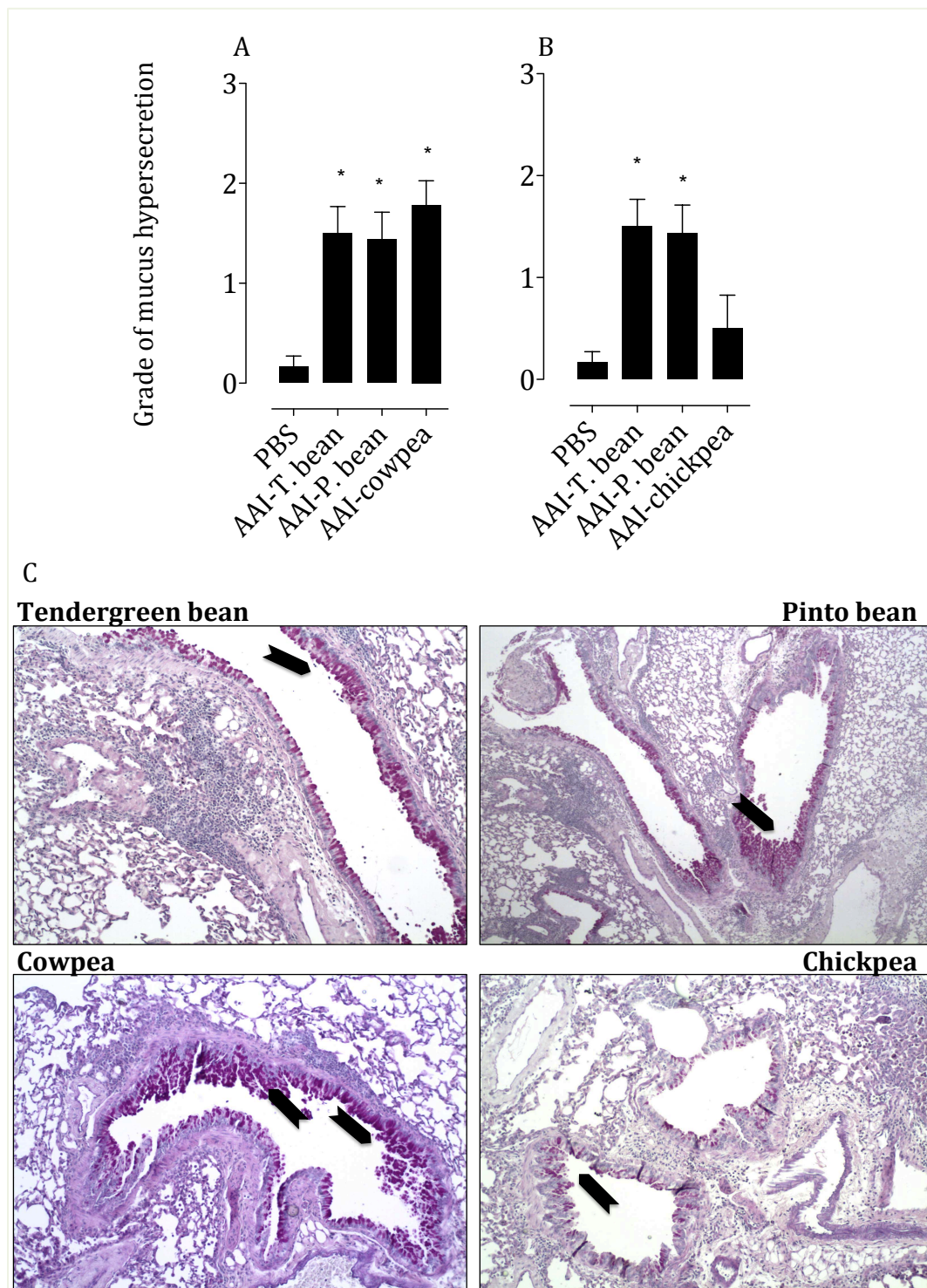
Figure 19. Histology of airways and lung parenchyma. *(A) and (B) demonstrate the grade of mucus production in lung parenchyma of mice challenged with different AAIs and PBS. (C) Representative photomicrographs of lung sections stained with PAS from mice challenged with Tendergreen AAI, Pinto AAI, cowpea AAI and chickpea AAI. Original magnification x 10. Data are presented as the grade of mucus production ± SD; n=8. Black arrows indicate mucus filled goblet cells. Statistical differences were determined using Wilcoxon test. *p value <0.05.*

3.2   Investigation of the effect of feeding GM chickpeas on the immune response in BALB/c female mice

3.2.1   ANALYSIS OF GM CHICKPEA SEEDS FOR THE PRESENCE OF THE TRANSGENE AAI

GM chickpea   bean
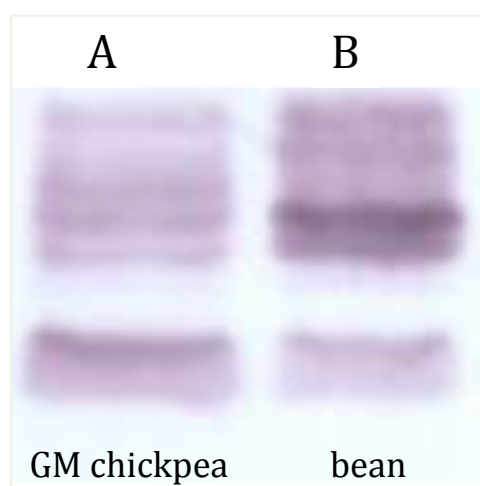
Immunoblot analysis of AAI protein from native Tendergreen bean seeds and transgenic chickpea seeds expressing AAI revealed that multiple polypeptides were produced in both cases and their profiles slightly differed in the materials from two crops (Figure 20).

Figure 20. Immunoblot of AAI protein *present in extracts of GM chickpea (lane a) and (lane b) Tendergreen bean. (courtesy of T.J. Higgins)*

3.2.2   CLINICAL OBSERVATION AND BODY WEIGHT

Table 8. Body weight of BALB/c female mice force feed Tendergreen bean, Pinto bean, GM- and nGM chickpea

Feeding groups	Body weight (g)	
	initial	final
PBS	16.24±0.468	21.41±0.878
Tendergreen bean	15.95±0.700	21.94±1.272
Pinto bean	15.64±0.5917	21.33±1.071
GM chickpea	17±0.622	21.36±1.66
nGM chickpea	15.96±0.5121	20.69±0.434

Control mice were fed PBS. Values are expressed as mean ± SD (n=8).

During the experimental period all mice survived and showed no adverse effects such as diarrhea or weight loss. Furthermore, the body weights did not significantly

differ in groups. The range was from initial weights of 16±0.125g to final weights of 21.32±0.19g (Table 8).

3.2.3   ANTIBODY RESPONSE INDUCED BY FEEDING WITH RAW GM- AND nGM CHICKPEAS IN MICE
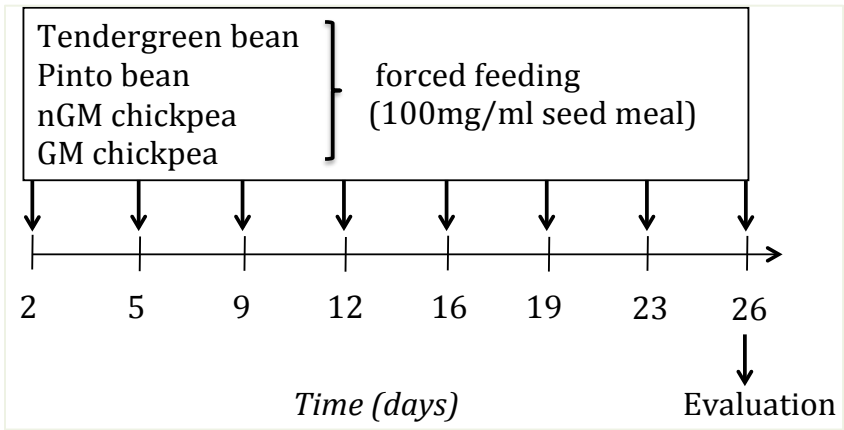
Figure 21: Experimental protocol to test GM chickpea feeding induction of allergy. BALB/c female mice were fed twice a week for 4 weeks with raw GM chickpeas, nGM chickpeas and control materials, native Tendergreen beans and Pinto beans. On day 26, blood was collected from tail vein and tested for AAI-specific antibodies.

To investigate whether GM chickpea feeding to mice induces allergy to the novel transgenic protein, we performed feeding studies. Previous investigations showed that oral exposure to food extracts in the absence of cholera toxin (CT) did not elicit food-specific antibody response, leading rather to oral tolerance [37]. In our experimental approach, female BALB/c mice were force fed eight times with 100 mg/ml (250 µl/mouse/dose) of raw GM- and nGM chickpea, Tendergreen and Pinto bean. Our results show that the levels of IgG1 were substantially increased after exposure to all food extracts, however both raw Tendergreen and Pinto bean meals were the most potent elicitor of IgG1 (Figure 22). Levels of this antibody titers following feeding Pinto bean to mice achieved 1/3200000, whereas those derived subsequent the exposure to Tendergreen were 20 fold lower (1/170000) (Table 9). In contrast, in mice fed GM chickpea, IgG1 reached a titer of only 1/50000.
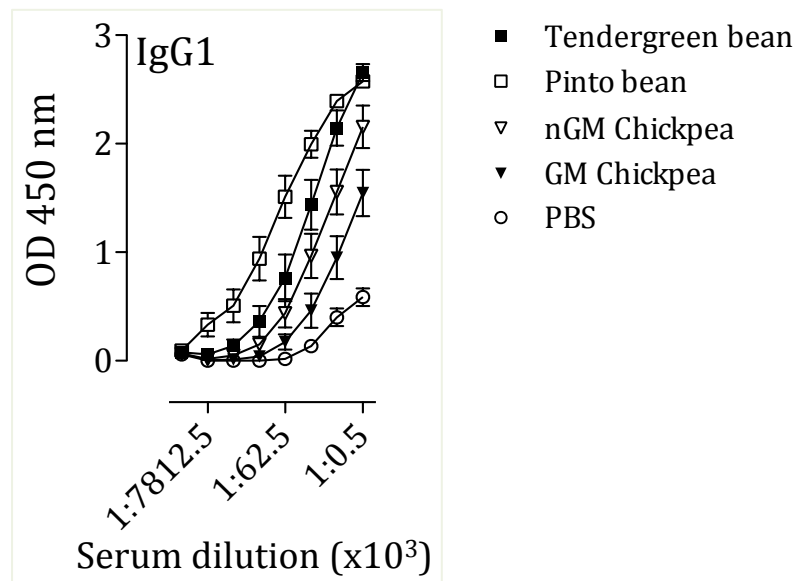
Figure 22. Antibody production secondary to pea and bean feeding. *Individual sera from different feeding groups were obtained after a total of 8 i.g. administrations. AAI specific IgG1 levels were determined by sandwich ELISA. Results are representative of at least 2 independent experiments and are reported as mean O.D. ± SEM (n=8).*

Table 9. Reciprocal titer values for IgG1 production following forced feeding with raw Tendergreen, Pinto bean, nGM- and GM chickpea.

Feeding:	IgG1	Statistics
	MEAN±SEM	One Way ANOVA
PBS	1167±666.7	
Tendergreen bean	170833±90753	
Pinto bean	3263000±1860000	**p<0.01 vs. PBS
GM chickpea	50000±38208	*p<0.05 vs. PB
nGM chickpea	92500±48440	

Statistical differences were determined using Kruskal-Wallis test. Values are presented as mean titer ± SEM. (n=8), *p<0.05, ** p<0.01 were considered significant.

3.2.4 INTRA-GASTRIC ADMINISTRATION OF PINTO BEAN BUT NOT TENDERGREEN BEAN OR CHICKPEA INDUCES IgG1 CROSS PRIMING ANTIBODIES TO PEA LECTIN

Remarcably, i.g. challenge of nGM chickpea, lacking AAI also induced a response to this protein (1/92500). We sought to investigate whether there was another component present in the chickpea that was closely related to AAI, which might induce a cross-reactive response. Lectins are carbohydrate-binding proteins present in beans and peas and play a role in the plant defense [87]. The genes encoding lectins and AAI exhibit an 82% degree of sequence similarity [88]. Immunization experiments described above (Figure 12) revealed that all AAIs extracted from beans, chickpea and cowpeas induced antibodies that crossreact to lectins. To determine whether feeding chickpea seed meal would also generate anti-lectin antibodies, we measured the levels of IgG1 generated against lectin. Our results indicated that only feeding Pinto bean, but not GM or nGM chickpea induced anti-lectin antibodies.

- ■ Tendergreen bean
- □ Pinto bean
- ▽ nGM Chickpea
- ▼ GM Chickpea
- ○ PBS
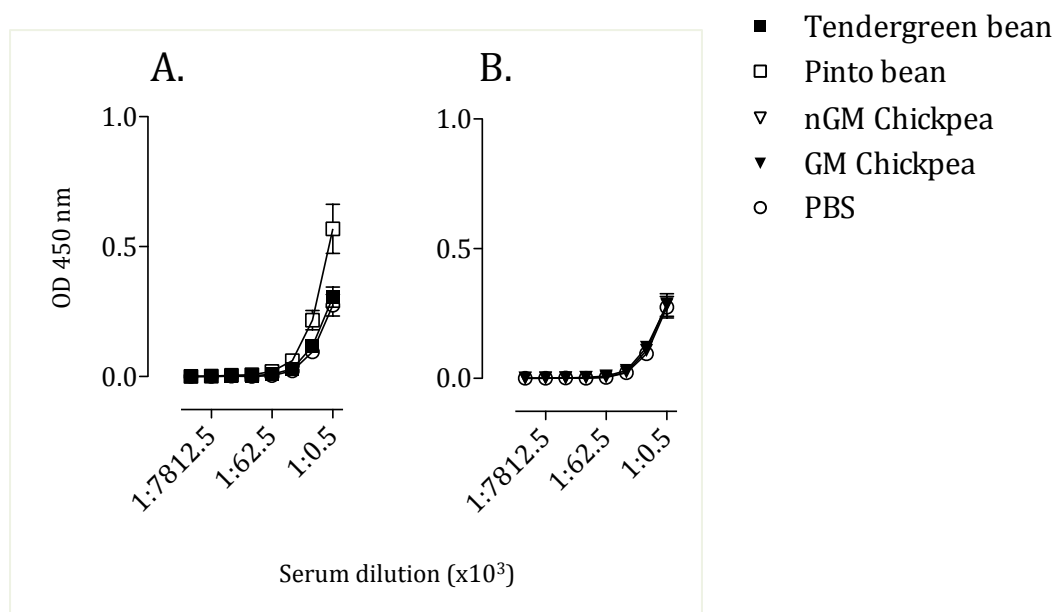
Figure 23. Consumption of Pinto bean but not chickpea induces cross-reactive IgG1 antibodies to pea lectin. Serum IgG1 anti-lectin antibody response in mice fed with (A) Tendergreen bean, Pinto bean, and (B) GM chickpea, nGM chickpea or PBS. Groups of mice (n=8) were fed twice a week for 4 weeks different seed meals. Seventy-two hours after the last feeding, blood was collected from the tail vein and individual serum samples were tested for the presence of anti lectin-IgG1, antibodies by sandwich ELISA. Data are expressed as the mean O.D. ± SEM.

3.2.5   ANTIBODY RESPONSE INDUCED BY GAVAGE & I.N CHALLENGE OF RAW CHICKPEAS IN MICE
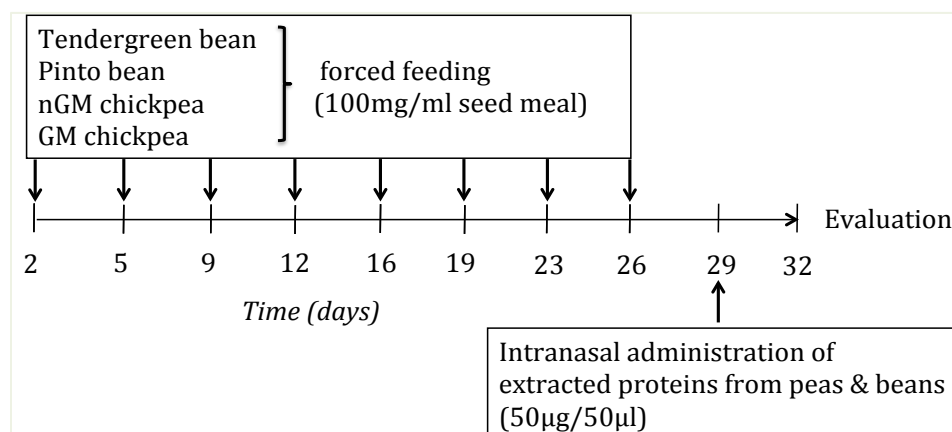
Figure 24: Experimental protocol to test if GM chickpea feeding induces allergy to the transgenic AAI protein*. BALB/c female mice were fed twice a week for 4 weeks with raw GM chickpeas, nGM chickpeas and control materials, native Tendergreen beans and Pinto beans. On day 29 mice were challenged i.n. with 50 μg/50 μl of the corresponding AAI protein. 72h after the last challenge we evaluated parameters characteristic of airway and lung allergic disease.*

Levels of Th2 and Th1 antibodies were measured after feeding and challenge with extracted AAI protein from the same source seed AAI (Figure 25). Our results show that levels of IgG1 were substantially increased after exposure to all food extracts and the subsequent challenge with AAI. The antibody titers following feeding with Pinto bean achieved 1/3938000 and those derived subsequent the exposure to Tendergreen were 13-fold lower (1/ 303929) (Table 10). In mice fed nGM chickpea and challenged AAI, anti-chickpea IgG1 reached a titer of only 1/97250 and only the half for mice fed GM chickpea (1/49125). Only Tendergreen bean feeding and challenge with AAI bean induced IgE antibody in mice (1/23). Unexpectedly, also nGM chickpea feeding and challenge was able to induce low, but detectable IgE (1/13). In contrast, feeding and challenge with GM chickpea only induced half of the IgE titers induced by feeding nGM chickpea.
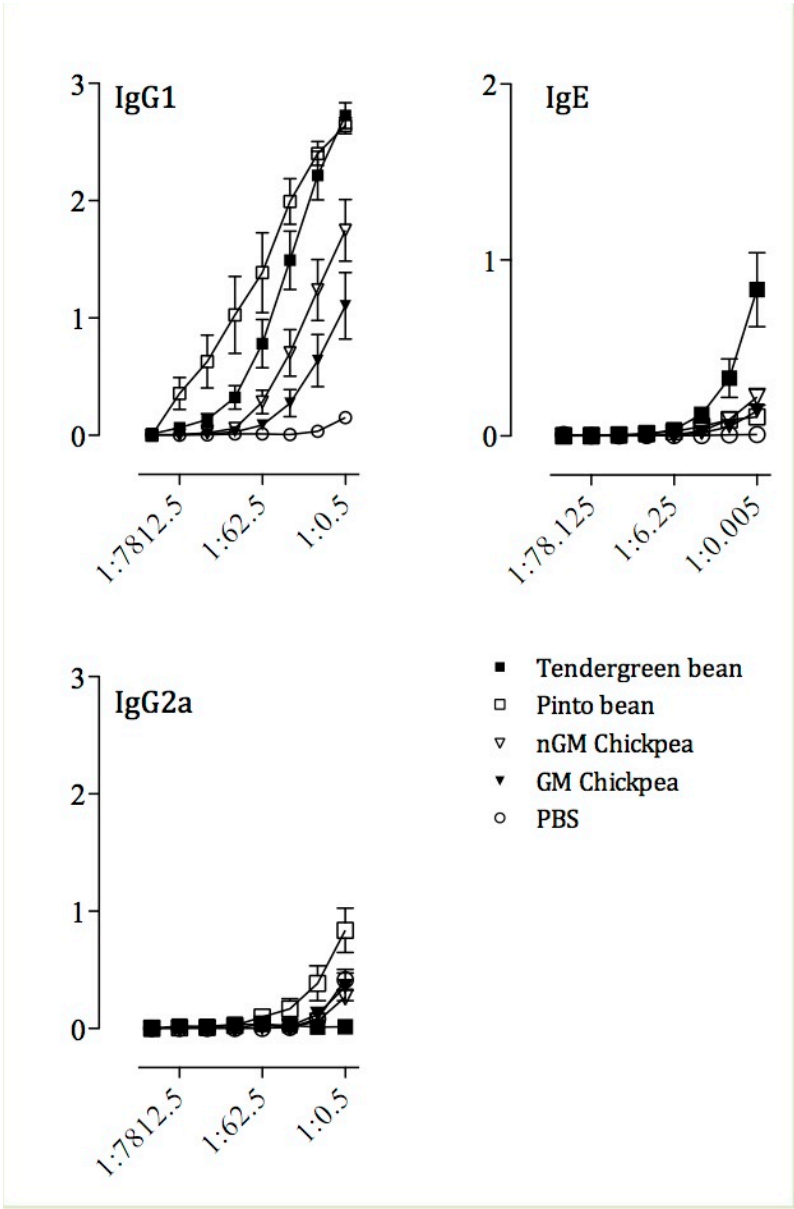
Figure 25. Antibody production secondary to chickpea and beans feeding and challenge with AAI protein. Oral consumption to raw Tendergreen bean, Pinto bean, GM chickpea, nGM chickpea or PBS predispose to AAI specific IgG1, IgE and IgG2a. Mice were sensitized by intra-gastric gavage with a total of 200 mg seed meal without adjuvant. At week 5, mice were i.n. challenged with 50 μg/50 μl of the corresponding purified AAI protein/mouse. Individual sera from different feeding groups were obtained 72h after the challenge and AAI-specific IgG1, IgE and IgG2a levels were determined by sandwich ELISA. Results are representative of at least two independent experiments and are reported as mean O.D. ± SEM (n=8).

Table 10: Reciprocal titer values for Th2-type-IgG1 and IgE antibodies and Th1 type-IgG2a antibody secretion following forced feeding with PBS, Tendergreen bean (TB), Pinto bean (PB), GM chickpea (GM ChP) and nGM chickpea (nGM ChP).

Group fed:	i.n. challenge with:	IgG1 MEAN±SEM	One Way ANOVA	IgE MEAN±SEM	One Way ANOVA
PBS	PBS	200.6±122.2	N.S.	1±0	N.S.
TB	AAI TB	303929±213145	* vs. PBS	23.29±17.26	N.S.
PB	AAI PB	3938000±2237000	** vs. PBS	1.667±0.666	N.S.
GM ChP	AAI ChP	49125±38365	N.S.	7.857±4.426	N.S.
nGM CoP	AAI CoP	97250±47769	N.S	13.5±4.371	N.S.

Group fed:	i.n. challenge with:	IgG2a MEAN±SEM	One Way ANOVA
PBS	PBS	1200±538.4	N.S.
TB	AAI TB	714.4±305.8	N.S.
PB	AAI PB	2357±1721	N.S.
GM ChP	AAI ChP	875.3±362.8	N.S.
nGM CoP	AAI CoP	875.3±362.8	N.S.

Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean titer ± SEM (n=8). *p<0.05, ** p<0.01, indicates statistical significance compared to mice fed PBS. N.S.: not significant.

3.2.6 FEEDING NATIVE BEANS AND ISOGENIC CHICKPEAS INDUCE FUNCTIONAL IgE ANTIBODIES

Feeding and challenge Tendergreen bean (1/23) and nGM chickpea (1/13.5) induced IgE. We, therefore, sought to determine whether the IgE antibodies are also biologically functional. For the measurement of AAI-specific degranulation of rat basophil leukemia (RBL)-2H3, cells were passively sensitized with sera from mice fed different seed meals and β-hexosaminidase release was measured after cross-linking of receptor-bound antibodies with different AAI proteins.
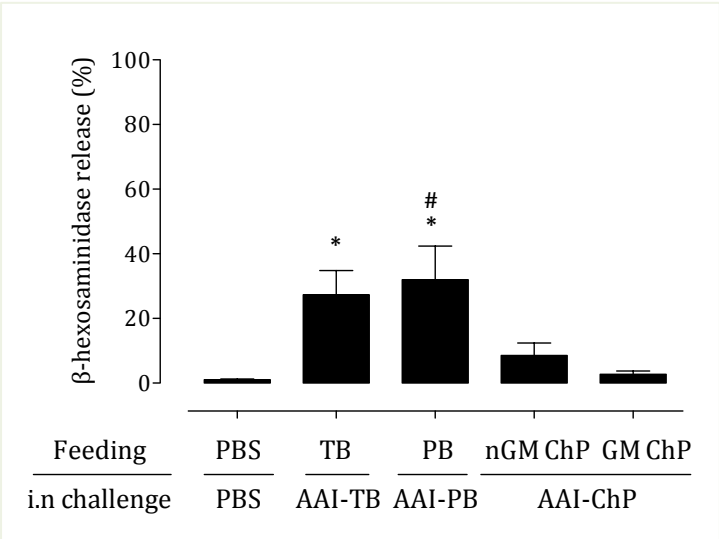
Figure 26. IgE functionality was assessed by RBL cell assay. *RBL-2H3 cells were passively sensitized with mouse sera obtained after feeding with seed meals and challenged with native or transgenic AAI (n=8). Control mice were fed and challenged with PBS. Results are reported as a percentage of total β-hexosaminidase release. One Way ANOVA, *indicates statistical significance compared with PBS fed mice; # indicates statistical significance compared with GM chickpea fed mice (p<0.05).*

Interestingly, our results demonstrated that sera from mice fed and challenged with Tendergreen and Pinto bean, but also with isogenic chickpeas induced AAI-specific degranulation. In contrast, sera collected from mice fed and challenged GM chickpea as well as PBS fed and challenged mice did not induce degranulation, meaning that only feeding native beans and isogenic control, but not AAI chickpeas generated functional IgE antibodies.

3.2.7   FEEDING NATIVE BEANS AND CHICKPEAS PRIMES FOR EOSINOPHILIC AIRWAY INFLAMMATION

Prescott et. al. [50] showed that mice develop eosinophilic airway and lung inflammation upon feeding GM peas and subsequent challenge with purified AAI. To test whether feeding GM chickpeas would also induce recruitment of eosinophils in BAL and lungs, we adhered to the same protocol and fed BALB/c female mice with raw Tendergreen and Pinto bean, GM chickpea and the isogenic control, and challenged them i.n. with the respective AAI proteins as *in vivo* read out of T cell priming that occurred during feeding to AAI.

Table 11: Characterization of the BAL fluid subsequent forced feeding with PBS, Tendergreen bean (TB), Pinto bean (PB), nGM chickpea and GM chickpea followed by i.n. challenge of the extracted AAI protein from the same source seed.

Feeding	PBS	TB	PB	nGM chickpea	GM chickpea
i.n. challenge	PBS	AAI-TB	AAI-PB	AAI-chickpea	AAI-chickpea
Eosinophils	1.378±0.5603	13.80±4.873	12.95±3.378	12.21±4.558	8.361±1.891
Lymphocytes	0.517±0.2051	2.816±0.9184	1.77±0.3245	2.636±0.5772	3.938±0.8614*
Macrophages	95.86±1.666	79±7.03	78.59±4.497	76.56±4.522*	82.67±2.943
Neutrophils	2.245±1.183	4.382±2.337	6.695±2.005	7.814±1.325	5.036±1.205

*Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean cell percentage ± SEM (n=8). *$p<0.05$ indicates statistical significance compared with mice fed PBS.*

Analysis of the cellular content of BAL fluid revealed an increase in eosinophil percentage in all groups of mice fed seed meal compared to mice fed PBS (Figure 27 and Table 11). Mice fed the isogenic chickpea revealed a slight increase in eosinophil percentages (12.21±4.558 vs. 8.361±1.891) compared to the transgenic variety. In contrast, the differential cell counts in the airway indicate that upon both native Tendergreen and Pinto bean feeding, the increase in eosinophils were comparable (13.80±4.873 vs. 12.95±3.378). Feeding native beans and chickpeas did not induce a significant increase in neutrophils or lymphocyte percentages in either group compared to the PBS control.
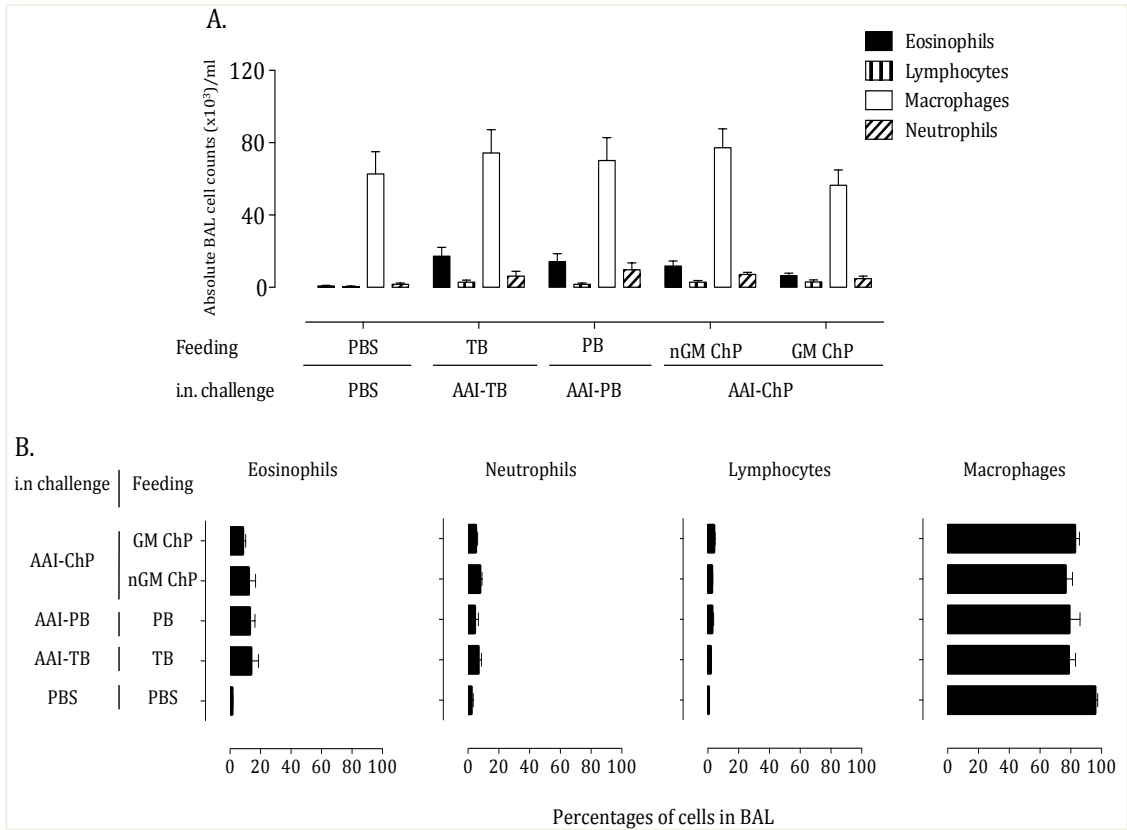
Figure 27. Cell inflammation in airways after feeding with Pinto bean, Tendergreen, nGM- and GM chickpea and challenge with AAI. *Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of raw seed meal. At week 5, mice were i.n. challenged with 50 μg/50 μl of the corresponding purified AAI. Seventy-two hours after the last i.n challenge BAL fluid was collected and differential cell counts were performed. Differential counts were derived from at least 300 cell counts and are expressed as absolute counts per mL (A) and percentages (B). Statistical differences were determined using Kruskal-Wallis test.*

3.2.8   FEEDING NATIVE BEANS AND CHICKPEAS PRIMES FOR EOSINOPHILIC LUNG INFLAMMATION

To examine whether feeding also primes for eosinophils in lungs, we determined the numbers of these cells in the lung tissue. In line with the results observed in the airway, feeding with all seed meals resulted in increase eosinophilic inflammation in lung tissue. In lung sections of mice fed Pinto beans, we detected low numbers of eosinophils (1.386±0.1881), whereas Tendergreen bean and the isogenic chickpea meals induced similar numbers of eosinophils (11±0.8376 vs. 11.16±1.142). In

contrast, the results obtained in the airways compared to lung sections, GM chickpea feeding induced an increase in eosinophil numbers (9.486±1.188).
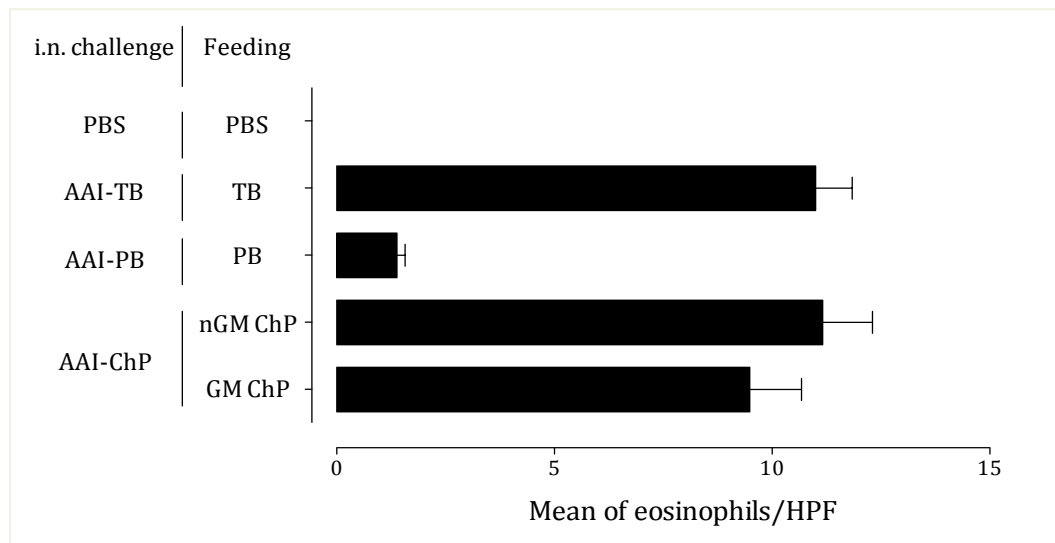
Figure 28. Mean eosinophil counts in lung parenchyma. *Quantification of eosinophils in lung parenchyma of mice fed nGM and GM chickpea compared with mice fed native Tendergreen or Pinto bean followed by an i.n. challenge with the extracted AAI protein from the same source seed. Control mice were fed and challenged with PBS.*

Table 12: Eosinophil counts in lung sections of mice fed Tendergreen bean (TB), Pinto bean (PB), GM chickpea and nGM chickpea.

Feeding:	i.n. challenge:	Eosinophils counts	One-Way ANOVA
		MEAN±SEM	
PBS	PBS	0±0	
TB	AAI-TB	11±0.8376	**vs. *Pinto* bean, ***vs. PBS
PB	AAI-PB	1.386±0.1881	**vs. chickpea
GM chickpea	AAI-chickpea	9.486±1.188	***vs. PBS
nGM chickpea	AAI-chickpea	11.16±1.142	***vs. PBS

*Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean eosinophil counts ± SEM (n=8). Kruskal-Wallis, * p<0.05, ** p<0.01, ***p<0.001 indicates statistical significance compared with mice fed PBS.*

3.2.9 FEEDING NATIVE BEANS AND CHICKPEAS INCREASES OVERALL INFLAMMATION AND INDUCES MUCUS HYPERSECRETION WITHIN THE GOBLET CELLS IN THE LUNGS

Since feeding with GM chickpeas, beans and isogenic control line primed for eosinophils in BAL and lungs, we assumed that these results would be mirrored by the overall inflammation and mucus production in lung parenchyma. The extent of cell infiltration around the central bronchi, alveoli and blood vessels was scored as described in Material and Methods. Lungs were processed for histological analysis 72h after the last i.n. challenge. In control mice challenged with PBS, lung tissue sections did not exhibit inflammatory cell infiltrates. Histological analysis of lung tissue stained with H&E revealed significant increase in airway inflammation in mice fed with Tendergreen bean but also nGM chickpea compared to PBS control group (Figure 30). Mucus hypersecretion is another important feature of allergic response in the lung. To assess the degree of mucus production within goblet cells, lung sections were stained with PAS reagent. Histological examination of lung revealed that PAS-positive stain in the lung section of conducting airways in all groups. However, the grades obtained for mucus hypersecretion were significantly increased in mice fed Tendergreen bean, GM chickpea and nGM chickpea compared to PBS fed mice (Figure 29 and Table 13).
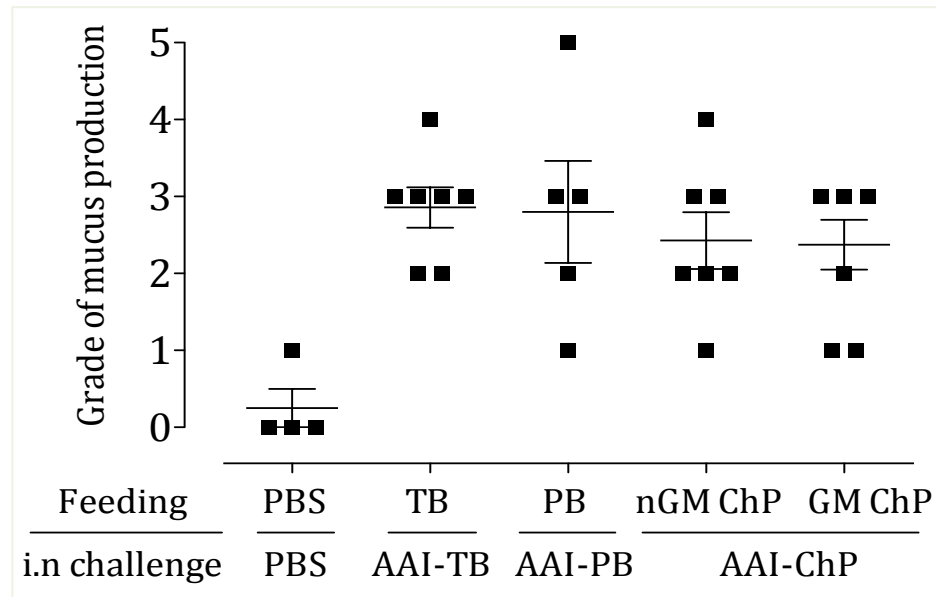
Figure 29. Mucus production in lung parenchyma of mice fed and challenged native and AAI chickpea. Control mice were fed and challenged PBS. Data are presented as the grade of mucus production ± SD; n=8. Statistical differences were determined using Wilcoxon test.

Table 13: Grade of cell infiltrates and mucus production in lung sections of mice fed PBS, Tendergreen bean, Pinto bean, GM chickpea and nGM chickpea and followed by i.n. challenge of extracted AAI protein from the same source seed.

Feeding	PBS	*TB*	*PB*	GM chickpea	nGM chickpea
i.n. challenge	PBS	AAI-TB	AAI-PB	AAI-chickpea	AAI-chickpea
Grade of cell infiltrates	0.3±0.122	0.720±0.183	0.833±0.380	0.4375±0.147	0.4375±0.113
Wilcoxon test	p=0.1489	*p=0.0350	p=0.1736	p=0.0533	*p=0.0263
Grade of mucus production	0.25±0.25	2.857±0.260	2.8±0.663	2.375±0.323	2.429±0.368
Wilcoxon test	p=0.850	*p=0.019	p=0.057	*p=0.011	*p=0.021

*Values are expressed as grade of cell infiltrates ± SEM, and mucus production ± SEM (n=8). Wilcoxon test, *p<0.05 indicates statistical significance compared with mice fed PBS.*
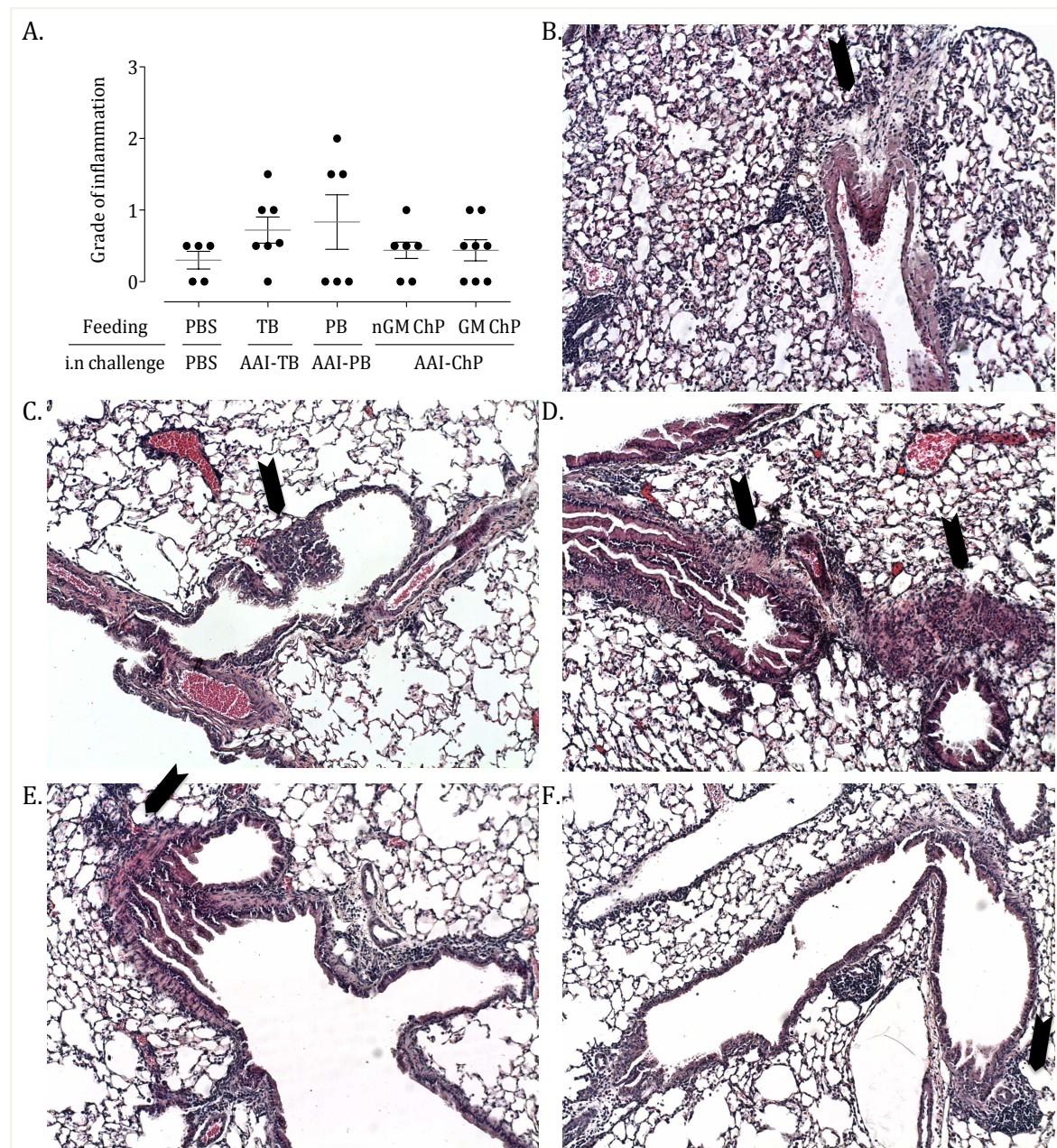
Figure 30. Lung histology of conducting airways and lung parenchyma. *(A) Grade of cell infiltrates in lung parenchyma of mice fed native beans, transgenic and isogenic chickpeas and i.n. challenged with AAIs. Data are presented as the grade of cell infiltrates ± SEM; n=8. Statistical differences were determined using Wilcoxon test, *p value <0.05. Control mice were fed and challenged PBS. Representative photomicrograph of lungs sections stained with H&E of mice fed and challenged with PBS (B), fed Tendergreen bean and challenged with TB-AAI (C), fed Pinto bean and challenged PB-AAI (D), fed GM chickpea and challenged chickpea AAI (E), fed nGM chickpea and challenged chickpea AAI (F). Original magnification x10. Black arrows indicate the cell infiltrates.*

3.2.10   HEAT-TREATMENT OF CHICKPEAS REDUCES THE IMMUNE RESPONSE AFTER INTRAGASTRIC ADMINISTRATION

Our results indicate that feeding of raw seed meal of native Tendergreen bean, Pinto bean, GM chickpea and isogenic chickpea induce allergic airway disease upon nasal challenge with purified AAI protein. However, beans and chickpeas undergo extensive boiling before consumption by the human population. Nevertheless, most food allergens are resistant to heating and enzymatic degradation and this enables the allergen to either sensitize the individual or to elicit and allergic reaction. To determine whether heat-treated seed meal would influence the response to native and transgenic legumes, we compared raw vs. heat-treated (100°C for 30 min) seed meals of Tendergreen bean, Pinto bean and chickpeas. Using the same experimental design, we fed mice and challenged them with one dose of 50 μg of AAI extracted either from the native or transgenic source. Overall inflammation, mucus hypersecretion and antibody titers were quantified 72h after i.n. challenge.
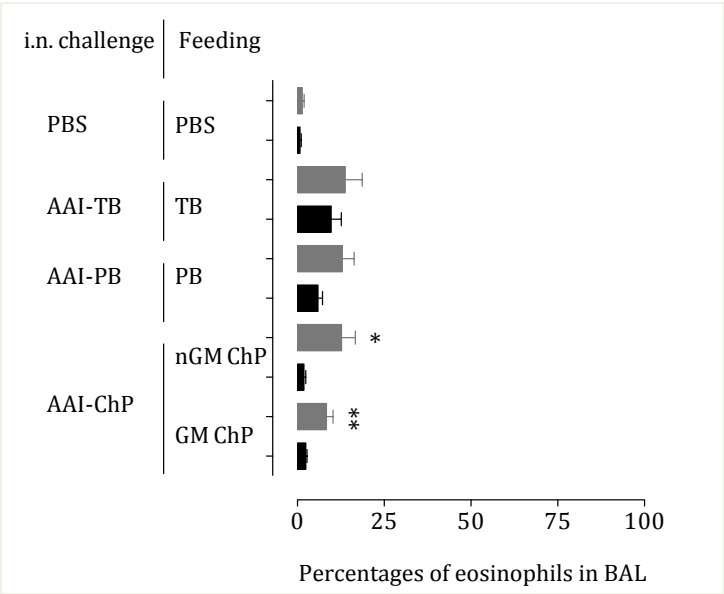
Figure 31. Percentages of eosinophils in the airways of BALB/c mice after feeding heat-treated and raw seed meal. Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of raw (grey bars) and heat-treated (black bars) seed meals. At week 5, mice were i.n. challenged with 50 μg/50 μl of AAI protein extracted from the same source seed. Seventy-two hours after the last i.n. challenge BAL fluid was collected and differential cell counts were performed. Differential counts were derived from at least 300 cell counts and are expressed as percentages. Statistical significance of difference between heat-treated and raw groups were determined using Student t test.

Airway cell counts revealed that mice fed heat-treated Tendergreen bean and Pinto bean showed lower, but not significantly decreased eosinophils compared to animals fed raw meals. In contrast, mice fed heat-treated GM and nGM chickpea had no significant decrease of airway eosinophilia compared to those fed raw seed meal (Figure 32).
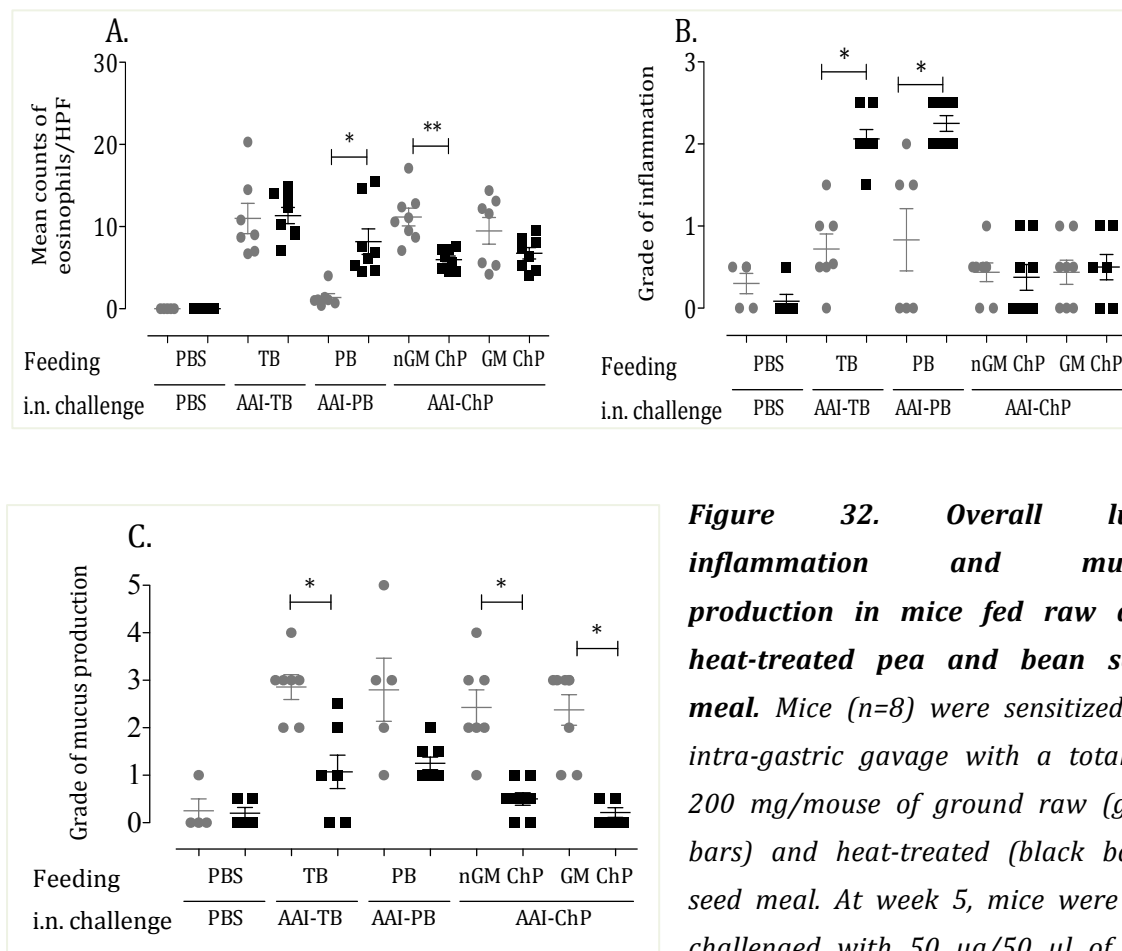
*Figure 32. Overall lung inflammation and mucus production in mice fed raw and heat-treated pea and bean seed meal. Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of ground raw (grey bars) and heat-treated (black bars) seed meal. At week 5, mice were i.n. challenged with 50 μg/50 μl of the corresponding purified AAI. Seventy-two hours after the last i.n. challenge, the mean of eosinophil counts (A), grade of cell infiltrates (B) and the grade of mucus hyper-production (C) were determined. Data are presented as the mean eosinophil counts ± SEM (A), grade of cell infiltrates ± SD (B), grade of mucus production ± SD (C). Statistical differences are calculated between heat-treated and raw groups as determined using a Student t test. *p<0.05 and ** p<0.01 were considered significant.*

Further investigation also showed that mucus production was significantly decreased in lung sections of mice fed heat-treated diets for all GM and nGM seed meal. In contrast, only small reduction in lung eosinophil counts was observed in heat-treated and raw seed meal fed groups. Interestingly, the grade of inflammation

did not correlate with other parameters in that there was an increase in heat-treated Tendergreen bean and Pinto bean fed groups compared to mice fed raw seed meals. The reason for this finding remains unclear (Figure 32). Analysis of Th2 (IgG1 and IgE) and Th1 (IgG2a) antibodies revealed that both raw and heat-treated seed meal from GM and nGM sources induced high levels of IgG1 and low levels of IgE and IgG2a. However, no significant differences were observed as a result of heat-treatment in either group (Figure 33).
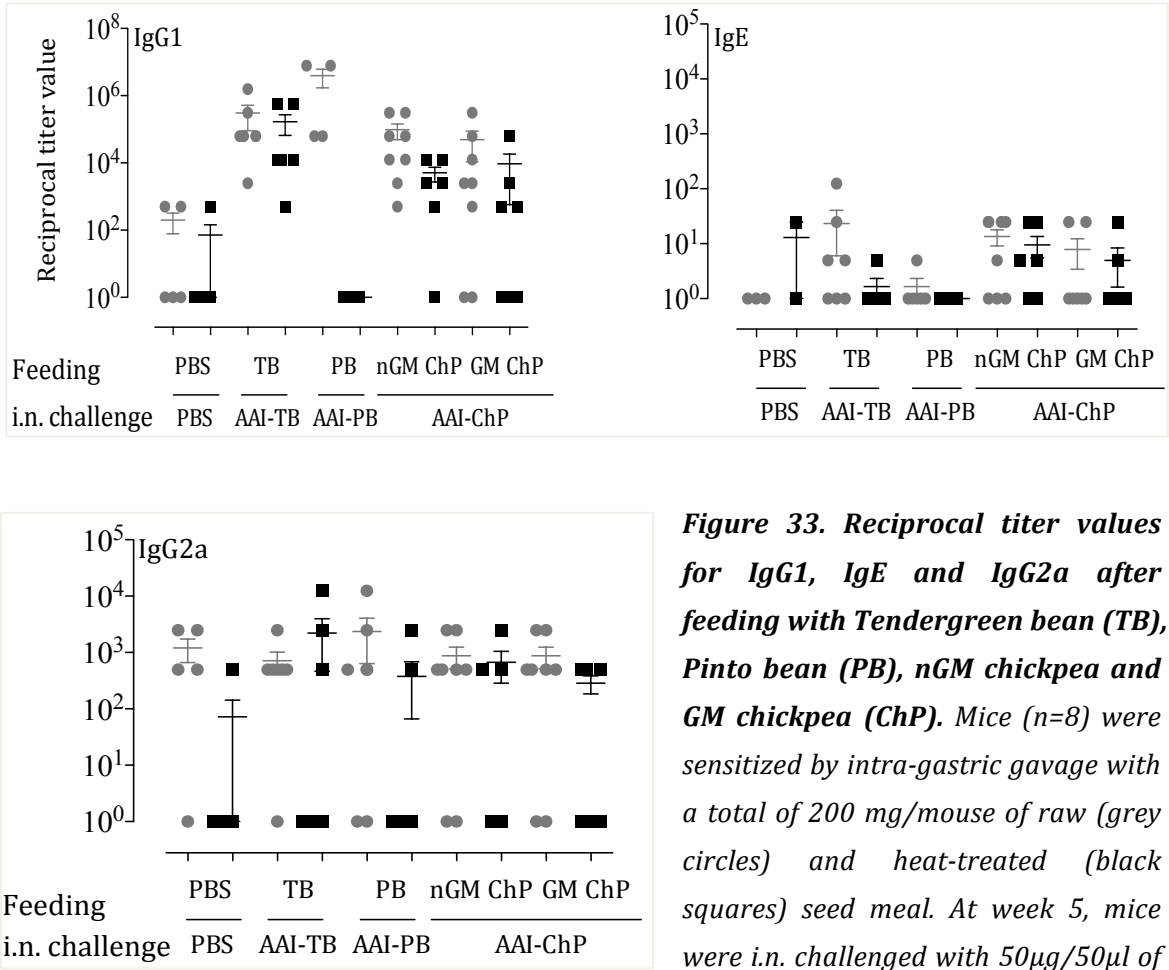
Figure 33. Reciprocal titer values for IgG1, IgE and IgG2a after feeding with Tendergreen bean (TB), Pinto bean (PB), nGM chickpea and GM chickpea (ChP). Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of raw (grey circles) and heat-treated (black squares) seed meal. At week 5, mice were i.n. challenged with 50μg/50μl of the corresponding purified AAIs. Seventy-two hours after the last i.n. challenge the antibody titers were measured by sandwich ELISA. The titers are expressed as the reciprocal titer value of the lower dilution where the last OD readings were still detected. Control mice were fed PBS Values are expressed as mean titer ± SEM Statistical significance of difference between heat-treated and raw groups were determined using Student t test.

3.3 Investigation of the effect of feeding GM cowpeas on the immune response in BALB/c female mice

3.3.1 ANALYSIS OF GM COWPEA SEEDS FOR THE PRESENCE OF THE TRANSGENE AAI

Immunoblot analysis of AAI protein from native Tendergreen bean seeds and transgenic cowpea seeds expressing AAI revealed that multiple polypeptides were produced in both cases and their profiles slightly differed for AAI produced in the 2 crops (Figure 34).
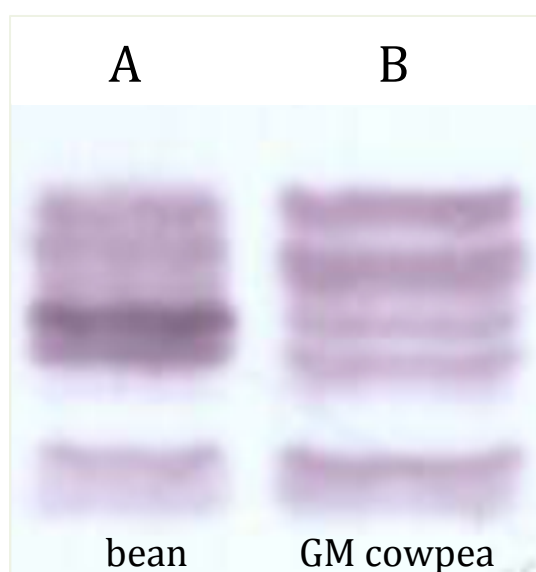
Figure 34. Immunoblot of AAI protein present in extracts of Tendergreen bean (lane a) and GM cowpea (lane b) (courtesy of T.J. Higgins).

3.3.2 CLINICAL OBSERVATION AND ABSOLUTE BODY WEIGHT

Table 14. Body weight of BALB/c female mice force-feed Tendergreen bean, Pinto bean, GM cowpea and two nGM cowpeas varieties.

Feeding groups	Body weight (g)	
	Initial	Final
PBS	16.24±0.468	21.41±0.878
Tendergreen bean	15.95±0.700	21.94±1.272
Pinto bean	15.64±0.5917	21.33±1.071
GM cowpea	15.7±0.806	22.06±1.043
nGM cowpea 1	16.28±0.843	21.02±0.979
nGM cowpea 2	15.68±0.457	21.33±1.071

Control mice were fed PBS. Values are expressed as mean ± SD (n=8).

During the experimental period, all mice survived and showed no adverse effects such as diarrhea or lost of weight. Additionally the body weights did not significantly differ among groups as evidenced by the body weights ranging from initial weights of 15.90±0.104 g to final weights of 21.52± 0.16 g (table 14).

3.3.3 ANTIBODY RESPONSE INDUCED BY GAVAGE ADMINISTRATION OF RAW TRANSGENIC AND TWO ISOGENIC COWPEA VARIETIES IN MICE
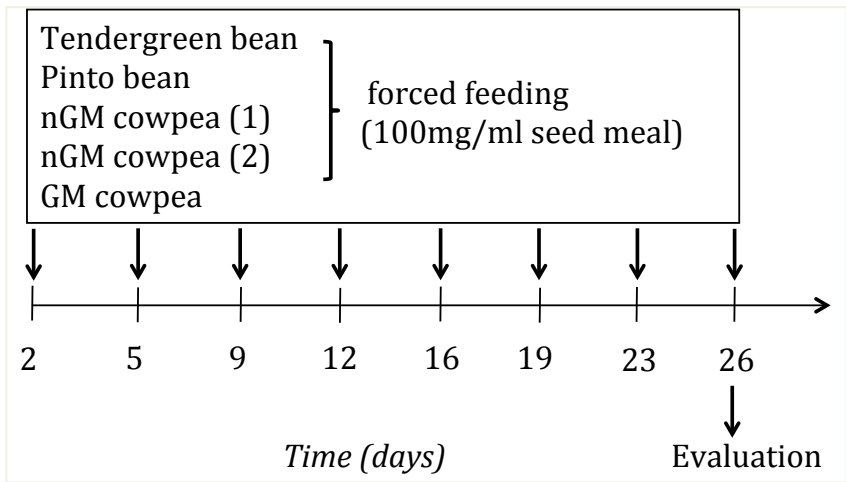
Figure 35: Experimental protocol for testing GM cowpea feeding induction of allergy to the transgenic AAI. BALB/c female mice were fed twice a week for 4 weeks with raw GM cowpeas, nGM cowpeas and control materials, native Tendergreen beans and Pinto beans. On day 26 blood was collected from tail vein and tested for AAI-specific antibodies.

To investigate whether GM cowpea feeding of mice induces allergy to the novel transgenic protein, we performed feeding studies. Previous studies showed that oral exposure to food extracts in the absence of cholera toxin (CT) did not elicit food-specific antibody response, leading rather to oral tolerance [37]. Using our experimental approach, female BALB/c mice were administered intra-gastric 8 times with 100 mg/ml (250 µl/mouse/dose) of raw GM-cowpea and nGM cowpea, Tendergreen and Pinto bean. As negative control mice were fed PBS. As expected PBS feeding failed to stimulate IgG1 antibody production. The exposure to all seed meals resulted in the production of high levels IgG1. Both raw native Tendergreen and Pinto bean meals were the most potent elicitors of anti-AAI IgG1. Levels of this Th2 antibody titer, following feeding with Tendergreen bean, achieved 1/170000, whereas those derived subsequent exposure Pinto bean were 19-fold higher

(1/3200000) (Table 15). The specific AAI IgG1 titers reached only 1/14000 for mice fed GM cowpea, 12-fold less when compared to mice fed Tendergreen bean.
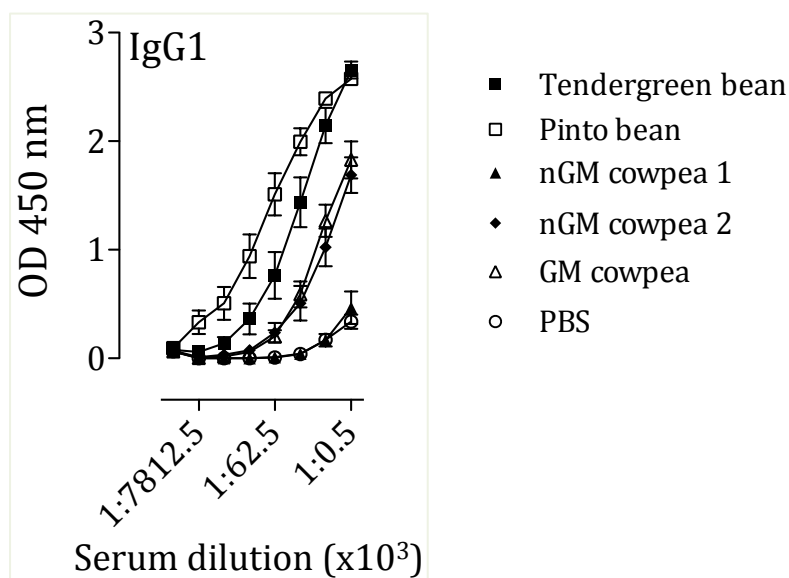
Figure 36. Serum anti-AAI IgG1 following feeding of raw and heat-treated GM cowpea. Individual sera from different feeding groups were obtained after a total of 8 forced feedings. AAI-specific IgG1 levels were determined by sandwich ELISA. Results are representative of at least 2 independent experiments and are reported as mean OD ± SEM (n=8).

Table 15: Reciprocal titer values for IgG1 production following forced feeding with raw Tendergreen, Pinto bean, nGM cowpeas 1 and 2 and GM cowpea.

Feeding:	IgG1	Statistics
	MEAN±SEM	One Way ANOVA
PBS	333.7±166.3	
Tendergreen bean	170833±90753	
Pinto bean	3263000±1860000	*p<0.05 vs. PBS
GM Cowpea	14688±7094	
nGM Cowpea 1	500.5±298.7	**p<0.01 vs. TB, ***p<0.001 vs. PB
nGM Cowpea 2	59688±37187	

*Statistical differences were determined using Kruskal-Wallis test. Values are presented as mean titer ± SEM. (n=8), *p<0.05, ** p<0.01, and ***p<0.001 were considered significant.*

3.3.4 Consumption of Pinto bean and nGM cowpea 2 induces IgG1 cross priming antibodies to pea lectin

Similar to nGM chickpea (Figure 22), feeding nGM cowpeas that lack AAI, also led to the production of anti-AAI antibodies (Figure 36). The titers upon intra-gastric administration of nGM cowpea 1 were 1/500, whereas mice fed nGM cowpea 2 induced a higher production of AAI-specific IgG1 (1/ 59000). We next investigated whether feeding whole cowpeas seed meal would generate antibodies against pea lectins due to the fact that lectins and AAI display high degree of amino acid sequence similarity [88]. Our results suggest that only consumption of Pinto beans and nGM cowpeas (2) induces antibodies to lectin whereas feeding GM or nGM cowpeas (1) did not (Figure 37).
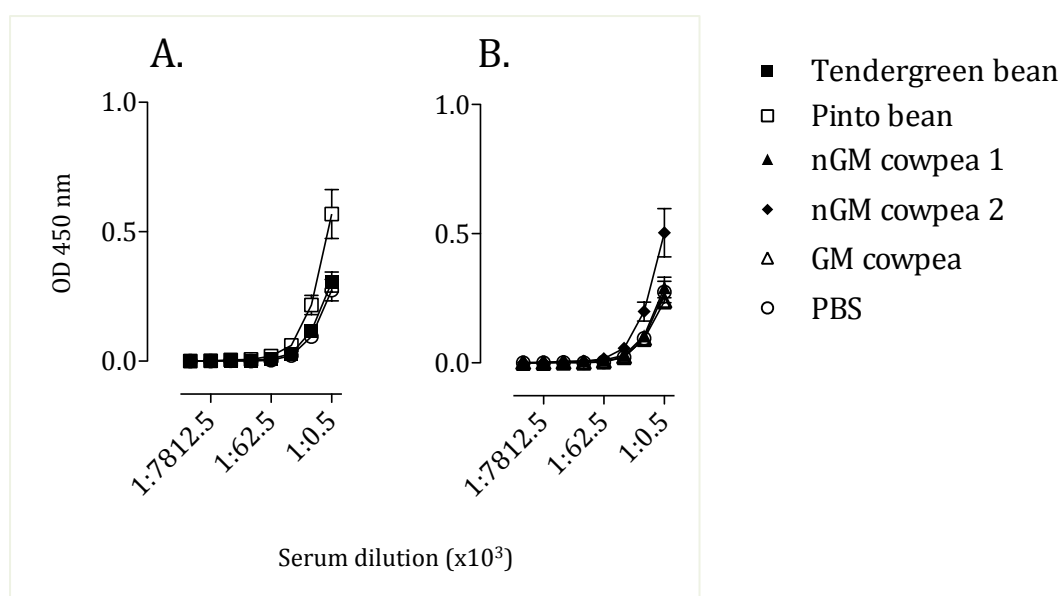
Figure 37. Feeding Pinto bean and nGM cowpea 2 induced a cross-reactive IgG1 antibody response to pea lectin. Groups of mice (n=8) were fed twice a week for 4 weeks different seed meals. 72h after the last feeding, blood was collected from the tail vein and individual serum samples were tested for the presence of anti lectin-IgG1, antibodies by sandwich ELISA. Data are expressed as the mean O.D. ± SEM.

3.3.5 ANTIBODY RESPONSE INDUCED BY GAVAGE AND I.N. ADMINISTRATION OF RAW COWPEAS AND AAI IN MICE
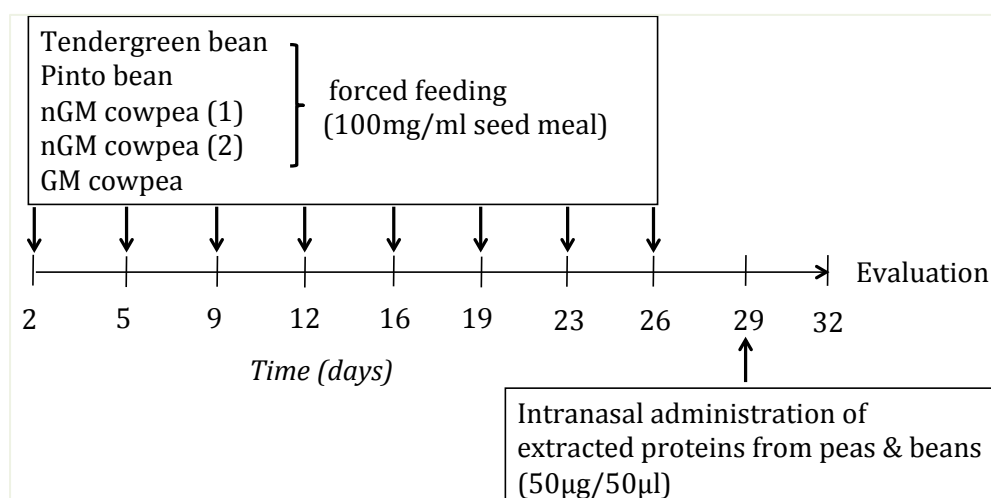
Figure 38: Experimental protocol testing GM cowpea feeding induction of allergy to the transgenic AAI. BALB/c female mice were fed twice a week for 4 weeks with raw GM cowpeas and two isogenic cowpea lines. As control materials we fed mice with native Tendergreen beans, the source of AAI in the transgenic cowpeas and as an additional control mice were fed Pinto beans. On day 29, mice were challenged i.n. with 50 µg/50 µl of the corresponding AAI protein. 72h after the last challenge we evaluated parameters characteristic of airway and lung allergic disease.

Levels of Th2 and Th1 antibodies were measured after feeding and challenge with extracted AAI protein from the same source seed AAI (Figure 38). Our results show that levels of IgG1 were substantially increased after exposure to all food extracts and the subsequent challenge with AAI, and in line with the results obtained after only feeding with seed meals (Figure 36) both raw Tendergreen and Pinto bean meals were the most potent elicitor of IgG1. Antibody titers following feeding with Pinto bean achieved 1/3938000 and those derived subsequent the exposure to Tendergreen were 13 fold lower (1/303929) (Table 16). Mice fed nGM cowpea 1 and challenged AAI cowpea IgG1 reached a titer of only 1/1626, whereas in mice fed nGM cowpea 2 and challenged AAI cowpea the titres were higher (1/59688). Feeding GM cowpea and challenge with cowpea AAI induced very low IgG1 titers (1/27000). However, Tendergreen bean feeding and challenge with AAI bean was able to induce IgE antibody in mice (1/23), whereas the other seed meals had very

low IgE titres. Interestingly, in sera of mice fed nGM cowpea 2 we measured low levels of IgE (1/2.5) while only titers of 1/1.5 were measured for GM cowpea feeding.
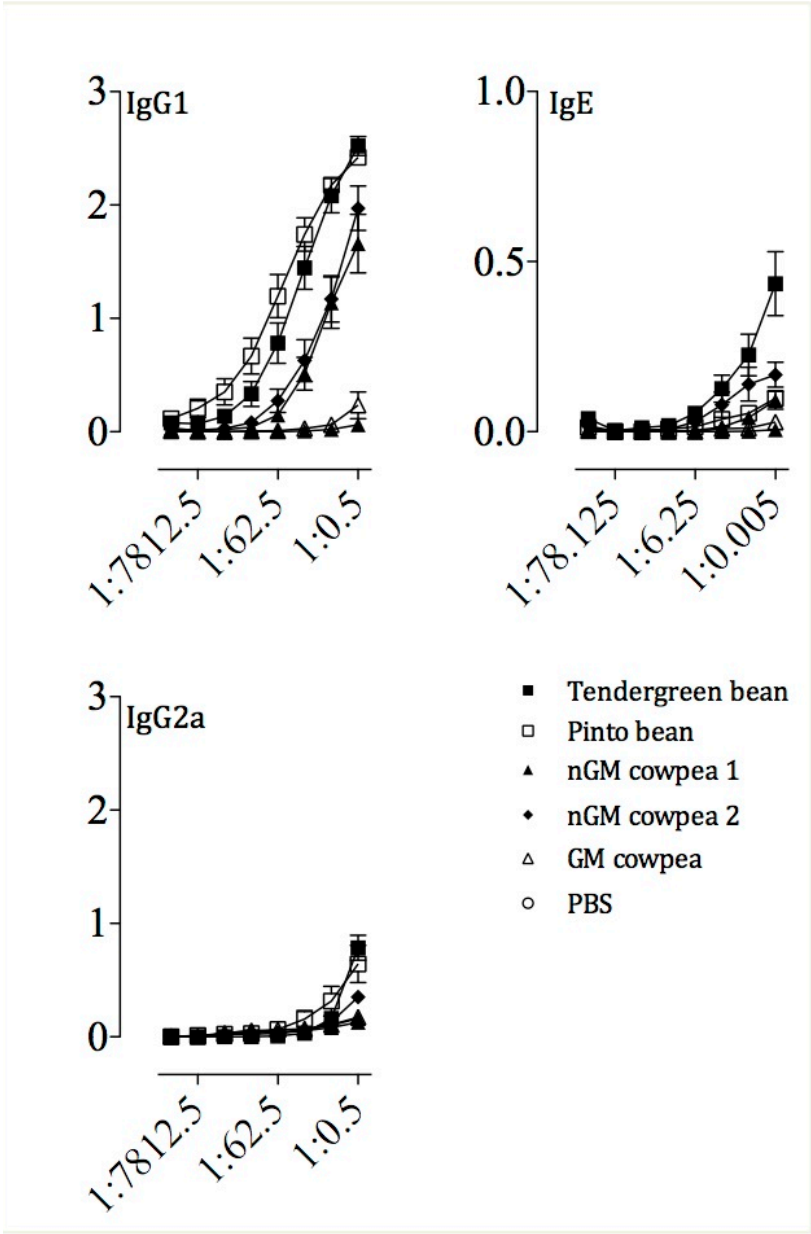
Figure 39. AAI specific IgG1, IgE and IgG2a. Mice were sensitized by intra-gastric gavage with a total of 200 mg of seed meal without adjuvant. At week 5, mice were i.n. challenged with 50 µg/50 µl of the corresponding purified AAI/mouse. Individual sera from different feeding groups were obtained 72h after the challenge and AAI-specific IgG1, IgE and IgG2a levels were determined by sandwich ELISA. *Results are representative of at least two independent experiments and are reported as mean OD ± SEM (n=8).*

Table 16: Reciprocal titer value for Th2 type-IgG1 and IgE and Th1 type-IgG2a antibody secretion following forced feeding with PBS, Tendergreen (TB), Pinto bean (PB), GM cowpea (GM CoP) and nGM cowpea 1 and nGM cowpea 2 (nGM CoP).

Group fed:	i.n. challenge with:	IgG1 MEAN±SEM	One Way ANOVA	IgE MEAN±SEM	One Way ANOVA
PBS	PBS	200.6±122.2	N.S.	1±0	N.S.
TB	AAI-TB	303929±213145	* vs. PBS	23.29±17.26	N.S.
PB	AAI-PB	3938000±2237000	** vs. PBS	1.667±0.66	N.S.
GM CoP	AAI-CoP	27000±10527		1.5±0.5	N.S.
nGM CoP 1	AAI-CoP	1626±1555	** vs. TB ** vs. PB	1±0	N.S.
nGM CoP 2	AAI-CoP	59688±37187	N.S.	2.5±0.73	N.S.

Group fed:	i.n. challenge	IgG2a MEAN±SEM	One Way ANOVA
PBS	PBS	1200±538.4	N.S.
TB	AAI TB	714.4±305.8	N.S.
PB	AAI PB	2357±1721	N.S.
GM CoP	AAI CoP	1±0	* vs. TB, * vs. PB
nGM CoP (1)	AAI CoP	1±0	* vs. TB, * vs. PB
nGM CoP (2)	AAI CoP	250.5±94.3	N.S.

*Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean titer ± SEM (n=8). *p<0.05, ** p<0.01, indicates statistical significance compared with mice fed PBS. N.S.: not significant.*

3.3.6 FEEDING BEANS AND ISOGENIC COWPEAS INDUCES FUNCTIONAL IGE ANTIBODIES

Next we aimed to determine whether IgE antibodies induced in response to cowpea feeding were biologically functional. Our results revealed that, feeding Tendergreen bean, Pinto bean, nGM chickpea and isogenic cowpea 1 produced IgE antibodies that induce AAI-specific degranulation. In contrast sera collected from mice fed and challenged GM cowpea, nGM cowpea 2 as well as PBS did not induce degranulation, meaning that only feeding native beans and one isogenic control variety, but not GM cowpeas generates functional IgE antibodies in mice.
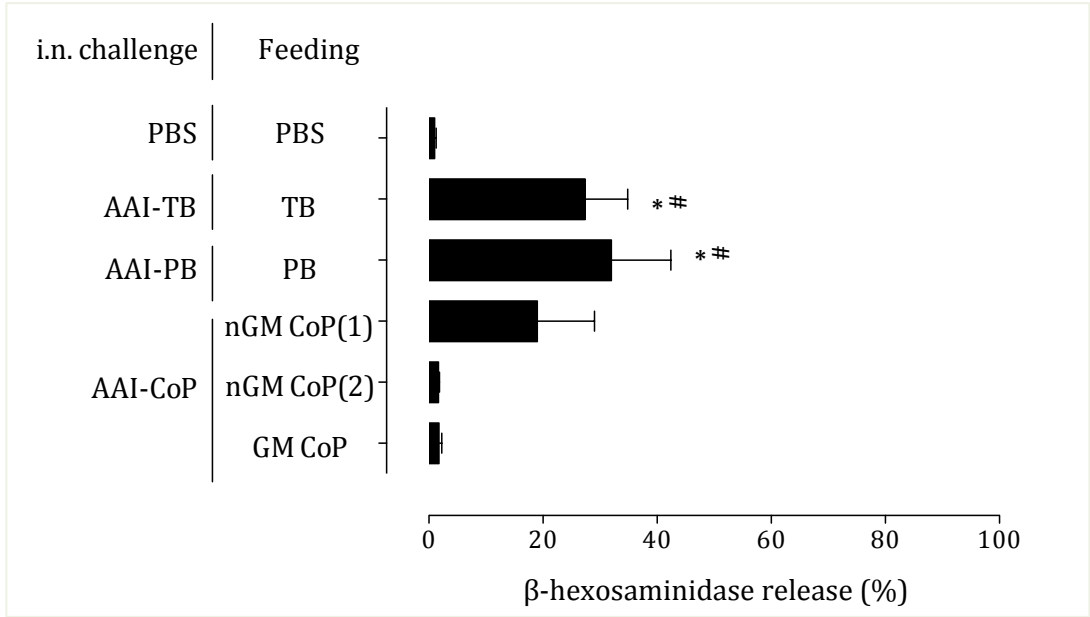
Figure 40. IgE functionality was assessed by RBL cell assay. *RBL-2H3 cells were passively sensitized with mouse sera obtained after feeding with different seed meals and challenge with the corresponding native or transgenic protein AAI (n=8). Control mice were fed and challenged with PBS. Results are reported as percentage of total β-hexosaminidase release. One Way ANOVA, *indicates statistical significance with PBS fed mice; # indicates statistical significance compared with GM cowpea fed mice (p<0.05).*

3.3.7 FEEDING WITH RAW NATIVE BEANS AND COWPEAS PRIMES FOR EOSINOPHILIC AIRWAY INFLAMMATION

Our previous results showed that mice developed airway and lung inflammation upon feeding GM chickpea, but also that nGM chickpea augmented the allergic responses in mice (Figure 27, 28 and 29). To test whether feeding GM cowpeas and nGM cowpeas also induced increased airway and lung eosinophilia, we fed BALB/c female mice with raw Tendergreen and Pinto bean, GM cowpea and the 2 isogenic lines, and challenged them with the corresponding AAI proteins as *in vivo* read out of T cell priming that occurred during feeding to AAI. Analysis of the cellular content of BAL fluid revealed an increase in percentages, of eosinophils in all groups of mice fed seed meals compared to mice fed PBS (Figure 41 and Table 17). In airways, the lowest percentage of eosinophils was recruited when mice were fed nGM cowpea 1 (4.43±1.523). In contrast, mice fed with the second isogenic cowpea line revealed a slight increase in eosinophils (7.37±2.09) similar to the transgenic cowpea variety

(7.70±2.94). In contrast, differential cell counts in lavage fluid revealed that upon Tendergreen and Pinto bean feeding the increase in eosinophils was comparable. (13.80±4.873 vs. 12.95±3.378). Feeding native beans and chickpeas did not induce a significant increase in neutrophils or lymphocyte percentages in either group compared to the PBS control (Figure 41 and Table 17).

Table 17: Characterization of the BAL fluid subsequent forced feeding and i.n. exposure to PBS, Tendergreen bean (TB), Pinto bean (PB), nGM cowpea (nGM CoP) and GM cowpea (GM CoP).

Feeding	PBS	TB	PB	nGM CoP (1)	nGM CoP (2)	GM CoP
i.n. challenge	PBS	AAI-TB	AAI-PB			AAI-CoP
				AAI-CoP	AAI-CoP	
Eosinophils	1.37±0.56	13.8±4.87	12.95±3.3*	4.43±1.52	7.37±2.09	7.70±2.94
Lymphocytes	0.51±0.2	2.81±0.91	1.77±0.32	2.51±0.67*	1.69±0.34	1.3±0.17
Macrophages	95.86±1.6	79±7.03	78.59±4.4*	88.49±1.81	86.49±2.9	86.15±2.6
Neutrophils	2.24±1.18	4.38±2.33	6.69±2	4.56±1.33	4.44±1.17	4.83±1.34

*Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean cell percentages ± SEM (n=8). *p<0.05 indicates statistical significance compared with mice fed PBS.*
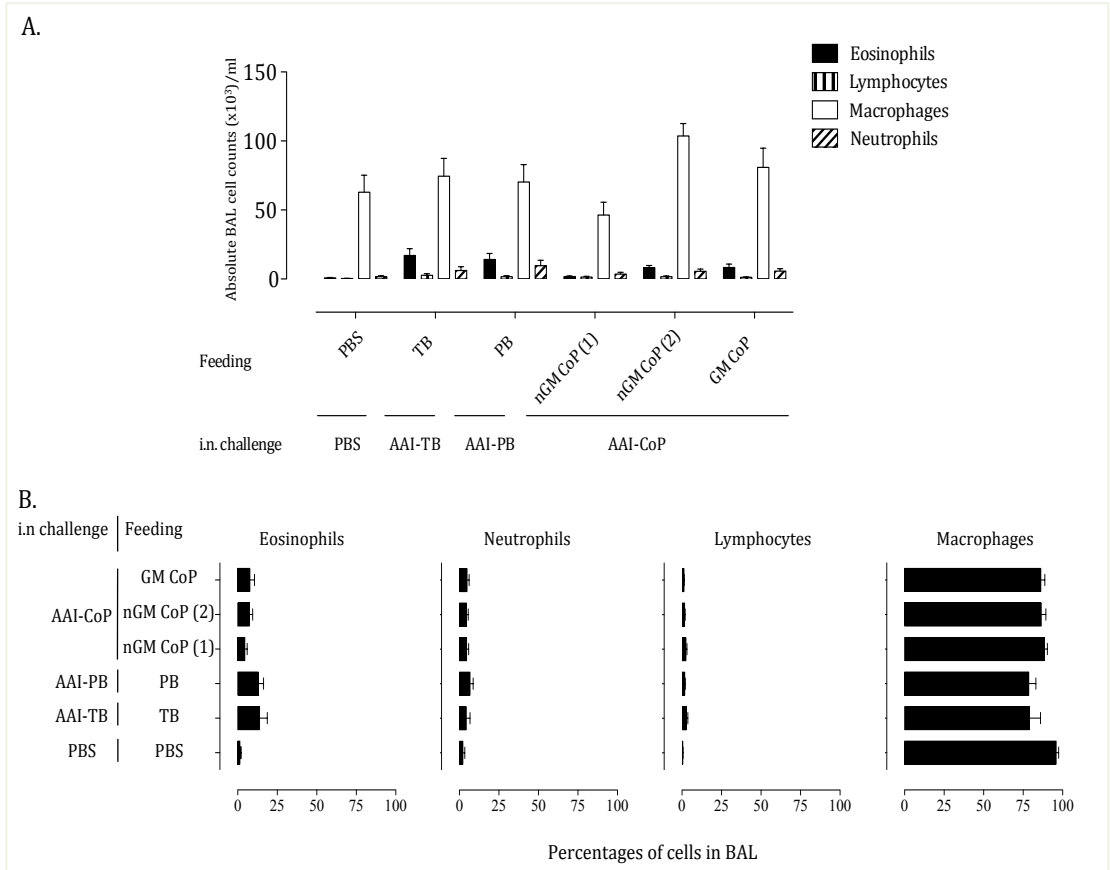
Figure 41. Cell inflammation in airways after feeding with Pinto bean, Tendergreen, nGM cowpea 1 and 2 and GM cowpea and challenge with AAI. *Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of raw seed meal. At week 5, mice were i.n. challenged with 50 µg/50 µl of the corresponding purified AAI. 72h after the last i.n challenge BAL fluid was collected and differential cell counts were performed. Differential counts were derived from at least 300 cell counts and are expressed as absolute counts per mL (A) and percentages (B). The results for each group are expressed as percentages ± SEM; n=8. Statistical differences were determined using Kruskal-Wallis test.*

3.3.8 FEEDING NATIVE BEANS AND COWPEAS PRIMES FOR EOSINOPHILIC LUNG INFLAMMATION

To examine whether feeding cowpeas also primed for eosinophils in lungs, we determined the numbers of these cells in lung tissue. As observed in the airway, feeding with all seed meals resulted in increased eosinophilic inflammation in lung tissue. In lung sections of mice fed Pinto beans we detected low numbers of eosinophils (1.386±0.1881), whereas Tendergreen bean and the 2 isogenic cowpea

seed meals induced higher numbers of eosinophils 11±0.8376 and 11.1±1.1, respectively. Similar to the results obtained in the airway, GM cowpea fed mice eosinophil numbers were increased (9.4±1.1).
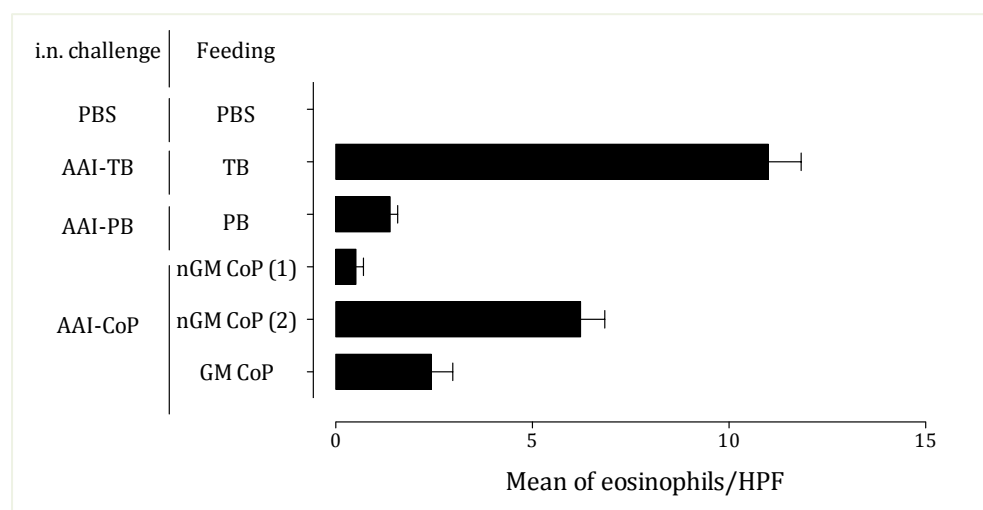
Figure 42. Mean eosinophils counts in lung parenchyma*. Quantification of eosinophils in lung parenchyma of mice fed two isogenic cowpea varieties and GM cowpea compared to mice fed native Tendergreen or Pinto bean. Subsequent feeding mice were challenged with the corresponding AAI protein. Control mice were fed and PBS.*

Table 18: Eosinophil counts in lung sections of mice fed Tendergreen bean, Pinto bean, GM cowpea and nGM cowpea 1 and 2.

Feeding groups	i.n. challenge	Eosinophils counts	Statistic
		Mean±SEM	One-Way ANOVA
PBS	PBS	0±0	
Tendergreen bean	AAI-TB	11±0.8	**vs. PB, PBS
Pinto bean	AAI-PB	1.3±0.1	**vs. chickpea
GM cowpea	AAI cowpea	9.4±1.1	***vs. PBS
nGM cowpea 1	AAI cowpea	11.1±1.1	***vs. PBS
nGM cowpea 2	AAI cowpea	11.1±1.14	***vs. PBS

*Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean eosinophil counts ± SEM (n=8). Kruskal-Wallis, * p<0.05, ** p<0.01, ***p<0.001 indicates statistical significance compared with mice fed PBS.*

3.3.9 FEEDING NATIVE BEANS AND COWPEAS INCREASES OVERALL INFLAMMATION AND INDUCES MUCUS HYPERSECRETION WITHIN THE GOBLET CELLS IN THE LUNGS

Because GM cowpeas, bean and isogenic control feeding primed for eosinophils in airway and lungs, we hypothesized that these results will be mirrored by the overall inflammation and mucus production in lung parenchyma. The severity of cell infiltrates and mucus production around the central bronchi, alveoli and blood vessels were scored as described in Material and Methods. Lungs were processed for histological analysis 72h after the last i.n. challenge. Lung tissue sections of control mice challenged with PBS exhibited minimal inflammation and mucus secretion. Histological analysis of lung tissue stained with H&E (Figure 44) and PAS (Figure 43) revealed that feeding Tendergreen bean and Pinto bean induced a significant increase of airway inflammation (0.720 ± 0.1834 and 0.833 ± 0.380) and mucus production (2.857 ± 0.2608 and 2.8 ± 0.663) compared to PBS groups. Similarly, feeding one isogenic cowpea variety increases lung inflammation and mucus production (2.250 ± 0.491 0.687 ± 0.282). In contrast, mice fed GM cowpea and the other isogenic cowpea variety exhibited lower inflammation and mucus production (Table 19).
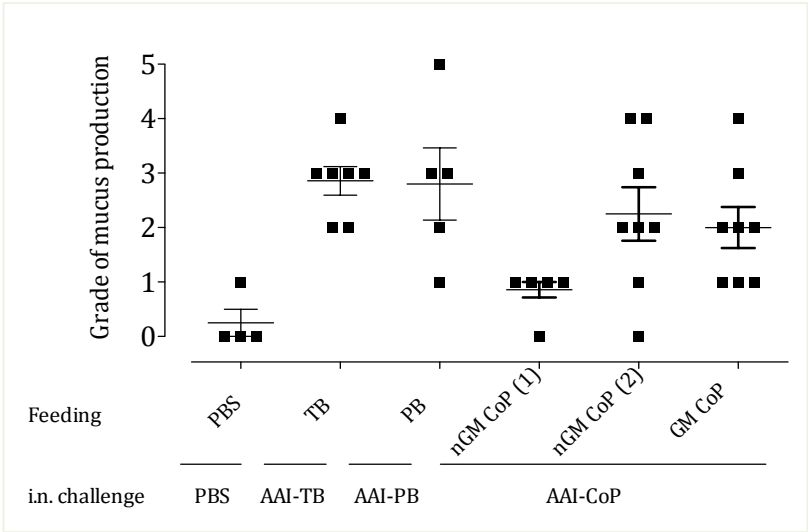
Figure 43. Mucus hypesecretion in lung parenchyma of mice fed GM and nGM cowpeas and challenged AAI. Control mice were fed and challenged PBS. Data are presented as the grade of mucus production ± SD; n=8. Statistical differences were determined using Wilcoxon test.
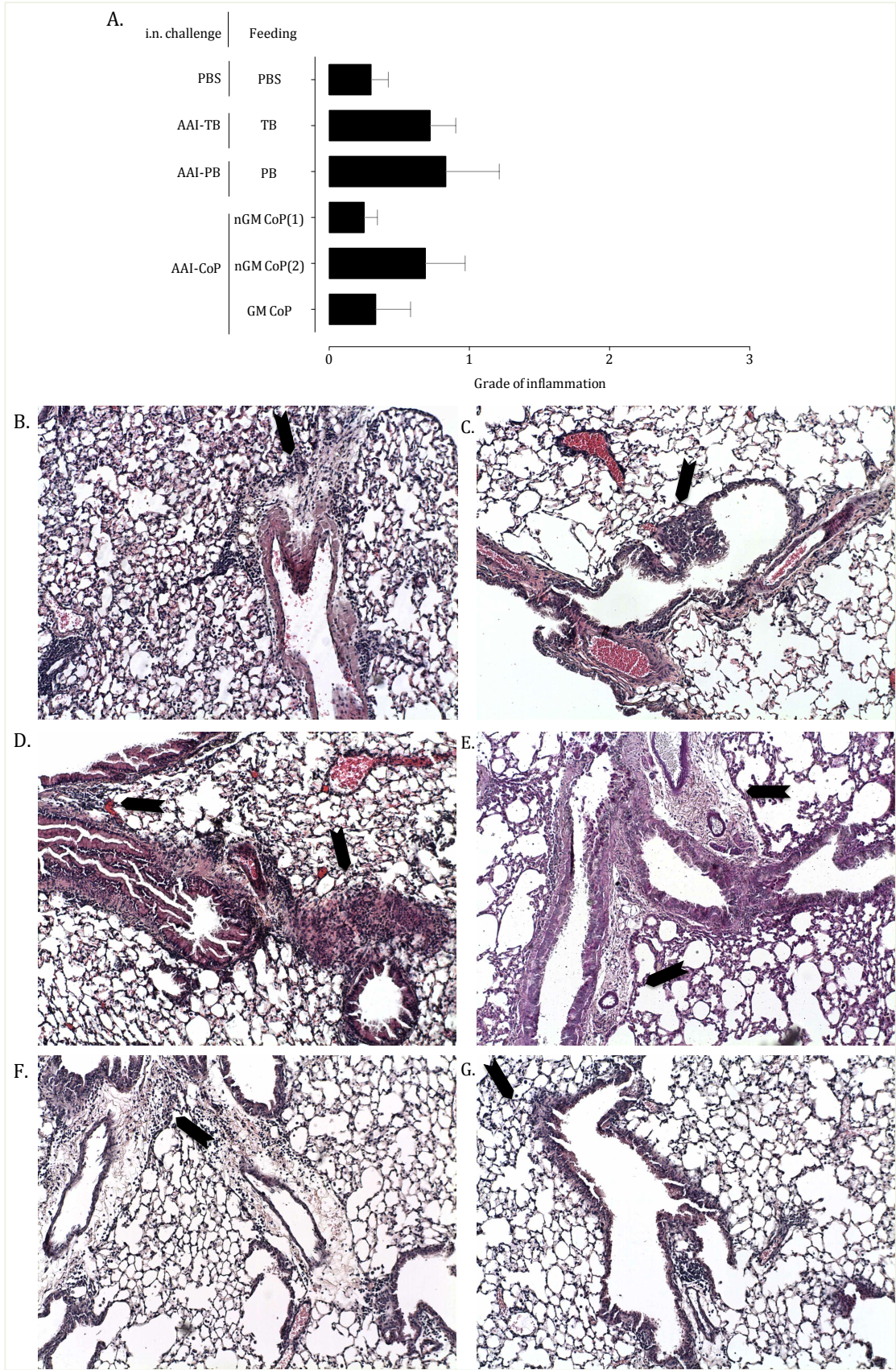
Figure 44. Overall lung inflammation in lung. *(A) Grade of cell infiltrates in lung parenchyma of mice fed native beans, transgenic and isogenic cowpeas and i.n. challenged with AAIs. Data are presented as the grade of cell infiltrates ± SEM from n=8 mice/group. Statistical*

*differences were determined using Wilcoxon test, *p value <0.05. Control mice were fed and challenged with PBS. Representative photomicrographs of lung sections stained with H&E of mice fed and challenged with PBS (B), fed Tendergreen bean and challenged with TB-AAI (C), fed Pinto bean and challenged PB-AAI (D), fed GM cowpea and challenged cowpea AAI (E), fed nGM cowpea 1 and challenged cowpea AAI (F) and fed nGM cowpea 2 and challenged cowpea AAI (G). Original magnification x 20. Black arrows indicate the cell infiltrates AAI.*

Table 19: Grade of cell infiltrates and mucus production in lung sections of mice fed Tendergreen bean (TB), Pinto bean (PB), GM cowpea (GM CoP) and nGM cowpea (nGM CoP).

	PBS	TB	PB	nGM CoP (1)	nGM CoP (2)	GM CoP
Grade of cell infiltrates	0.3±0.12	0.72±0.18	0.83±0.38	0.25±0.09	0.68±0.28	0.33±0.24
Wilcoxon test	p=0.148	*p=0.035	p=0.173	p=0.071	*p=0.031	p=0.50
Grade of mucus production	0.25±0.25	2.85±0.26	2.8±0.66	0.87±0.14	2.25±0.49	2.0±0.37
Wilcoxon test	p=0.850	*p=0.019	p=0.057	*p=0.023	*p=0.02	*p=0.013

*Values are expressed as grade of cell infiltrates ± SEM, and mucus production ± SEM (n=8). Wilcoxon test, *p<0.05 indicates statistical significance compared with mice fed PBS.*

3.3.10 EFFECT OF HEAT-TREATMENT OF COWPEA ON THE IMMUNE RESPONSE AFTER INTRAGASTRIC ADMINISTRATION

In the experiments described above we observed that feeding heat-treated seed meals from Tendergreen bean, Pinto bean and chickpea to mice induces a decrease in the overall inflammation and antibody production. To determine whether heat-treatment of the seed meal would also influence the response to native and transgenic cowpeas we compared 4 weeks feeding trial with seed meals that were raw vs. those heat-treatment at 100°C for 30 min with Tendergreen bean, Pinto bean, isogenic and AAI cowpeas. Using the same experimental design after feeding mice were challenged with one dose of 50 µg of AAI proteins extracted either from the native source or from the transgenic one. Overall inflammation, mucus hypersecretion and antibody titers were quantified seventy-two hours after the i.n. challenge. After heat treatment we observed a decrease in the overall immune response, however we still detect eosinophil in BAL. Mice fed native Tendergreen

bean and Pinto bean heat-treatment materials showed a lower but not significant decrease of eosinophils in BAL compared to the raw diets. The same trend was observed for mice fed heat-treated GM cowpea diet. In contrast, in mice fed cooked nGM cowpea 2, we detected a significant decrease of airway eosinophilia compared to those in the groups fed the raw diets, although this response was not detected in the first isogenic cowpea line.
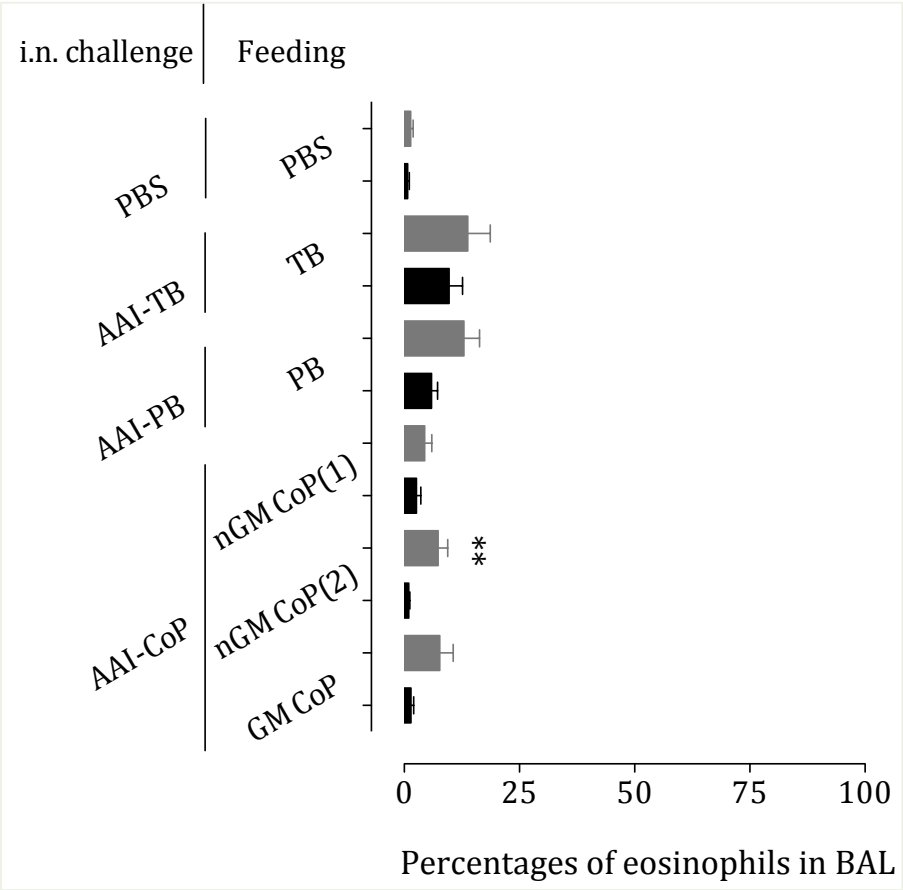
Figure 45. Percentages eosinophils in BAL of BALB/c mice after feeding heat-treated and raw seed meals. *Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of raw (grey bars) and heat-treated (black bars) seed meal. At week 5, mice were i.n. challenged with 50 μg/50 μl of the corresponding purified AAI. Seventy-two hours after the last i.n challenge BAL fluid was collected and differential cell counts were performed. Differential counts were derived from at least 300 cell counts and are expressed as percentages. Statistical significance of difference between heat-treated and raw groups were determined using Student t test.*
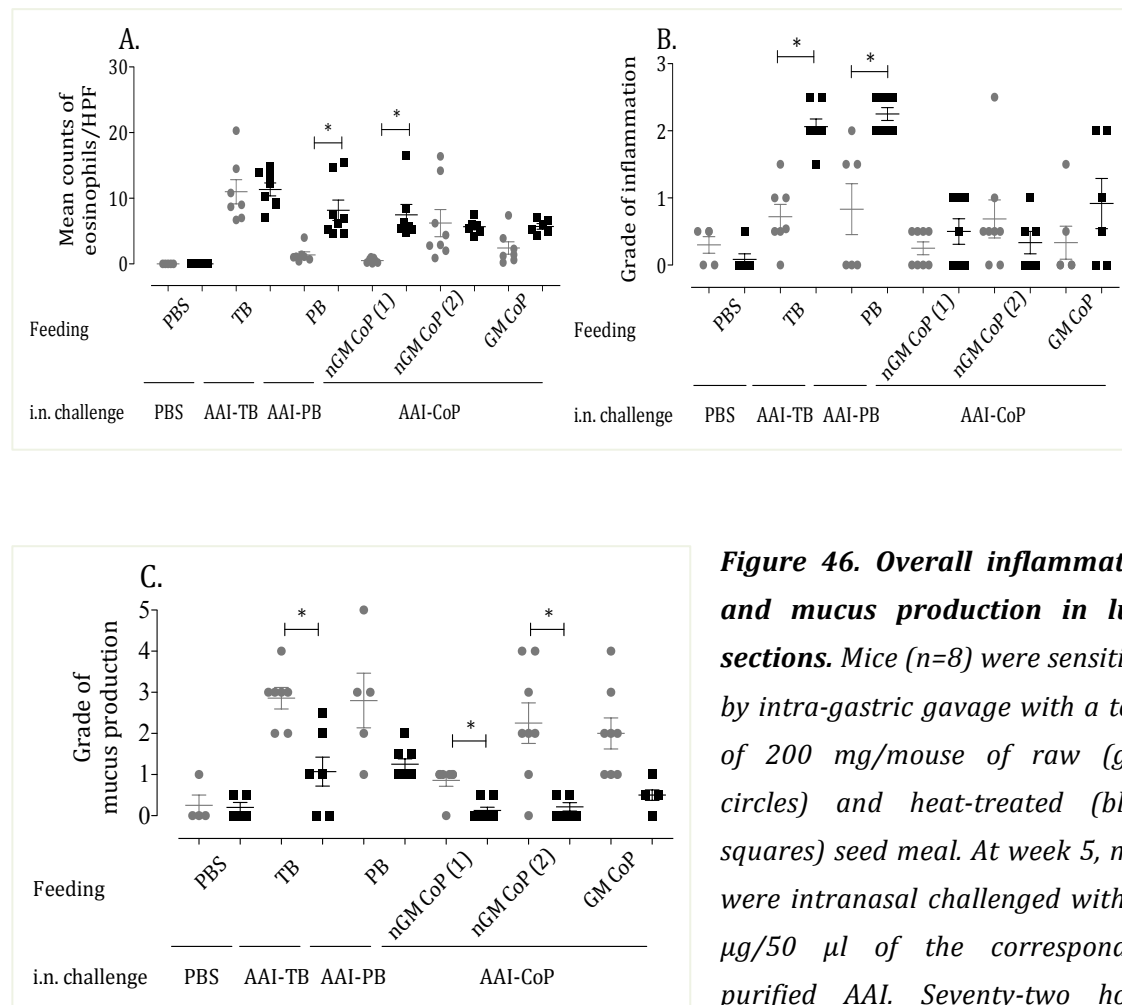
*Figure 46. Overall inflammation and mucus production in lung sections. Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of raw (grey circles) and heat-treated (black squares) seed meal. At week 5, mice were intranasal challenged with 50 µg/50 µl of the corresponding purified AAI. Seventy-two hours after the last i.n. challenge the mean of eosinophils (A), grade of cell infiltrates (B) and the grade of mucus production (C) were determined. Data are presented as the mean eosinophil counts ± SEM (A), grade of cell infiltrates ± SD (B), grade of mucus production ± SD (C). Statistical differences between heat-treated and raw groups were determined using Student t test. *p<0.05 was considered significant.*

In line with the results observed in the airways, the grade of mucus production was also decreased in all groups fed heat-treated diets compared to raw fed mice. In contrast, the lung eosinophil counts and inflammation were significantly increased in mice fed raw native *Pinto* bean compared to raw seed meals.

Serum titers of anti-AAI-specific IgG1 were markedly increased in response to all feeding with raw meals compared to heat-treated seed meals. Interestingly, the titers of anti-AAI-specific IgG1 were reduced upon feeding with heat-treated *Pinto* bean and isogenic cowpeas but not upon feeding with Tendergreen bean and GM cowpea. Feeding raw Tendergreen bean also induced IgE antibodies directed against

AAI in 4 out of 8 mice, but was lost upon heat treatement and heat-treated Tendergreen bean seed meal primed for Th1-type IgG2a responses. Likewise, feeding with one of the raw isogenic cowpeas induced low anti-AAI-specific IgE antibody titers with some variation in individual titers, which were lost after heat-treatment. Intra-gastric exposure to AAI cowpea failed to elicit detectable IgE titers and lower levels of IgG1.
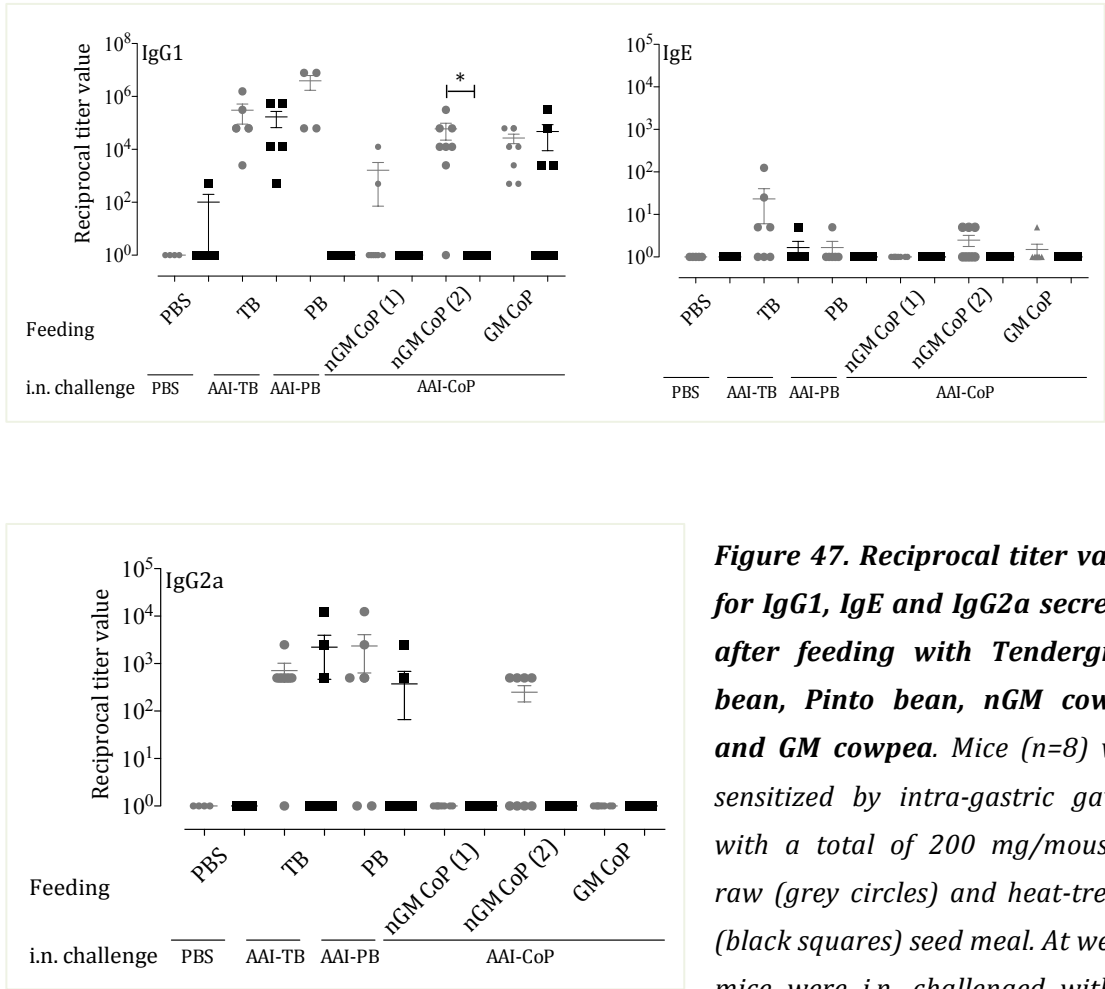
Figure 47. Reciprocal titer values for IgG1, IgE and IgG2a secretion after feeding with Tendergreen bean, Pinto bean, nGM cowpea and GM cowpea. Mice (n=8) were sensitized by intra-gastric gavage with a total of 200 mg/mouse of raw (grey circles) and heat-treated (black squares) seed meal. At week 5, mice were i.n. challenged with 50 µg/50 µl of the corresponding purified AAIs. Seventy-two hours after the last i.n. challenge the antibody titers were measured by sandwich ELISA. The titers are expressed as the reciprocal value of the lower dilution where the last O.D. readings were still detected. Control mice were fed PBS Values are expressed as mean titer ± SEM Statistical differences between heat-treated and raw groups were determined using Student t test.

3.4   Investigation of the adjuvant effect of feeding GM corn (MON810) on the initiation of OVA-induced allergic disease in BALB/c female mice

In parallel experiments, we analyzed whether *ad libitum* feeding of MON810 would influence the allergenicity to a non-cross-reactive allergen. We induced allergic asthma by injecting female BALB/c mice on days 0 and 21 with OVA in PBS and nebulized them with OVA on 2 consecutive days and the mice developed acute allergic asthma (acute onset). To examine the influence of MON810 feeding on the initiation disease phase of OVA-induced allergic asthma, we fed mice pellets containing 33% GM- or isogenic-corn vs. normal mouse corn-free mouse food twice a week for 4 weeks before inducing initial disease as illustrated in Figure 48. Before administration to the mice MON810 maize was analyzed for the presence of Cry1Ab, and PCR analysis confirmed the presence of a 211 bp fragment in the GM diet (Figure 48).
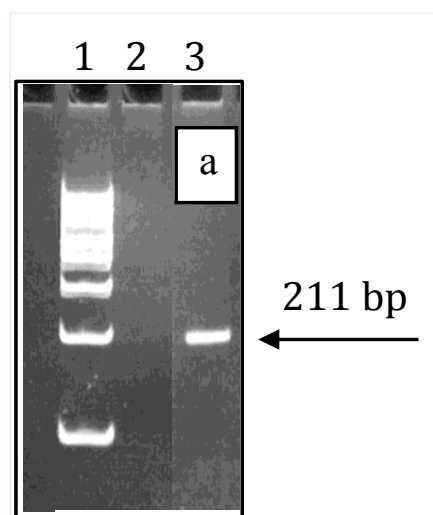
*Figure 48. Confirmation for the presence of Cry1Ab gene in MON 810 maize. Purified genomic DNA from MON810 maize was used in PCR assay designed to amplify a 211-bp region of the cry1Ab coding region. **Lane 1** molecule standard; **lane 2** negative control (isogenic maize); **lane 3** Mon810 DNA (courtesy of Dr. Éva Gelencsér, Central Food Research Institute – Budapest, Hungary)*
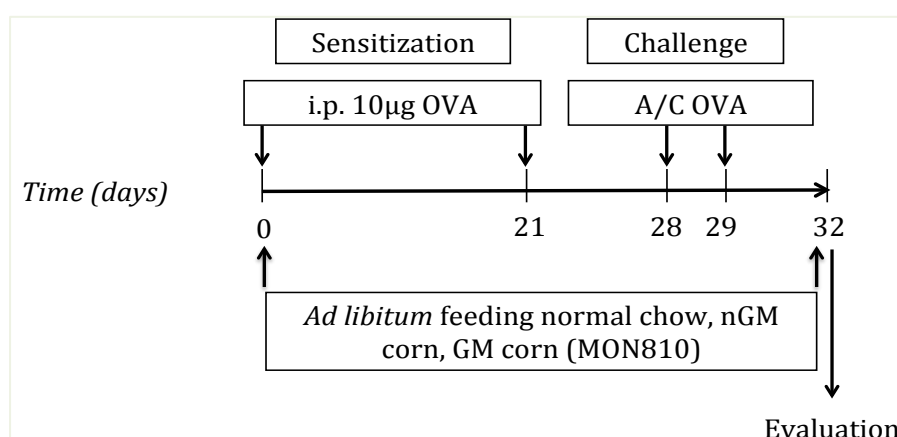
***Figure 49. Experimental protocol to investigate whether ad libitum feeding with GM corn would influence the allergenicity to OVA**. BALB/c female mice were fed pellets containing 33% isogenic or transgenic corn inclusion. As additional control mice were fed corn free diet. On days 0 and 21 mice were sensitized i.p. with 10 µg of OVA. To simulate an acute asthma attack mice were challenged with 1% OVA solution on days 28 and 29. Seventy-two hours after the last challenge we evaluated parameters characteristic of airway and lung allergic disease.*

Table 20. Experimental details for the initiation experiments

Groups	Corn inclusion in diet	OVA immunization	Mice number/group	Repeats
MON810	33%	+	8	2
Isogenic corn	33%	+	8	2
Mouse chow	0%	+	8	2
Naïve	0%	-	6	2

Mice were fed three different diets on the initiation phase of OVA-induced-allergic asthma. Age matched naïve mice were not sensitized and challenged with OVA and their diet remained free from corn.

3.4.1 CLINICAL OBSERVATION AND BODY WEIGHTS

Organ weights were recorded as potential indicator of a dietary effect on the organism. Liver and kidneys are central metabolic organs and are important for metabolic and excretory processes and are therefore often regarded as indicator organs for toxic effects. Liver and kidneys are central metabolic organs and are

important for metabolic and excretory processes and are therefore often regarded as indicator organs for toxic effects. Our results indicate that there were no significant differences in the body, spleen, kidney or liver weight between GM maize fed groups and nGM fed corn controls (Table 21)

Table 21. Body weight of BALB/c female mice force-feed corn free chow, GM and isogenic corn.

Relative weights	MON810	Isogenic corn	Mouse chow	Naive
Body	21.19±1.59	21.93±0.48	24.05±1.51	22.82±1.07
One-Way ANOVA			** vs. MON810	
Spleen	0.86±0.23	0.66±0.11	0.47±0.07	0.57±0.09
One-Way ANOVA	** vs. mouse chow			
Liver	5.67±1.28	4.53±0.43	4.33±0.27	4.23±044
One-Way ANOVA	*vs. mouse chow			
Kidney	1.33±0.24	1.38±0.15	1.16±0.05	1.41±0.06
One-Way ANOVA		* vs. mouse chow		

Values are expressed as mean weight ± SD (n=8). Statistical significance of difference between the groups was determined using Kruskal-Wallis test.

Mice fed corn-free chow revealed better feed uptake that led to a significant increase in body weight compared to the mice fed the corn diets. In contrast, the relative spleen, liver and kidney weights were increased in animals fed both corn diets compared to mice fed corn free pellets.

3.4.2 ANALYSIS OF THE ADJUVANT EFFECT OF *AD LIBITUM* FEEDING OF THERMAL PROCESSED CORN PELLETS ON THE ANTIBODY RESPONSE

To determine the antibody response to feeding, sera were taken 48 hours after the last OVA-aerosol challenge. In disease initiation, feeding corn free chow induces slightly higher levels of IgG1, IgE and IgG2a antibodies, compared to other groups. Interestingly, feeding GM corn did not increase antibody titers, compared to isogenic corn-fed groups. Our results suggest that OVA-specific IgG1, IgG2a and IgE antibodies were unaffected regardless whether mice were fed GM or non-GM diets.
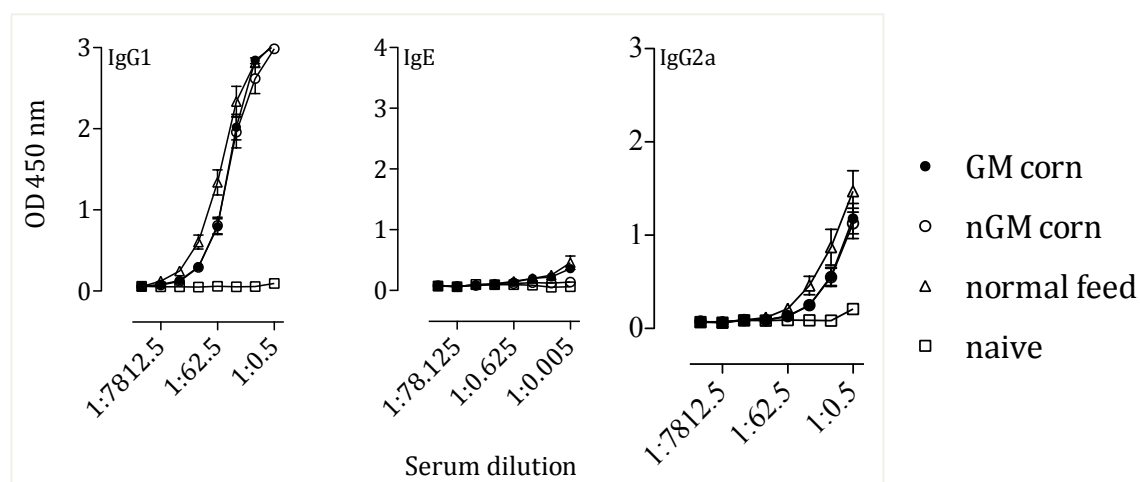
Figure 50. Evaluation of anti-OVA IgG1, IgE (Th2 phenotype) and IgG2a (Th1 phenotype) in individual serum with sandwich ELISA*. OVA-specific antibodies were determined in OVA-induced asthma mice fed MON810, isogenic corn and corn free mouse chow. Age matched control mice were kept naïve and fed corn free pellets.*

3.4.3 ANALYSIS OF THE ADJUVANT EFFECT OF *AD LIBITUM* FEEDING OF THERMAL PROCESSED CORN PELLETS ON THE AIRWAY AND LUNG EOSINOPHILIC INFLAMMATION

As expected, naïve mice did not develope either airway or lung inflammation. At the time of acute onset control mice fed corn-free chow and GM corn fed mice had similar numbers of airway eosinophils (22209 ± 6791 vs. 24558 ± 5501). In contrast, feeding isogenic corn induced slightly, but not significant increase in BAL eosinophilia (35598 ± 13103). To investigate the effects of GM corn feeding upon lung inflammation, lungs were removed and processed 72h after the last aerosol challenge and stained specifically for eosinophils with LUNA. Eosinophils were absent in lung tissue sections of naïve mice. All groups sensitized and challenged with OVA had significant increase in eosinophil numbers in mice consuming *Bt*- and isogenic corn having higher numbers of inflammatory cells compared to corn-free fed animals. These results were similar to those obtained in BAL.
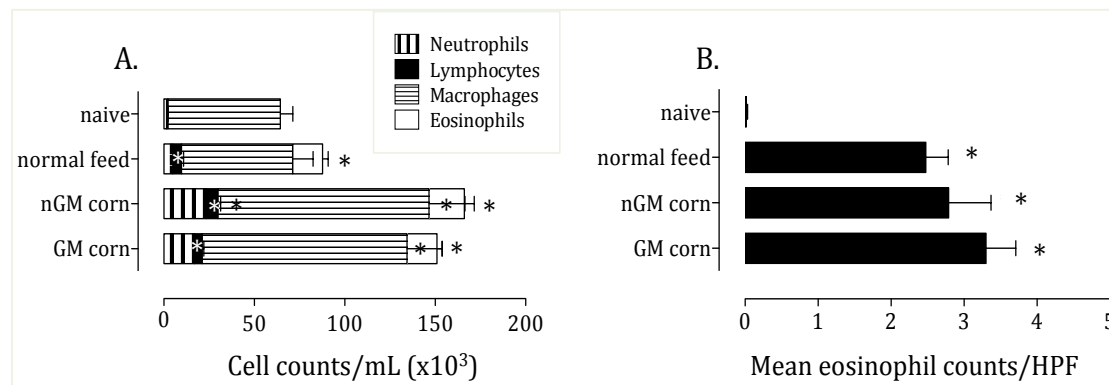
Figure 51. Effect of ad libitum feeding mice with MON810 (GM corn) or the isogenic control maize for 30 days on total BAL cell numbers. Mice (n=8) were fed ad libitum pellets containing 33% GM- or isogenic corn for 4 weeks during the initiation of an acute asthma attack. Data represent absolute cell counts of airway cells (A) and eosinophil counts within lung infiltrates (B) and reported as means ± SEM from 8 mice per group. Statistical differences were determined using Kruskal-Wallis test followed by Dunn's multiple comparison test, $^*p < 0.05$ vs. naïve. Absolute airway cell counts were determined by morphological examination of at least 300 cells in cyto-centrifuged preparations. The numbers of eosinophils in lung tissue were determined by counting 10 high power fields (40x) randomly throughout the central and peripheral lungs.

Table 22: Effect of transgenic and non-transgenic maize consumption on different cell population in BAL

Cell counts / Statistics	MON810	Isogenic corn	Mouse chow	Naïve
Eosinophils	24558 ± 5501**	35598 ± 13103**	22209 ± 6791*	80.08 ± 30.55
Lymphocytes	7762 ± 1739	11682 ± 2377**	8272 ± 2421	1226 ± 296.1
Macrophages	198720 ± 32740*	210587 ± 30847*	98938 ± 18392	70274 ± 12209
Neutrophils	31461 ± 7701**	46508 ± 6401*	8937 ± 2529***	1754 ± 935.8
Total cell counts	262500 ± 44631*	297500 ± 48135*	155000 ± 44280	155000 ± 44280

Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean cell percentages ± SEM (n=8). One-way ANOVA, $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ indicates statistical significance compared with naïve mice.

3.4.4 ANALYSIS OF THE ADJUVANT EFFECT OF *AD LIBITUM* FEEDING OF THERMAL
       PROCESSED CORN PELLETS ON THE OVERALL INFLAMMATION AND MUCUS
       HYPERSECRETION

To investigate the effects of GM corn feeding upon lung inflammation and mucus hypesecretion, lungs were removed 72h after the last aerosol challenge and stained specifically for cell infiltrates (H&E) and muco-polysaccarides secreted by the goblet cells (PAS). Subsequently the lung sections were evaluated and graded for disease severity as described in Material and Methods. The grades of inflammation and mucus production and counts of eosinophils in the lung tissue are recorded in Table 23. Tissue sections from all mice sensitized and challenged with OVA showed significant increase in both infitrates and mucus hypersecretion compared with the control naïve mice (Figures 52 and 53). However, no difference was detected between the groups fed GM, nGM and mouse chow in either parameter.

Table 23: OVA-induced overall inflammation in lung sections of mice fed MON810, isogenic corn and corn free mouse chow.

	MON810	Isogenic corn	Mouse chow	Naive
Grade of cell infiltrates	1.87±0.79***	2.18±0.37***	2.64±0.47***	0.0±0.0
Grade of mucus production	2.6±0.91***	2.8±0.94***	2.89±0.62***	0.1±0
Counts of eosinophils	3.3±0.40***	2.78±0.58*	2.47±0.30*	0.016±0.016

*Values are expressed as grade of cell infiltrates ± SD, mucus production ± SD and mean eosinophil counts ± SEM. Statistical significance of difference between the groups was determined using Wilcoxon test for the first two parameters, and Kruskal-Wallis test for the last parameter. *p<0.05, *** p<0.001 indicate statistical significance compared with naïve group.*
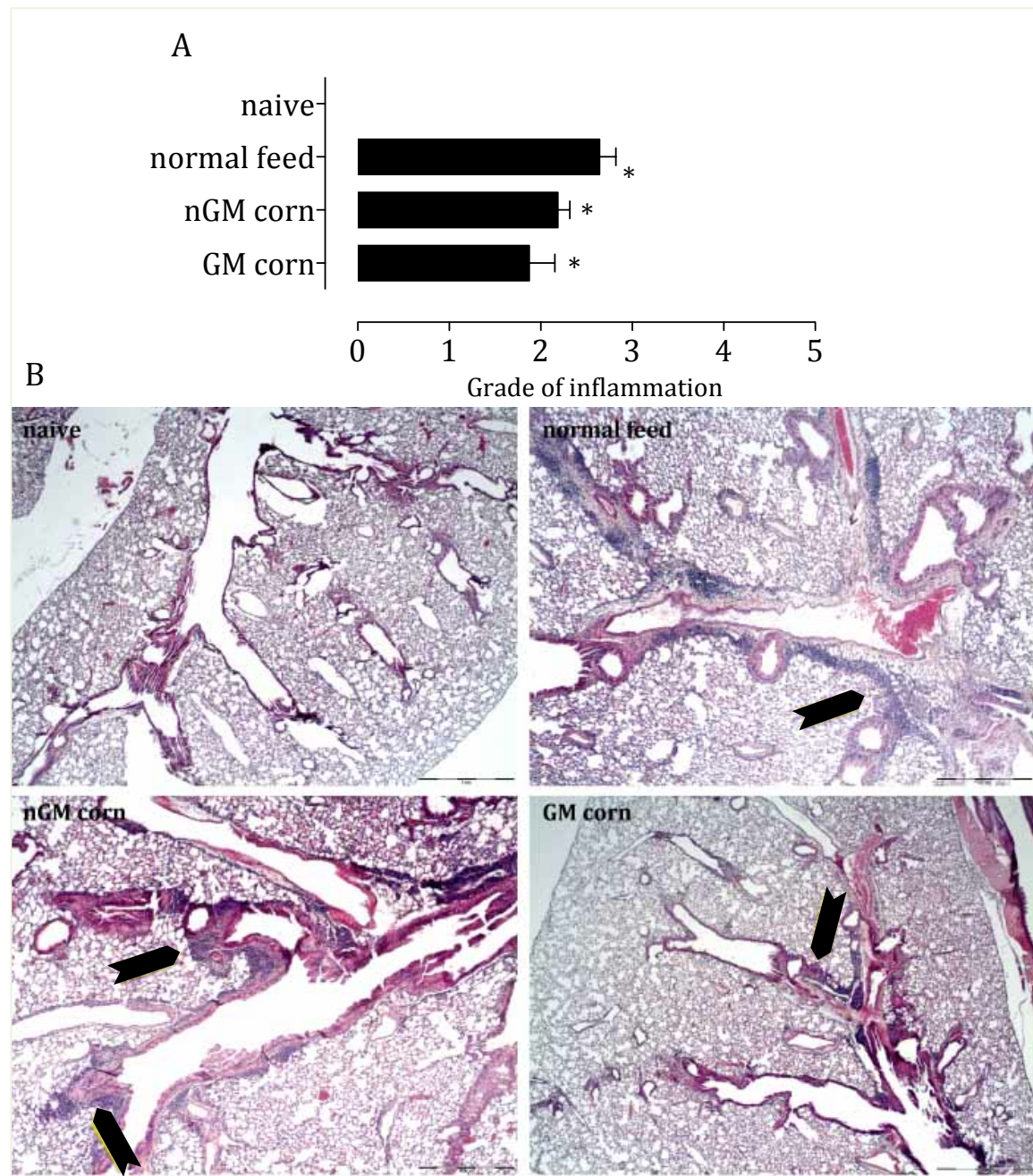
Figure 52. OVA-induced lung inflammation. (A) Grade of cell infiltrates in lung parenchyma of mice in the acute phase of allergic asthma and fed 33% GM- or isogenic corn. Data are presented as the grade of cell infiltrates ± SD; n=8. (B) Representative photomicrographs of lungs stained with H&E. Statistical differences were determined using Wilcoxon test. Control mice were fed corn-free pellets. Representative photomicrographs of lung sections stained with H&E. Original magnification x 20. Black arrows indicate the cell infiltrates.
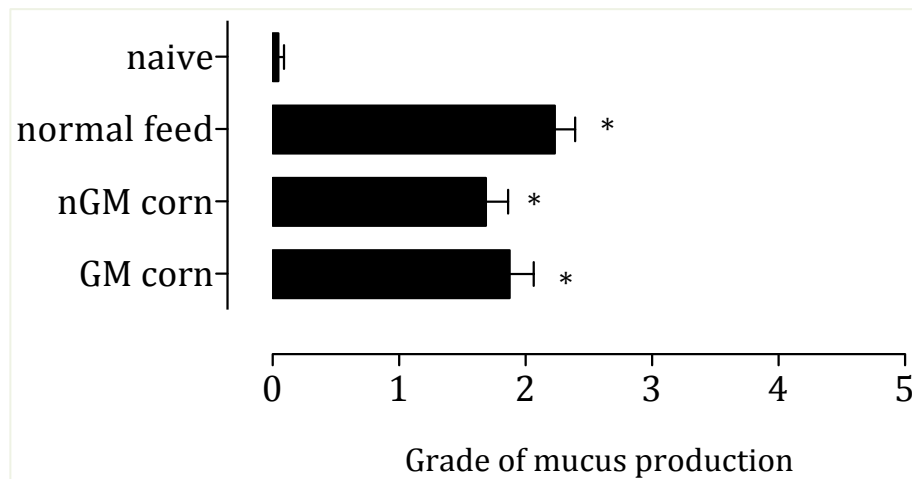
Figure 53. Mucus hypersecretion in lung parenchyma of mice fed GM- or isogenic corn.
Data are presented as the grade of mucus production ± SD from n=8 mice/group. Statistical differences were determined using Wilcoxon test.

3.5 Investigation of the adjuvant effect of feeding GM corn (MON810) on the exaceration of OVA-induced allergic disease in BALB/c female mice

We next investigated the adjuvant effect of feeding of pellets containing 33% MON810 inclusion on the exacerbation of OVA-induced disease. We induced allergic asthma by injecting female BALB/c mice on days 0 and 21 with OVA in PBS and nebulized them with OVA on 2 consecutive days. Mice developed acute allergic asthma (acute onset) and were then allowed to recover for 1 month before re-exposing them to nebulized OVA to induce a disease exacerbation (relapse). To examine the influence of MON810 feeding on the relapse phase of OVA-induced allergic asthma, we fed mice with pellets containing 33% GM or isogenic-corn vs. normal mouse corn-free mouse food twice a week for 4 weeks before inducing exacerbation (Figure 54).
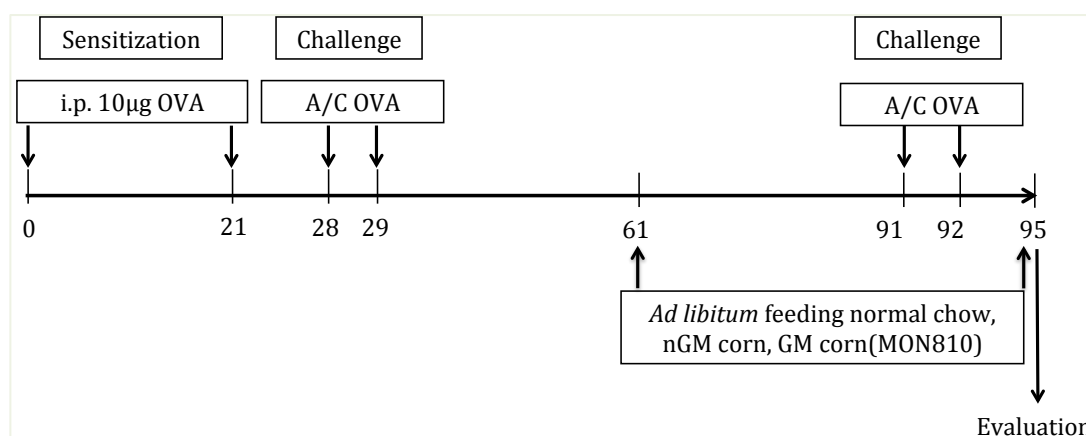
Figure 54. Experimental protocol to investigate whether ad libitum feeding with GM corn would influence the allergenicity to non-crossreactive allergen. BALB/c female mice were fed pellets containing 33% isogenic or transgenic corn inclusion. As an additional control, mice were fed corn free pellets. On days 0 and 21 mice were sensitized i.p. with 10 µg of OVA. To simulate an acute asthma attack mice were challenged with 1% OVA solution on days 28 and 29. Mice developed acute allergic asthma and were then allowed to recover for 3 months before re-exposing them to nebulized OVA, on days 91 and 92 to induce a disease exacerbation (relapse). Seventy-two hours after the last challenge we evaluated parameters characteristic of airway and lung allergic disease.

Table 24. Experimental details for the relapse experiments

Groups	Corn inclusion in diet	OVA immunization	Mice number/group	Repeats
MON810	33%	+	8	2
Isogenic corn	33%	+	8	2
Mouse chow	0%	+	8	2
Naïve	0%	-	6	2

Mice were fed different diets before inducing disease exacerbation to OVA. Age matched naïve mice were not sensitized and challenged with OVA and their diet was free from corn.

3.5.1 CLINICAL OBSERVATION AND BODY WEIGHTS

Organ weights were recorded as potential indicator of a dietary effect on the organism. Our results indicate that there were no significant differences in the body, spleen, kidney or liver weight between GM maize fed groups and nGM fed corn controls. However, induction of OVA induced disease relapse lead to a significant increase in liver weights compared to naïve mice as illustrated in Table 25. No further differences were detected in organ weights between the groups.

Table 25. Body weight of BALB/c female mice force-feed corn free chow, GM and isogenic corn.

Relative weights	MON810	Isogenic corn	Mouse chow	Naïve
Body	24±1.24	23.61±0.42	24.51±0.91	24.31±0.93
One-Way ANOVA				
Spleen	0.67±0.14	0.62±0.12	0.79±0.10	0.67±0.11
One-Way ANOVA				
Liver	4.41±0.26	4.36±0.12	4.4±0.2	3.87±0.18
One-Way ANOVA	* vs. naive	* vs. naive	* vs. naive	
Kidney	1.20±0.13	1.20±0.15	1.02±0.19	1.25±0.08
One-Way ANOVA				

Values are expressed as mean weight ± SD (n=8). Statistical significance of difference between the groups was determined using Kruskal-Wallis test.

3.5.2 ANALYSIS OF THE ADJUVANT EFFECT OF *AD LIBITUM* FEEDING OF THERMAL PROCESSED CORN PELLETS ON THE ANTIBODY RESPONSE

To determine whether feeding MON810 would increase the antibodies levels to OVA allergen in the exacerbation phase of the disease, we collected sera 72 h after the last OVA aerosol challenge and levels of Th2 and Th1 antibodies were measured. Measurements of OVA-specific IgG1, IgE and IgG2a revealed that, similar with the results obtained in the initiation experiments feeding MON810 did not increase the levels of OVA specific antibodies (Figure 55).
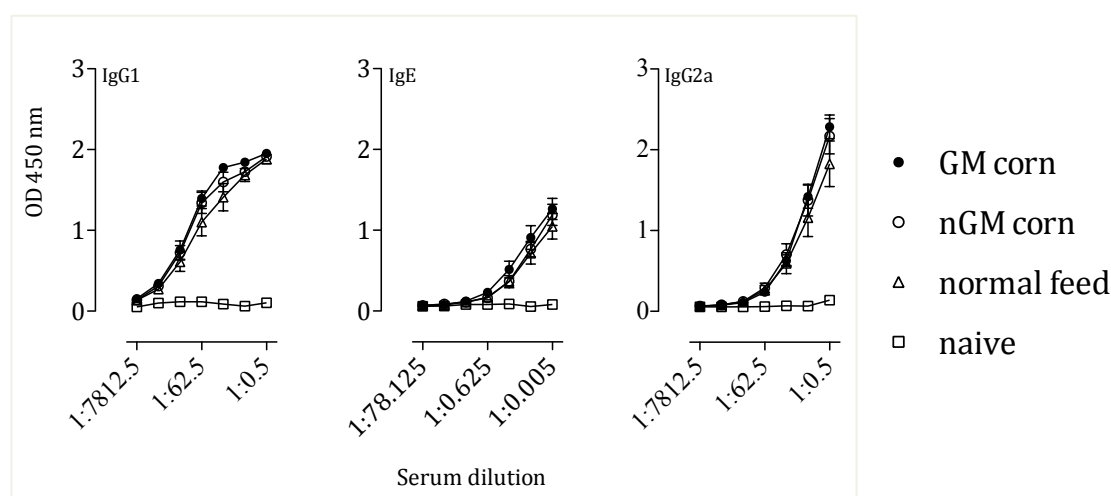
***Figure 55. Evaluation of anti-OVA IgG1, IgE (Th2 phenotype) and IgG2a (Th1 phenotype) in individual serum with sandwich ELISA**. OVA specific antibodies were determined in animals with established allergic asthma and fed MON810, isogenic corn and corn free mouse chow. Age matched control mice were kept naïve and fed corn free pellets.*

3.5.3 ANALYSIS OF THE ADJUVANT EFFECT OF *AD LIBITUM* FEEDING OF THERMAL PROCESSED CORN PELLETS ON THE EOSINOPHILIC LUNG INFLAMMATION

To assess whether the MON810 maize consumption would have immunological consequences, we performed the phenotypic analysis of BAL fluid isolated from mice fed GM maize and the isogenic control line. To exclude any other influence than that caused by the Cry1Ab toxin (corn effect in mice diet), we also analyzed the cellular content in airway inflammation of mice fed a standard pellet diet without corn

inclusion. As expected, in naïve mice, fed corn-free pellets, the total cell numbers were 1.6-fold lower when compared to mice with OVA-induced relapse disease fed the same diet. At disease relapse, mice fed GM corn had similar numbers of total airway cells as the mice fed nGM corn (405625 ± 47701 vs. 409375 ± 64562). Likewise, no differences in airway and lung eosinophilia, between mice fed MON810 and isogenic control line were detected. Although airway eosinophilia of mice fed both GM and nGM corn pellets was 3-fold higher compared to the mice fed corn-free chow, this difference was not detected in the eosinophils counts in lung. In addition, the numbers of lymphocyte, macrophages and neutrophils were also higher in mice fed GM and nGM corn diets (Figure 56 and Table 26) indicating a corn effect rather then a GM corn effect on the airway inflammatory cell population.
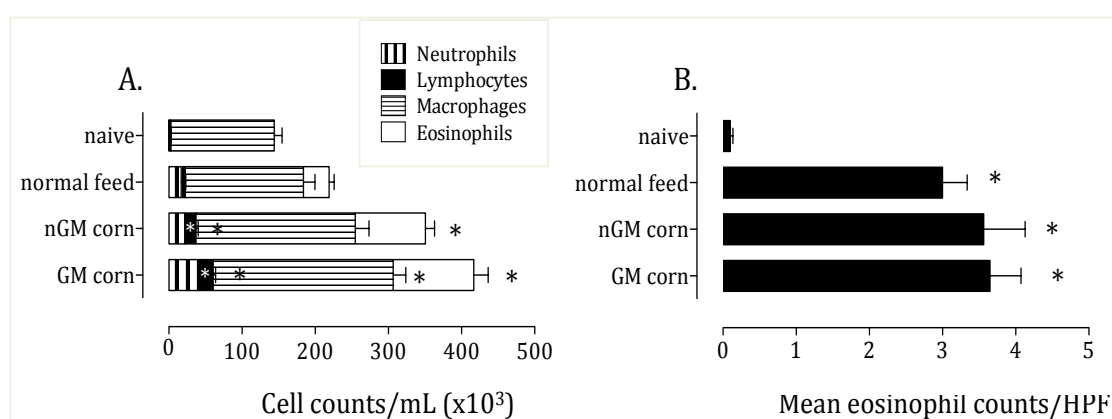
Figure 56. Effect of the transgenic and nontransgenic maize consumption on different cell population in the airway. Mice (n=8) were fed ad libitum pellets containing 33% GM- or isogenic corn for 4 weeks during the relapse phase of asthma disease. Data represent absolute cell counts of airway cells (A) and eosinophil counts within lung infiltrates (B) and reported as means ± SEM from 8 mice per group. Statistical differences were determined using Kruskal-Wallis test followed by Dunn's multiple comparison test, *p < 0.05 vs. naïve. Absolute airway cell counts were determined by morphological examination of at least 300 cells in cyto-centrifuged preparations. The numbers of eosinophils in lung tissue were determined by counting 10 high power fields (40x) randomly throughout the central and peripheral lungs.

Table 26: Effect of the transgenic and non-transgenic maize consumption on different cell population in BAL

	MON810	Isogenic corn	Mouse chow	Naive
E	82760 ± 16497**	96336 ± 21693**	29848 ± 13216	172.3 ± 133.2
L	18094 ± 3941**	18094 ± 3941**	7199 ± 1524	1267 ± 283.3
M	279675 ± 27424**	251137 ± 38078	157567 ± 28223	141401 ± 14652
N	25097 ± 5348****	17288 ± 5028**	9671 ± 3592	493.8 ± 161
Total	405625 ± 47701	409375 ± 64562	220000 ± 67447	136667± 17401

Statistical differences were determined using Kruskal-Wallis test. Values are expressed as mean cell percentages ± SEM (n=8). *p<0.05, **p<0.01 and ***p<0.001 indicates statistical differences compared mice fed PBS.

3.5.4 ANALYSIS OF THE ADJUVANT EFFECT OF *AD LIBITUM* FEEDING OF THERMAL PROCESSED CORN PELLETS ON THE OVERALL INFLAMMATION AND MUCUS HYPERSECRETION

Histological analysis of lung tissue stained with PAS and H&E (Figure 57) revealed a significant increase in airway inflammation and mucus production in mice fed both corn and corn free diets when compared to the naive control group (Table 27). However, no differences in either parameter were detected between GM and nGM corn-fed groups.
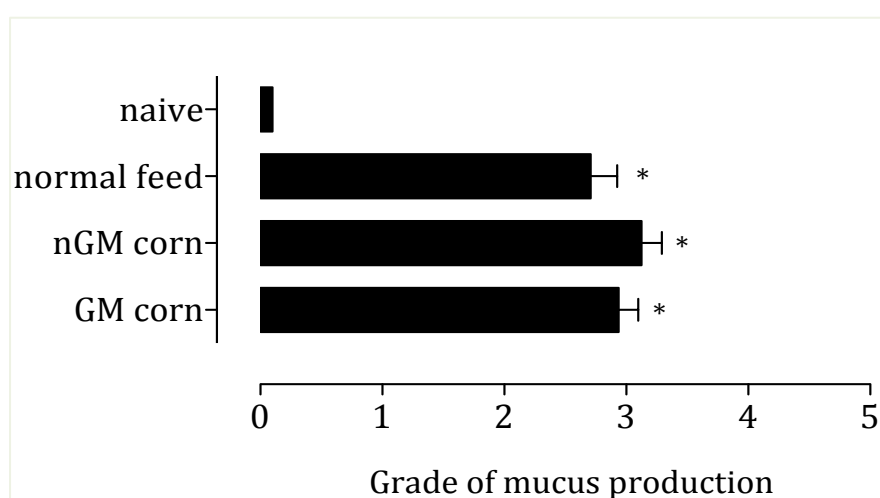
Figure 57. Mucus production in lung parenchyma of mice fed ad libitum pellets containing 33% GM- or isogenic corn for 4 weeks during the relapse phase of asthma disease. Data are presented as the grade of cell infiltrates ± SD from n=8 mice/group. Statistical differences were determined using Wilcoxon test. Control mice were fed corn free pellets.

Table 27: OVA-induced overall inflammation in lungs of mice fed MON810, isogenic corn and corn free mouse chow.

	MON810	Isogenic corn	Mouse chow	Naive
Grade of cell infiltrates	1.56±0.56	2.28±0.36	2.0±0.88	0.2±0.2
Wilcoxon test		***vs. naive	* vs. naive	
Grade of mucus production	2.93±0.64	3.12±0.67	2.70±0.75	0.1±0
Wilcoxon test	*** vs. naive	*** vs. naive	**vs. naive	
Counts of eosinophils	1.87±0.76	1.68±0.70	2.23±0.62	0.04±0.15
Kruskal-Wallis	*** vs. naive	*** vs. naive	*** vs. naive	

Values are expressed as grade of cell infiltrates ± SD, mucus production ± SD and mean eosinophil counts ± SEM. Statistical significance of difference between the groups was determined using Wilcoxon test for the first two parameters, and Kruskal-Wallis test for the last parameter. *p<0.05, *** p<0.001 indicates statistical significance increase compared with naïve group.
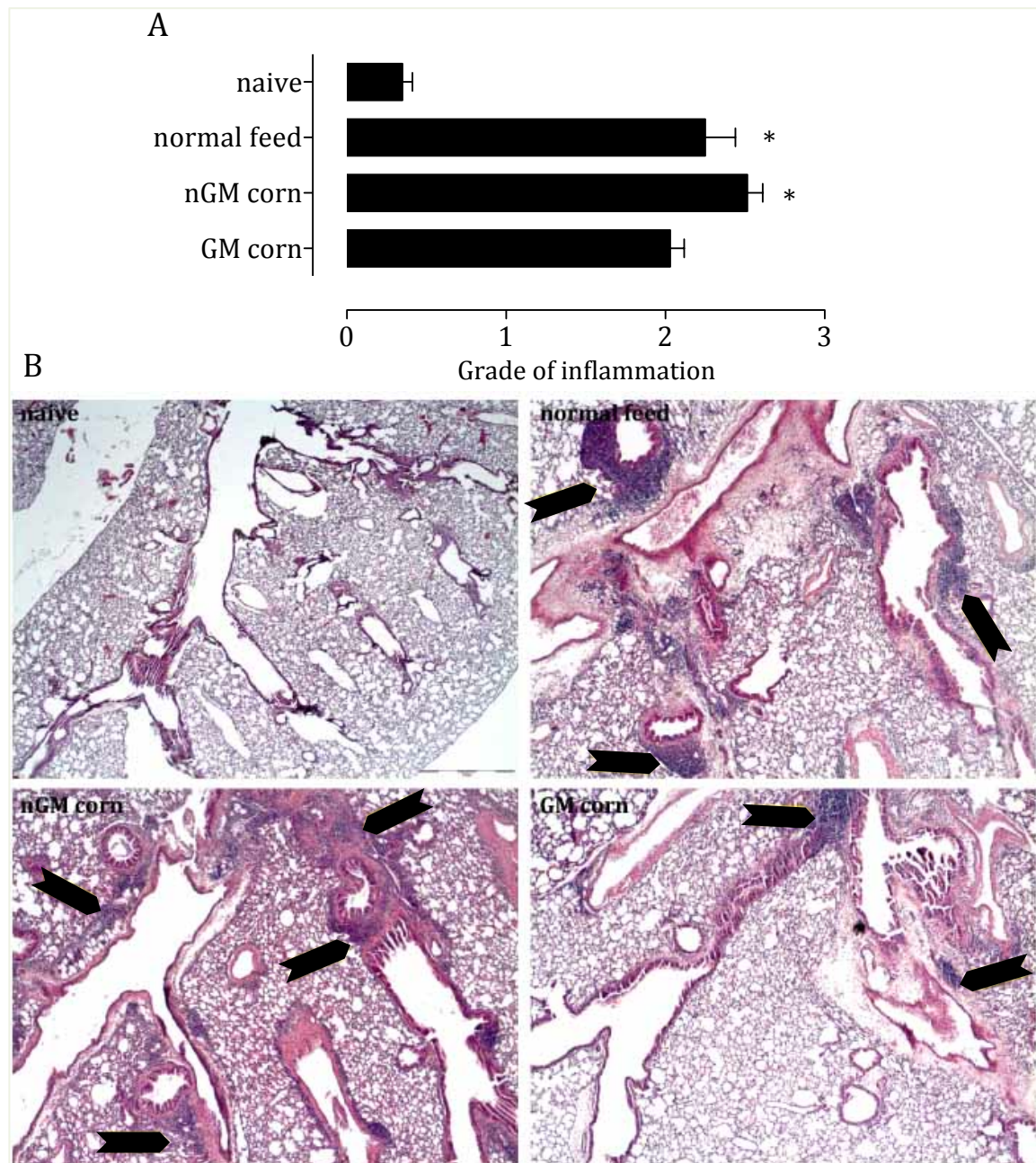
Figure 58. Severity of OVA-induced lung inflammation in BALB/c mice. *(A) Grade of cell infiltrates in lung parenchyma of mice fed ad libitum pellets containing 33% GM- or isogenic corn for 4 weeks during the relapse phase of asthma disease. Data are presented as the grade of cell infiltrates ± SD from n=8 mice/group. (B) Representative photomicrographs of lungs stained with H&E. Statistical differences were determined using Wilcoxon test. Control mice were fed corn free pellets. Representative photomicrograph of lung sections stained with H&E. Original magnification x 20. Black arrows indicate the cell infiltrates.*

The digestive fates of the transgenic DNA raised questions regarding to human and animal consumption. For this purpose, total DNA was extracted from quadriceps muscle from mice fed twice a week for 4 weeks a diet including either GM corn, nGM corn or PBS. DNA preparation were analysed by PCR followed by Southern blot hybridization for the presence of a 211-bp fragment of the *Bacillus thuringiensis* Cry1Ab gene. Using PCR method fragments of transgenic and endogenous plant DNA were not detected in the quadriceps muscle sample from animals fed MON810. By using 1μg of input DNA per reaction none of the extracted samples was positive for cry1Ab (Figure 59).
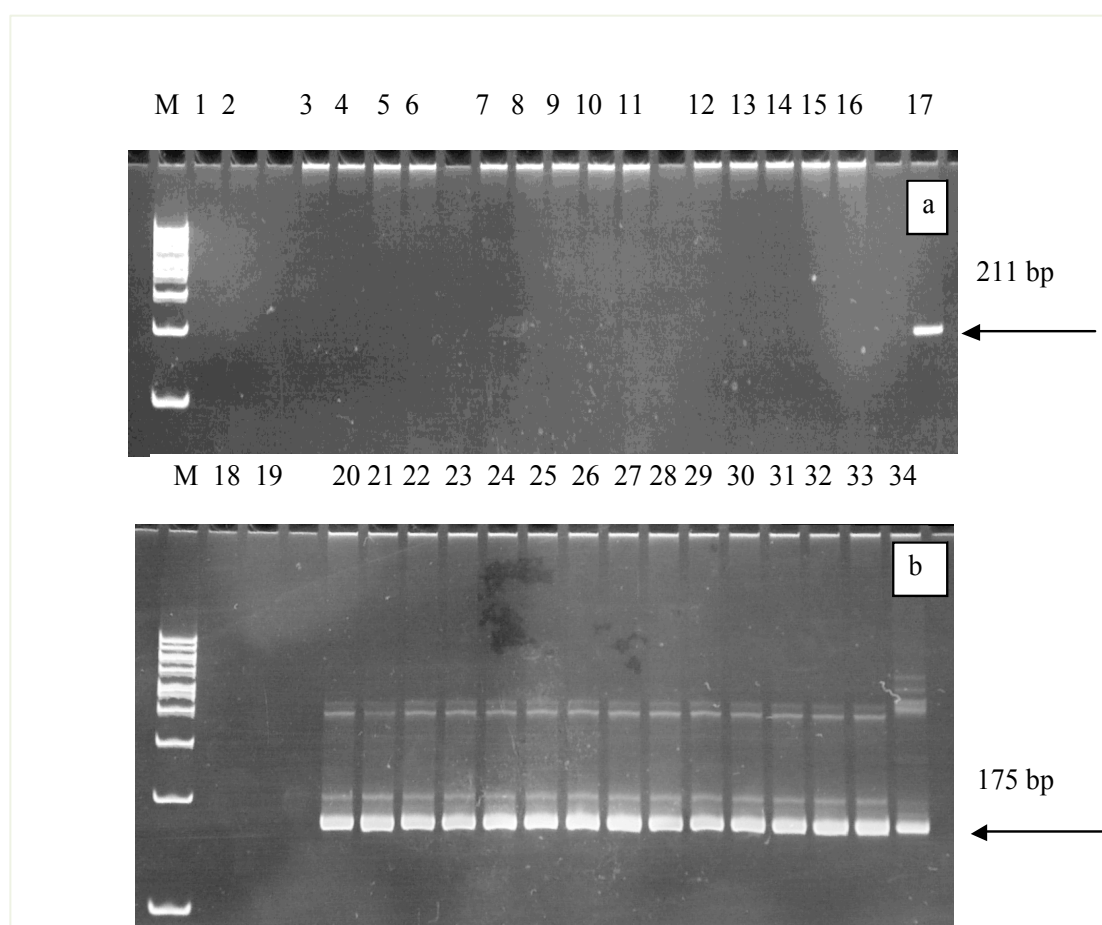
Figure 59. PCR products of genomic DNA from mouse muscle samples for the Cry1Ab gene fragment*. Purified genomic DNA from MON810 maize was used in PCR assay designed to amplify a 211-bp region of the cry1Ab coding region. Lane M molecule standard; lanes 1,18 negative control (without DNA); lanes 2,19 DNA isolation control; lanes 3-6, 20-23 muscle samples of mice fed PBS; lanes 7-11, 24-28 muscle samples of mice fed isogenic corn; lanes 12-16, 29-33 muscle samples of mice fed GM corn, lane 17 MON810 DNA, lane 34 pig DNA.*

4   DISCUSSION

In this study, we evaluated the potential allergenicity of two GM legumes and the adjuvanticity of a already commercialzed GM (Bt-maize), in an allergic asthma mouse model. Firstly, we observed two important features characterising GMO chickpeas and cowpeas. On one hand, transgenic AAIs expressed in GMO chickpeas and cowpeas are as immunogenic and allergenic as native bean AAI and on the other hand, both GMO chickpea and cowpea consumption induced an allergic response to AAI that was similar to Tendergreen bean. Secondly, in short term feeding experiments testing adjuvanticity of Bt-maize (MON810), we observed no influence on either disease onset or exacerbation of allergic asthma.

4.1.1   DIFFERENCES BETWEEN ANTIBODY TITRES WHEN IMMUNIZING WITH DIFFERENT AAIS I.P.

Field peas, cowpeas and chickpeas were genetically modified with AAI gene from the Tendergreen bean and are therefore protected against weevils [65], [67]. The use of this gene was attractive for food crops because allergies to bean in humans are rare. In addition, it has been shown that the native AAI in beans undergoes compex posttranslational modifications [76]. Characterization of native and transgenic AAIs expressed in chickpeas and cowpeas by structural analysis revealed that, transgenic β-chains in both crops displayed less overall glycosylation and significant C-terminal truncations thus, differing from Tendergreen bean AAI [73]. In a previous study, Prescott et. al., suggested that, genetically modified field peas, receiving AAI gene from the Tendergreen bean, have a higher immunogenic potential [50]. In our study, using the intraperitoneal route of administration, we investigated and compared the immune response to transgenic AAIs extracted from GMO chickpea and GMO cowpea and the native AAI from Tendergreen and Pinto bean. This route of administration was successfully used previously to establish differences between allergens and non-allergens in previous studies [42, 93]. When we evaluated the results of the i.p. immunization with AAIs, we found that differences in posttranslational modifications alters imunogenicity. Transgenic but also native AAIs stimulated the production of a mixed Th2/Th1 response with low IgE, high IgG1 and IgG2a. To our surprise, i.p. immunization with AAI extracted from transgenic chickpea induced

high IgE titers. However, these results were not observed when mice were immunized with the transgenic cowpea AAI, where IgE titers remained low. Moreover, immunization with native bean AAI also induced an IgE response. In summary, we found no correlation between different glycosylation patterns and the immunogenicity of the AAIs. Furthermore, the transgenic expression of AAI in chickpea and cowpea did not led to an increase in immunogenicity as the native AAI lead to a higher immune response in mice.

4.1.2 Differences between antibody titres when immunizing with different AAIs i.n.

We further investigated the potential differences between native and transgenic AAIs in their ability to initiate an allergic immune response. We treated the mice with repeated i.n. challenges with various AAIs derived from GM and native legumes, and examined the lungs for evidence of allergic inflammation and the serum for antigen-specific Ab. Similar to the response induced by intraperitoneal route, upon i.n. challenge all AAIs were able to induce a mixed Th2/Th1 response. This response was characterized by a strong production of specific IgG1 and IgG2a antibodies and low levels of IgE. However, our results revealed that antibody levels subsequent to i.n. administration of transgenic and native AAIs, are much higher than those generated via i.p. route. These differences are probably due to a higher dose of AAIs used in for the i.n. compared to the i.p. protocol. Comparisons between the immune responses to transgenic and non-transgenic AAIs using different routes of antigen exposure led to divergent IgE responses. Even though the amount of AAI isolated from GM chickpea was 15 times higher when delivered i.n. compared to i.p., local administration of this protein did not induce antigen-specific IgE. Thus, it is important to consider that the variation in glycosylation that occurs in AAIs and the administration route may be associated with changes in allergenicity. However, our results show that transgenic expression of AAI in GMO chickpea and GM cowpea is not specifically associated with increased in allergenicity.

4.1.3 MICE DEVELOP IMMUNE RESPONSE TO THE GM PROTEIN WHEN FED BEANS AND TRANSGENIC SEED MEALS

Potential increase in the allergenicity of GM plants may occur due to possible unintended effects during the transformation process. Feeding experiments revealed that intra-gastric administration of both GMs and native seed meals primed for AAI-induced eosinophilia in airways and lungs. The oral route of administration of potential allergenic foods is largely considered to be the most appropriate route of exposure when developing models for testing food allergy. This route of administration has been previously evaluated as a method for assessing the induction of allergic responses to dietary proteins administered orally, using CT for peanut [94, 99], cow´s milk [99], shrimp [100], and soy antigens [101] as well as without adjuvant for cashew nut [102], milk whey protein [103] and hazelnut [104]. Our results revealed that feeding raw GM chickpeas and GM cowpeas induced the development of allergic airway disease upon i.n. challenge with purified AAI protein. However, this response was lower when compared with their isogenic counterparts. Moreover the response varies within the two isogenic cowpeas varieties probably due to natural variation. Interestingly, feeding Pinto bean and Tendergreen bean also resulted in secretion of Th2 antibodies, eosinophil recruitment in BAL and lungs, and cell infiltrates. Notably, feeding native beans led to an antibody response to AAI, which was more robust than those induced by feeding GM legumes. Prescott et al. [50] and Smart et. al [111] using a similar feeding protocol with subsequent i.n. protein challenge, reported that Pinto bean did not induce in mice any Th2-associated inflammation and these results are in contradiction with our observations. Considering that the feeding protocols, dose and materials that we used in our experiments were similar with those used in this earlier study, we can not explain the discrepancy in the results [50]. We speculate that one of the main factors responsible for these these differences between results might be the composition of the daily diets that the mice consumed in Australia versus Austria. Thus, in our study, feeding both GM legumes and native beans induces allergic response to AAI protein, and the consumtion of transgenic varieties did not enhanced the immune response.

Previously, it has been also shown that SSA-lupin, a GM crop, promotes a Th1 type response with high IgG2a antibody production [111] and authors speculate that this

particular GM plant could supress allergic inflammation. In our study, we could not detect any increase in IgG2a antibody production upon feeding transgenic meals to the mice. Herein we tested 2 GM varieties, chickpeas and cowpeas.However, we were unable to distinguish allergenic from non-allergenic food extracts using the oral route of exposure, with the mice developing an immune response to all meals regardless whether isogenic or transgenic.

4.1.4 FEEDING BEANS AND ISOGENIC SEED MEALS INDUCE FUNCTIONAL IGE ABS

Unexpectedly, native Tendergreen and Pinto beans feeding induced a higher immune response when compared with the response generated to the GM chickpeas and GM cowpeas. Moreover, feeding native beans and isogenic legumes generated functional IgE antibodies. These results may be partially explained by the presence of high PHA content in beans [109]. In a 28 and 90 day feeding trial, PHA-E lectin was found to induce immunomodulating effects with increase in IgA levels and mesenteric lymph node weight in Wistar rats [31]. Another study [110] showed that 16 common lectins, particularly PHA and ConA were able to induce human basophils to secrete IL-4 and IL-13, the key promotors for Th2 responses and IgE synthesis. Another explanation of these unexpected results may be the route of exposure. Some authors consider that i.g. administration followed by i.n. challenge of pure protein may support Th2 inflammation [7]. In light of these findings we think that the response seen in mice may not be relevant in humans, as consumption of isogenic cowpeas and chickpeas do not usually lead to IgE production in healthy individuals .

4.1.5 MICE RESPONSE TO ISOGENIC SEED MEALS

Surprisingly, in our feeding experiments we have also found that the antibodies to AAI developed not only in mice fed GM chickpeas, GM cowpeas and native beans, but also in mice fed isogenic varieties, which do not express AAI. We speculate that the AAI response obtained to the isogenic controls may be due to a crossreactive protein with a amino-acid sequence homologous with AAI. Indeed, our results demonstrate that the immunization with transgenic and native AAIs, induced antibodies that cross-react with pea lectin. This results corelates with the fact that in peas and beans, different lectins share high degree of amino acid sequence identity with AAI [108].

4.1.6 COOKING PEAS OR BEANS CHANGES RESPONSES TO AAI

To investigate whether heat treatment alters allergenicity to GM chickpeas and GM cowpeas, we performed additional experiments where mice were fed heat-treated GM and nGM chickpeas and cowpeas. Usually, beans, chickpeas and cowpeas are extensively cooked, before being consumed by the human population. However, it is known that most food allergens are resistant to heating and enzymatic degradation [114]. Moreover, recent data revealed an increase in allergenicity to peanut, upon roasting [115]. Bean AAI has been shown to be a heat-stable protein, which is partially resistant to proteolytic degradation [116]. Studies revealed that cooking, although significantly reduced the inhibitory activity of AAI, failed to totally alter the ability of the transgenic pea to prime for Th2-inflammation when challenged in the lung [50]. Here, we were able to detect a decrease in the percentages of airway eosinophilia, as well as a reduction in both Th1 and Th2 antibody levels compared to mice fed raw diets. Similar to previous findings [50], our results suggest that heat treatment reduced, but not entirely altered the ability of GM chickpeas and GM cowpeas to prime for Th2 inflammation.

4.1.7 BT MAIZE (MON810) CONSUMPTION DURING THE INITIATION PHASE OF ALLERGIC ASTHMA DEVELOPMENT AND RELAPSE, DOES NOT WORSEN ALLERGENICITY TO A UNRELATED ALLERGEN

In parallel experiments, we investigated the effect of feeding GM maize at the time of the first episode of allergic asthma and during asthma attacks with OVA. Widely used crops as maize, potato, tobacco, soybean, and tomato have been engineered to express Bt gene, but not all of these plants were evaluated for potential allergenicity. The results obtained in this study show that upon consumption of the commercialized maize line, MON810, no increase the allergic immune response to OVA could be detected. In addition, no other adverse effects were observed.

4.1.8 FEEDING GM MAIZE DOES NOT INCREASE THE ALLERGIC RESPONSES TO OVA

Previous studies have shown that consumption of GM peas enhanced the OVA-specific allergic response in a BALB/c model. As a consequence, we sought to

investigate the adjuvant effect of MON810 in OVA induce allergic disease. Here, we found increase in Th2 parameters, BAL eosinophils and lung inflammation as a consequence of OVA sensitization. We also measured anti-OVA IgE and IgG1 antibodies as an indicator of allergy and Th2 response. Our results revealed that in OVA-induced mice, antigen-specific IgE and IgG1 (Th2 type) and IgG2a (Th1 type) were significantly increased compared with the naïve mice. However, food supplementation with transgenic maize did not alter the titer of either of the antibodies mentioned above. In line with our results, *Chen et. al.* showed that oral fruit supplementation with transgenic TPY10-4 papaya, did not increase the allergic potential of OVA in sensitized BALB/c mice [123]. In addition, Skin prick test in sensitive subjects suffering for asthma-rhinitis or IgE antibodies secretion against pure Cry1Ab protein in individuals with food allergy confirmed the lack of allergenicity of MON810 maize [124]. Similarly, in our mouse study, Bt-maize administration did not increase either parameters associated with Th2 response compared to that of mice fed the nGM diet, suggesting that dietary Bt-maize, containing cry1Ab toxin may not have any influence on allergic asthma phenotype.

4.1.9 FEEDING GM MAIZE DOES NOT LEAD TO INCREASE IN CRY 1AB ANTIBODY LEVELS IN SERA

Measurements of anti-Cry1Ab specific humoral immune response detected no specific antibodies in the serum of mice fed the Bt meal, and this finding was in agreement with other published studies [122].

4.1.10 FEEDING GM MAIZE DOES NOT AFFECT BODY AND ORGAN WEIGHTS

Induction of OVA-mediated disease in the relapse phase, leads to a significant increase in liver weights in mice fed all diets compared to naïve mice suggesting that OVA immunization itself has an effect on this organ. However, no difference in the body, spleen or liver weights were detected in either initiation or relapse of disease in mice fed MON810 compared to its isogenic control. Different studies on rats, mice, pigs, and poultry were performed where body and organs weights were monitored as an indicator of overall animal health upon feeding GM corn. Similar to our findings, no differences on the body weights were found in studies with rats fed MON810 for 90 days [78] and broilers fed the same GM for 42 days [118]. The same results were

seen when we determined the final organ weights. In other studies, contradictory results were obtained referring to spleen, kidney or liver weights [119-121].

4.1.11 Consumption of MON810 does not lead to detection of Cry1Ab gene in muscle

In the last part of this study, we tried to detect the presence of the transgenic DNA in the mouse tissue muscle after feeding Bt-maize. One of the main concerns of including GM food and feed on the market is the potential horizontal transfer of the transgenic DNA to animal tissues [126]. At this point there are few studies where highly sensitive PCR methods were used to detect fragments of ingested transgenic DNA in animal tissues [125, 127]. In the present study, we were not able to detect a small sized fragment of 211 bp of the Cry1Ab coding region in quadriceps muscle from mice fed MON810 maize. These results correlate with previous published studies in which transgenic DNA was not detected in the organs or blood of animals fed GM crops [128, 129].

Conclusions

The experimental evidence summarized above suggests that although differences in glycosylation leads to modified AAI structure in beans, GMO cowpeas and GMO chickpeas, it did not trigger any increase in immunogenicity and allergenicity upon GMO consumption.Moreover, feeding GMO led to a crossreactive response to a isogenic protein in mice. Due to the complex genetic diversity that predisposes humans to allergy [131], it is still not clear if any animal model, used in the safety evaluation process is suitable to predict the allergic potential of newly engineered GM crop. Furthermore, the study of the GM maize adjuvanticity infers that Bt corn does not influence allergic response to other allergens and appears to be safe for consumption in atopic individuals.

SUMMARY

GM plants are developed to protect against insect pests and aims to improve yield. However, there is increasing concern associated with the genetically modified (GM) crops such as toxicity and the potential of the introduced proteins to elicit a potentially harmful immunological response including allergenic hypersensitivity. The consortium focused on identifying biomarkers that can be followed after a GM food is released on the market. Using two prototype allergenic alpha-amylase inhibitor (AAI) pea (*data not shown*), cowpea and chickpea produced by Dr. T.J. Higgins at Commonwealth Scientific and Industrial Research Organization (CSIRO) Plant Industry Division in Australia, we determined how this particularly GM foods influence the allergenic responses in various experimental models. The cowpeas and chickpeas used in this study express the AAI gene normally found in the common bean (*Phaseolus vulgaris* L. cv. *Tendergreen*), which completely protects both pea varieties from damage by inhibiting the α-amylase enzyme in the pea weevil (*Bruchus pisorum*). In this study we addressed the immunogenicity of transgenic AAI and the allergenicity of the GM cowpea and chickpea in a mouse model of allergic disease. Our results show that even though the transgenic AAI proteins are differentially glycosylated in chickpea and cowpeas compared to the AAI in the native beans, they are not more immunogenic and allergenic. Furthermore, feeding experiments revealed that GM cowpeas and chickpeas but also the isogenic varieties and more important the native beans induce functional antibodies to AAI which are crossreactive with pea lectin. In parallel experiments, we investigated the adjuvant effect of the authorized GM corn (MON810) on the onset of allergic disease or disease exacerbation. Our results show that mice did not develop more severe allergic disease when fed GM maize compared to normal mouse food and non-GM maize. These results indicate that there is no increase in allergenicity to other unrelated allergens upon eating GM maize.

ZUSAMMENFASSUNG

Genetisch modifizierte Organismen (GMO) werden entwickelt um Schädlinge zu bekämpfen und dadurch den Ertrag zu verbessern. Seit Jahren gibt es allerdings, Diskussionen darüber, ob genetisch veränderte Nahrungsmitteln auch ein Gefahrenpotenzial in sich bergen z.B. Toxizität oder neu eingeführte, transgene Proteine die Potentiale Überempfindlichkeitsreaktionen hervorrufen. Eine Risikoeinschätzung des Verzehrs von GMO vor deren Vermarktung kann mit derzeitigen Kenntnissen noch nicht angemessen evaluiert werden und ist daher mit nicht kalkulierbaren Konsequenzen verbunden. Weder eine Quantifizierung der GMO-Belastung, noch eine Korrelation zwischen GMO und Krankheitsausbrüchen können, wegen des fehlens von Meldesystemen und einer mangelhaften Kennzeichnung von GMO-Inhalten berechnet werden. Als eines der Studienobjekte dienten Erbsen (*Pisum sativum)*, Augenbohnen (*Vigna unguiculata*) und Kichererbsen (*Cicer arietinum*) die mit genetisch modifiziertem α-Amylase-Inhibitor (AAI) Gen von einer Bohnensorte (*Tendergreen bean*) versehen werden, Die zum Schutz gegen Schädlinge (Erbsensamenkäfer) dienen. Verseuchungen von gelagerten Samen durch Getreiderüsselkäfer führen vor allem in den Entwicklungsländern zu Verlusten dieser Nahrungsmittelkulturen. Samen von *Phaseolus vulgaris* sind aufgrund ihrer hemmenden AAI Protein, weitgehend resistent gegen diese Schädlinge. *Die* transgenen *Pisum sativum, Vigna unguiculata und Cicer arietinum* enthalten das aus dem *Phaseolus vulgaris* stammende Gen für den AAI. Das Gen exprimiert das α- Amylase–Enzym, welches GMO-Samen vor dem Rüsselkäfer schützten.

Eine 2005 veröffentlichte Studie impliziert, dass die GM Erbse das Potenzial hat, nach dem Konsum, allergische Reaktionen hervorzurufen. Ziel des Projektes ist es, die Ergebnisse dieser Studie einer objektiven Prüfung zu unterziehen und spezielle Biomarker zu finden, die Aufschlüsse über mögliche Langzeitwirkungen dieses Nahrungsmittels auf die Gesundheit geben können. Der Schwerpunkt des Projekts lag darin, Effekte dieser GMO in Mäusen, zu überprüfen. Unsere Ergebnisse zeigen, dass, obwohl die transgenen AAI Proteine unterschiedlich glykosyliert werden in vergleich zum ursprüngliche AAI Proteine die sich in Bohne befindet, diese sind nicht mehr Immunogen und Allergen. Weitere Versuche haben gezeigt, dass sowohl die GMOs wie auch die isogenen Sorten und noch wichtiger die Bohnensorte

funktionale Antikörper zu AAI induzieren, Verantworlich für dieses Ergebniss ist nicht die genetische Modifikation mit dem Protein AAI sondern eine Kreutzreaktion mit einem in Erbsen vorhandene Proteine, sogenannte Erbsen-Lektine. In parallel durchgeführten Experimenten haben wir die beistehende Wirkungen des genehmigten GM-Mais (MON810) auf den Beginn der allergischen Erkrankung und während der Krankheit Exazerbation untersucht. Unsere Ergebnisse zeigen, dass Fütterung von GM-Mais, und die isogene-Mais Sorte keine Verschliemmerung der allergische Reaktionen verursachen.

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**Europass
Curriculum Vitae**

Personal information

First name(s) / S¹urname(s)	Daniela Reiner
Address(es)	
Telephone(s)	Mobile:
E-mail	daniela.reiner@meduniwien.ac.at; daniela.reiner@gmail.com
Nationality	
Date of birth	
Gender	Female

**Desired employment /
Occupational field**

Researcher

Work experience

Dates	1/10/2008 untill 1/04/2013
Occupation or position held	Scientific researcher
Main activities and responsibilities	*In vivo* and *in vitro* tehniques: Mice handling: intranasal and intragastric administartion; intraperitoneal and intravenous injection Immunohistochemistry Immunofluorescence staining ELISA Cell culture Cell isolation from murine spleen and lungs AHR
Type of business or sector	Medical University of Vienna, Experimental Allergy
Additional work experiences	▪ GMSAFOOD project: one week at Teagasc (Irish Agriculture and Food Development Authority). ▪ Participation in EU-project: "Public Private Partnership for Asthma Imagin and Genomics-P3AGI". 3 months Secondment in CBM (Consorzio per il centro di biomedicina molecolare), Triest, Italy, 2010; Gained knowledge in Optical imaging techniques and genomic biomarkers for severe Allergic Asthma. ▪ Participation in EU-project: "Public Private Partnership for Asthma Imagin and Genomics-P3AGI". 2 months Secondment in Marinomed Biotechnology GmbH, 2010 and 2011.

Education and training

Dates	01/08/2008-31/10/2012 Doctoral studies Genetics-Microbiology 01/10/2007-01/06/2008 Masters degree Genetics-Microbiology
Title of qualification awarded	Master of Science
Principal subjects/occupational skills covered	phD-"Study of genetic modified peas in a mouse model" masters-"Characterization of airway APC involved in the initiation of allergic asthma"
Name and type of organisation providing education	University of Vienna
Name and type of organisation providing training	Medical University of Vienna

Personal skills and competences

Mother tongue(s)	Romanian
Other language(s)	German, English, Italian

Self-assessment	Understanding		Speaking		Writing
European level (*)	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C2	C1	C1	C1
German	C2	C2	C1	C1	C1

Social skills and competences	Team spirit and ability to adapt to multicultural environment gained through my work abroad
Organisational skills and competences	Sense of organizations
Computer skills and competences	Microsoft Office (Word Excel and PowerPoint); Adobe Photoshop; GraphPad Prism 5;

Additional information

Posters and Oral Presentations

Austrian Society for Allergy and Immunology, Alpbach, Austria, December 2007:
Characterization of lung APCs taking up soluble and aeroantigens.
Daniela Reiner, Rui-Yun Lee, Pallavi Singh, Gerhard Dekan, Oskar Hoffmann,
Georg Stingl, Michelle M. Epstein.

Austrian Society for Allergy and Immunology, Vienna, Austria, December, 2010:
Feeding Genetically Modified (GM) Bt-maize MON810 does not influence allergic
disease to other allergens in mice.
Daniela Reiner, Rui-Yun Lee, Michelle M. Epstein.

European Academy of Allergy and Clinical Immunology, Venice, Italy, February, 2011:
Effect of feeding genetically modified Bt-corn on allergic disease.
Daniela Reiner, Rui-Yun Lee, Michelle M. Epstein.

Young Scientist Association of Medical University of Vienna, Austria, June, 2011:
Effect of feeding genetically modified Bt-corn on allergic disease.
Daniela Reiner, Rui-Yun Lee, Michelle M. Epstein.

Public private partnership for asthma imaging and genomics, Summer school, Vienna, Austria, June 2011:
Genetically modified alpha-amylase inhibitor peas do not influence allergenicity to other unrelated allergens in mice.
Daniela Reiner, Rui-Yun Lee, Eva Gelencsér, Michelle M. Epstein.

„GMSAFOOD GMO safety & Post market monitoring Conference March, Vienna, Austria, 2012
Are mice a good model for testing GMO allergenicity?
Daniela Reiner, Rui-Yun Lee, Michelle M. Epstein.

Österreichische Gesellschaft für Allergologie und Immunologie, November, Vienna, Austria, 2012
Genetically modified α-amylase inhibitor peas are not specifically allergenic in mice
Daniela Reiner, Rui-Yun Lee, T.J.V. Higgins, Michelle M. Epstein.

Research publication:

Comparison of the alpha-amylase inhibitor-1 from common bean (Phaseolus vulgaris) varieties and transgenic expression in other legumes post-translational modifications and immunogenicity.
Peter M Campbell, Daniela Reiner, Andrew E Moore, Rui-Yun Lee, Michelle M. Epstein, T J V Higgins. *Agric Food Chem., 2012*

Genetically modified α-amylase inhibitor peas are not specifically allergenic in mice. Rui-Yun Lee*, Daniela Reiner*, Andy Moore, T. J. V. Higgins, Michelle M. Epstein*, Plos one, 2013*
*Authors contributed equally to this study

Award Travel award to European Academy of Allergy and Clinical Immunology, Venice, Italy

Annexes References supplied on request

Genetically Modified α-Amylase Inhibitor Peas Are Not Specifically Allergenic in Mice

Rui-Yun Lee[1,9], Daniela Reiner[1,9], Gerhard Dekan[2], Andrew E. Moore[3], T. J. V. Higgins[3], Michelle M. Epstein[1]*

1 Division of Immunology, Allergy and Infectious Diseases, Experimental Allergy, Department of Dermatology, Medical University of Vienna, Vienna, Austria, 2 Institute of Clinical Pathology, Medical University of Vienna, Vienna, Austria, 3 CSIRO Plant Industry, Canberra, ACT, Australia

Abstract

Weevils can devastate food legumes in developing countries, but genetically modified peas (*Pisum sativum*), chickpeas and cowpeas expressing the gene for alpha-amylase inhibitor-1 (αAI) from the common bean (*Phaseolus vulgaris*) are completely protected from weevil destruction. αAI is seed-specific, accumulated at high levels and undergoes post-translational modification as it traverses the seed endomembrane system. This modification was thought to be responsible for the reported allergenicity in mice of the transgenic pea but not the bean. Here, we observed that transgenic αAI peas, chickpeas and cowpeas as well as non-transgenic beans were all allergenic in BALB/c mice. Even consuming non-transgenic peas lacking αAI led to an anti-αAI response due to a cross-reactive response to pea lectin. Our data demonstrate that αAI transgenic peas are not more allergenic than beans or non-transgenic peas in mice. This study illustrates the importance of repeat experiments in independent laboratories and the potential for unexpected cross-reactive allergic responses upon consumption of plant products in mice.

Introduction

Genetically modified (GM) crop areas have increased rapidly since their introduction in 1996 [1]. New approaches to generate plants that are resistant to insect infestation are being actively sought, especially to reduce reliance on chemical insecticides. For example, genetically modified peas (*Pisum sativum*), chickpeas (*Cicer arietinum*) and cowpeas (*Vigna unguiculata*) expressing the gene for alpha-amylase inhibitor-1 (αAI) from the common bean (*Phaseolus vulgaris*) cultivar Tendergreen are completely protected from weevil destruction [2,3,4]. αAI is seed-specific, accumulated at high levels and undergoes post-translational modification as it traverses the seed endomembrane system [5]. The excellent insecticidal effect of αAI [6] and the long-term safe consumption of beans containing αAI [7] make it a promising gene to insert into insect-susceptible legumes. However, one study suggested that αAI peas expressed a variant protein resulting in allergic responses in mice to the peas but not the beans [8]. They found that mice consuming αAI peas developed elevated levels of αAI-specific IgG1 but not IgE antibodies, had enhanced delayed-type hypersensitivity responses and increased reactivity to other allergens (adjuvant effect) whereas mice fed non-transgenic peas and Pinto beans had no αAI reaction. Mass spectrometry results revealed differences in post-translational modifications, which the authors suggested led to the reported allergenicity. These results were received with some skepticism including an editorial in Nature Biotechnology [9].

More recently, a comparison using high-resolution mass spectrometry of αAI from bean and transgenic legume sources revealed heterogeneous structural variations in peas and beans due to differences in glycan and carboxypeptidase processing, but the transgenic versions were within the range of those observed from several bean varieties [5]. Moreover, when purified αAIs from beans and transgenic peas were used to immunize mice, all elicited Th1 and Th2- type αAI-specific antibodies [5]. This questions the reported enhanced αAI transgenic pea-specific immunogenicity and allergenicity compared with the naturally occurring protein in beans.

The objective of this study was to evaluate allergenicity of αAI peas, cowpeas and chickpeas and compare them to non-transgenic controls, Pinto and Tendergreen beans (the latter was the source of αAI gene) in mice. To achieve this aim, we evaluated the immunogenicity and allergenicity of αAIs from these transgenic legumes to determine whether the transgenic αAIs were more allergenic than the αAIs from Pinto and Tendergreen beans. The evaluation included a comparison of antibody titres to αAIs from each source. Additionally, we tested the antibody response to twice weekly consumption of the pea, cowpea, chickpea and bean meals for 4 weeks. After the feeding period, we challenged the respiratory tract with αAI to evaluate *in vivo* T lymphocyte responses. Lastly, we assessed the adjuvant effect of αAI pea consumption on the initiation and exacerbation of non-cross-reactive ovalbumin (OVA)-induced allergic lung disease.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the guidelines for the care and use of laboratory animals of the Austrian Ministry of Science. The protocol was approved by the Committee on the Ethics of the Austrian Ministry of Science (Number: GZ: 68.205/0237-II/3b/2010). All painful procedures were performed under anesthesia, and all efforts were made to minimize suffering.

Mice

Female BALB/c mice (6–8 week old) were purchased from Charles River (Germany). Mice were provided with tap water ad libitum throughout the study and were maintained in the University of Veterinary Medicine animal facility in Vienna, Austria. We accommodated 8 mice per Type III cage with stainless steel covers using a 12 h light/dark schedule, at temperature of approximately 22°C. Mice were observed two times daily. The basal diet was OVA-free autoclaved SSNIFF V1126-000, from Soest, Germany: (http://www.ssniff.de/documents/03_katalog_dt_maus_ratte.pdf) provided *ad libitum*. All experiments used 8 animals per group.

Isolation of α-Amylase Inhibitors

The transformation of peas, chickpeas and cowpeas for seed-specific expression of the αAI gene from the common bean (*P. vulgaris*, cv Tendergreen) has been described previously [2,3,10]. Seed meals from the transgenic legumes, Pinto and Tendergreen beans have approximately the same concentration of αAI and are in the range 2–4% of total seed protein [5]. αAIs from the seeds of the various beans and transgenic legumes were purified as previously described [11]. Briefly, seed meals from Pinto bean, Tendergreen bean, and transgenic peas, cowpeas and chickpeas were extracted with a NaCl solution (1%) followed by a heat treatment (70°C), dialysis and centrifugation. The inhibitors were enriched by anion exchange (DEAE-Sepharose CL-6B, Pharmacia) and gel filtration (Sephacryl S-200, Pharmacia) chromatography. Active fractions were determined by inhibition of porcine pancreatic α-amylase (Ceralpha: α-Amylase Assay Kit, Megazyme International, Ireland) and the most pure fractions were determined by inspection of Coomassie-stained 15–25% SDS-PAGE gels. Finally, the appropriate pooled fractions were dialysed against water, lyophilized and stored at 4°C. The proteins were highly purified as can be assessed from the mass spectrometric analyses described earlier [5]. Pea lectin was purified as described earlier [12]. The level of pea lectin in peas [13] is comparable to the level of αAI in the peas [5]. Pea lectin is structurally related to αAI [14] and their amino acid sequences are 38% identical and 54% similar to each other as determined by Blast® analysis. Purified proteins contained low or undetectable levels of endotoxin (Andrew Moore, unpublished data).

αAI feeding and immunization protocols

Intraperitoneal immunization: Naïve mice received i.p. injections of 10 μg of purified αAIs from either αAI pea, Tendergreen bean, Pinto bean, or pea lectin in 200 μl PBS on days 0 and 21. One week later, sera were taken and stored at −20°C until use in ELISAs measuring anti-αAI or pea lectin-specific antibody titres. *Intranasal immunization:* In separate experiments, we instilled naïve mice with 50 μg of purified αAI dissolved in 50 μl PBS into the nares, so that it reaches the lungs, on days 0, 2, 4, 14, 16, 18 and tested for anti-αAI-specific antibody titres and allergic lung inflammation and mucus production on day 21. *Pea and bean feeding for the evaluation of allergic responses to αAI:* Feeding experiments were done by gavage (intragastric administration). Mice were gavaged suspensions of 100 mg/ml in 250 μl of PBS raw or 100°C heat-treated seed meals of αAI -pea, -cowpea, -chickpea and non-transgenic pea, Pinto bean and Tendergreen bean twice weekly for 4 consecutive weeks using the same protocol as in Prescott et al. [8]. As a read out of allergic sensitization during feeding, at 96 h after the final gavage, mice received one intranasal instillation of 50 μg of αAI purified from αAI pea or Tendergreen bean dissolved in 50 μl of PBS as a lung challenge. The mice were then evaluated 72 h later for antibody titres, allergic lung inflammation and mucus production. *Adjuvant studies:* Mice were gavaged suspensions of 100 mg raw seed meals of αAI pea, non-transgenic pea, Pinto bean and Tendergreen bean in 250 μl of PBS twice weekly for 4 consecutive weeks, 1 month before the initiation and exacerbation of OVA-induced allergic asthma (see protocol below). Both heat-treated and raw seed meals were used in these studies to determine whether there were differences between seed meals with denatured proteins.

Induction of OVA-induced allergic asthma

Mice were immunized with 10 μg of OVA (Sigma Chemical Co., St. Louis, MO) i.p. on days 0 and 21. Mice were challenged 1 week later with nebulized 1% OVA in PBS in a Plexiglas chamber by an ultrasonic nebulizer (Aerodyne, Kendall, Neustadt, Germany) for 60 min twice daily on days 28, 29 for disease initiation. For disease exacerbation, mice were allowed to recuperate from acute disease and were then nebulized on days 91 and 92. Three days after the last aerosol challenges, the mice were evaluated for antibody titres, allergic lung inflammation and mucus production.

Lung inflammation and mucus hypersecretion

Airway inflammation: Mice were terminally anesthetized 72 h after the last antigen challenge. The mice were then subjected to tracheotomy followed by the lavage of the lungs 3 times with PBS for a total volume of 1 ml to collect bronchoalveolar lavage fluid (BAL). The total number of cells in BAL was enumerated (Neubauer hemocytometer) and the differential cell counts were determined by morphological examination of at least 300 cells in cytocentrifuged preparations (Cytospin-4, Shandon Instruments, UK), stained with Kwik-Diff (Thermo Fisher Scientific Inc., Pittsburgh, PA).

After BAL, lungs were fixed by immersion in 4% paraformaldehyde and then embedded in paraplast. Lung sections of 3 μm were stained with hematoxylin and eosin (H&E) for morphological evaluation, with Luna stain for eosinophil enumeration and with Periodic-acid-Schiff reagent (PAS) for detection of mucus within the lung epithelium. For scoring of inflammatory cell infiltration, sections containing main stem bronchi from each lung specimen stained with H&E were used. Blinded observers graded the extent of inflammation in the lungs according to a semi-quantitative scoring system: Grade 0: no inflammatory infiltrates; Grade 1: inflammatory infiltrates in central airways; Grade 2: inflammatory infiltrates extending to middle third of lung parenchyma; and Grade 3: inflammatory infiltrates extending to periphery of the lung. We enumerated eosinophil counts in lung sections stained with Luna by counting ten random fields (40× magnification) containing alveoli but without major airways or vessels on low power magnification, and averaged the counts for each lung section. For detection of mucus-secreting cells, adjacent lung sections were stained with PAS and counter stained with hematoxylin. We used the following scoring system for mucus production: Grade 0 – no mucus producing cells in airways; Grade

1: 0–20%; Grade 2: 21–40%; Grade 3: 41–60%; Grade 4: 61–80 and Grade 5: 81–100% mucus producing cells in airway walls stained for mucopolysaccharide.

Serum OVA- and αAI-specific immunoglobulin

For the measurement of antigen-specific immunoglobulin IgG1, IgG2a and IgE, ELISA plates were coated with OVA, purified αAI or pea lectin at 10 µg/ml overnight at 4°C. The plates were then washed and blocked with 2% bovine serum albumin in PBS for 2 h at RT. The plates were washed and sera were added and incubated for 24 h at 4°C. Plates were washed again and then incubated with biotinylated anti-IgG1 for an additional 2 h at 4°C (Southernbiotech, Birmingham, AL), anti-IgE (Becton Dickinson Biosciences, Franklin Lakes, NJ) or anti-IgG2a (Southernbiotech) detection mAbs, followed by incubation with streptavidin horseradish peroxidase (Southernbiotech) for 1 h at RT. Plates were washed and incubated with a TMB substrate solution (100 µl/well, BD OptEIATM, Becton Dickinson Biosciences) for 10 min at RT. The reaction was stopped with 100 µl of 0.18 N H_2SO_4 and absorbance was measured at 450 nm.

Statistical analysis

Groups were compared with the Kruskal-Wallis test followed by the Dunn's multiple comparison test and the Mann Whitney test for grading histology using GraphPad Instat v.5.0 (GraphPad Software Inc.). p values were considered significant at <0.05.

Results and Discussion

The scheme in Figure 1 illustrates the experimental protocols. We first tested the hypothesis that different post-translational modifications to αAI in pea alters immunogenicity and allergenicity compared to αAI in bean. To directly investigate αAI immunogenicity, we immunized mice with purified αAI from Pinto bean, Tendergreen bean and transgenic pea, cowpea and chickpea by i.p. (Fig. 1A) or i.n. (Fig. 1B) routes. We administered 10 µg of αAI without adjuvant i.p. 3 weeks apart and measured anti-αAI-specific IgG1, IgG2a and IgE serum titres one week later (Fig. 2A). To further assess the *in vivo* allergic response induced by αAI, we immunized mice i.n. with 50 µg of αAI 6 times over a 3-week period and then evaluated antibody titres and lung responses (Fig. 2B).

Intraperitoneal immunization with all αAIs led to increased allergic isotype, anti-αAI-specific IgG1 responses (Fig. 2A) and confirmed previous data [5]. Cowpea, Pinto bean and chickpea αAIs generated the highest IgG1 titres, whereas Tendergreen bean αAI resulted in a slightly lower titre and pea αAI was the least immunogenic. Anti-αAI-specific IgE levels were low for all groups with chickpea αAI having the highest titre. Generally, the allergy IgE antibody isotype responses are 10–100 fold lower than allergic IgG1 isotype in mice (M. Epstein, unpublished results). Because we used protocols intended to skew responses towards allergic Th2 isotypes, IgG2a titres were, as expected, lower than IgG1. Tendergreen bean, chickpea and cowpea αAIs induced the highest IgG2a titres. Although there are distinct patterns of glycosylation of αAIs [5] that may explain the magnitude of antibody responses, there was no apparent correlation between anti-αAI titres and the source of the αAI.

Intranasal αAI administration led to high anti-αAI-specific IgG1 titres against cowpea and Tendergreen bean αAIs, followed by lower titres against Pinto bean and chickpea αAIs and the lowest titres were against pea αAI (Fig. 2B). Anti-αAI IgE responses were low for all αAIs. Interestingly, IgG2a titres were higher for i.n. compared to i.p. αAI administration. This is probably related to

the higher total i.n. dose of 300 µg of αAI compared with a total αAI i.p. dose of 20 µg. Thus, both IgG1 and IgG2a isotype titres were higher in i.n. compared to i.p. experiments. Cowpea and Tendergreen bean αAIs induced the highest anti-αAI-specific IgG2a titres followed, in order, by Pinto bean, chickpea and pea. Immunization by i.n. and i.p. routes demonstrated that antibody responses to αAI from beans and transgenic peas differed but the transgenic proteins were not more immunogenic or allergenic than bean αAIs.

Except for chickpea αAI, intranasal administration of all αAIs induced significant airway and lung inflammation when compared to PBS (Fig. 2C–E). Pinto bean and cowpea αAI induced the highest eosinophil infiltration in the airways with approximately 20 and 12% eosinophils within the infiltrates, respectively. αAI from pea, Tendergreen bean and chickpea induced approximately 11, 5 and 3% eosinophils in BAL fluid, respectively (Fig. 2C). Pinto bean αAI-induced airway eosinophilia is statistically greater than eosinophilia induced by Tendergreen bean and chickpea αAIs. Enumeration of eosinophils in lung tissue sections revealed that immunization with all αAIs induced significant allergic inflammation compared to PBS controls (Fig. 2D). Tendergreen and chickpea αAIs appeared to induce more allergic inflammation in lungs, but there were no statistical differences between any of the αAI-immunized groups. Similarly, all αAI-immunized mice developed extensive inflammatory infiltrates in contrast to PBS control sections that had low or no inflammation (Fig. 2E and Fig. S1). Analysis of PAS-stained lung sections revealed that all groups had similar mucus secretion responses to i.n. protein immunization compared to low or no mucus production in PBS controls (Fig. 2F and Fig. S1). Taken together, these data illustrate that when administered as per our protocols, αAI, irrespective of source is immunogenic and allergenic in mice. Variations in immune responses may be related to differential post-translational modifications such as glycosylation as previously reported [5]. However, no correlation could be made between immunogenicity and allergenicity of αAIs from bean and the transgenic legumes.

To evaluate whether consumption of bean and αAI pea seed meals generated allergic responses to αAI, we fed mice αAI transgenic peas, non-transgenic (nGM) peas, Tendergreen bean and Pinto bean (Fig. 1C). Mice received raw or heat-treated seed meal diluted in PBS twice weekly for 4 consecutive weeks, followed by 50 µg of αAI i.n. This intranasal exposure was added as an indication of *in vivo* T lymphocyte activation following ingestion of seed meal containing αAI. We then measured allergic airway and lung inflammation, mucus hypersecretion and antibody production as a readout for an αAI-specific immune response.

Serum antibody titres tested 72 hours after the i.n. instillation showed that consumption of all raw seed meal suspensions including nGM seed meal plus αAI i.n. exposure led to the production of anti-αAI-specific antibodies (Fig. 3A). Serum titres measured from mice before and after i.n. αAI were similar (data not shown) and naïve mice administered one i.n. dose of αAI did not induce immune responses (data not shown). The titres were highest for Tendergreen bean>Pinto bean>nGM chickpea>αAI cowpea>αAI chickpea>nGM cowpea = αAI pea = nGM pea. Indeed, nGM chickpea serum titres were even higher than the titres in serum from animals fed transgenic seed meals. Anti-αAI IgE and IgG2a titres were lower than that of IgG1 and IgE and IgG2a titres were highest in mice fed bean seed meal.

Due to the antibody response observed upon feeding nGM peas, we sought to identify whether there was a protein in the nGM pea that was crossreactive with αAI. Because of the known homology of pea lectin with αAI, we compared antibody reactivity of pea lectin from nGM peas with bean αAI using separate
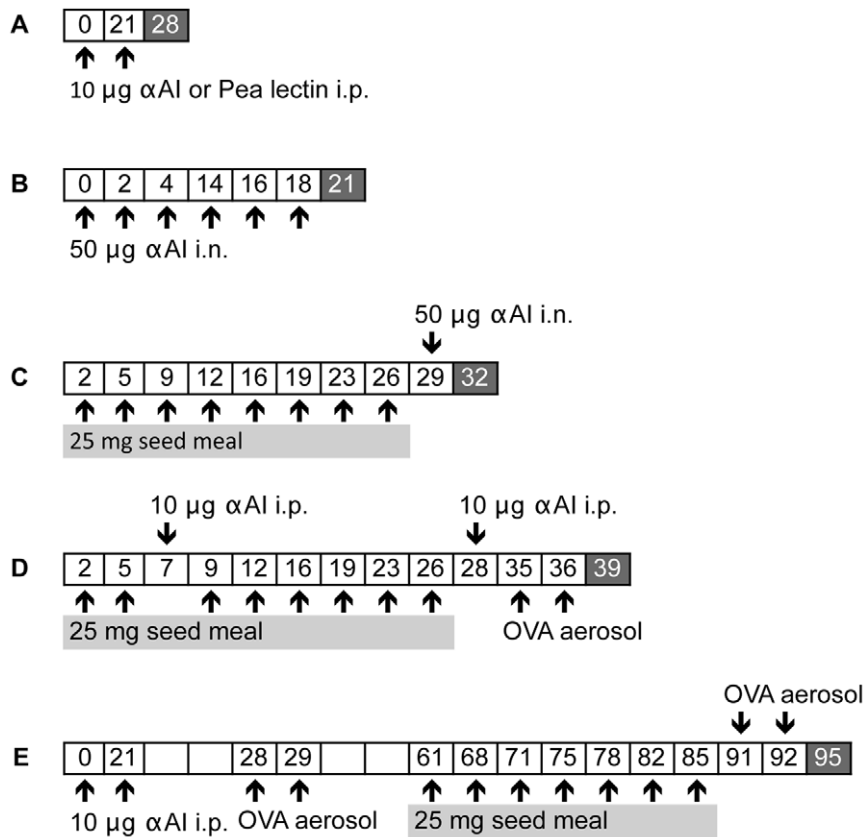
Figure 1. Experimental protocols. A. *Intraperitoneal immunization with purified proteins to assess protein immunogenicity.* On days 0 and 21 mice were immunized with 10 μg of purified αAIs from the transgenic peas, Tendergreen bean, Pinto bean, or pea lectin purified from non-transgenic peas. On day 28, sera were harvested and evaluated for IgG1, IgE and IgGa2 antibodies to αAI. B. *Intranasal immunization with purified proteins to assess differences in protein allergenicity.* On the indicated days, mice were instilled with 50 μg of purified αAIs from transgenic peas, Tendergreen bean, Pinto bean and tested for antibody titres and allergic lung responses on day 21. C. *Seed meal feeding for the evaluation of immune responses to αAI upon ingestion.* Mice were gavaged with 25 mg seed meals from αAI -pea, -cowpea, -chickpea, non-transgenic peas, Pinto bean and Tendergreen bean 8 times on the indicated days. On day 29, mice received an intranasal instillation of 50 μg of αAI purified from αAI pea or Tendergreen bean, and were evaluated on day 32 for antibody titres and allergic lung responses. D. *Adjuvant effect of peas and beans on the initiation of OVA-induced allergic lung disease.* Mice were gavaged with 25 mg raw or cooked seed meals from αAI -pea, -cowpea, -chickpea, non-transgenic peas, Pinto bean and Tendergreen bean 8 times on the indicated days. Mice were immunized to induce allergic disease with 10 μg of OVA on days 7 and 28. After one week, the mice were nebulized with OVA on days 35 and 36. On day 39, antibody titres and allergic lung responses were measured. E. *Adjuvant effect of peas and beans on the exacerbation of OVA-induced allergic lung disease.* Mice were induced with allergic disease on days 0 and 21 and aerosolized on days 28 and 29 and then allowed to recuperate. On the indicated days mice were gavaged 8 times with 25 mg raw or cooked seed meals. One day later, mice were nebulized with OVA on 2 consecutive days to induce a disease exacerbation. On day 90, they were evaluated for antibody titres and allergic lung responses.
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approaches. Firstly, we measured anti-pea lectin IgG1 in sera from mice fed beans and peas and found that transgenic αAI and nGM peas produced high anti-pea lectin antibody titres that were higher than the other bean and pea seed meal fed-mice (Fig. 3B). These results indicated that the consumption of peas led to pea lectin antibody production. Secondly, we immunized mice i.p. with pea lectin and measured the anti-pea lectin IgG1 response (Fig. 3C) and also tested pea lectin immune sera against αAIs (Fig. 3D). As expected, immunization with pea lectin induced high serum titres when reacting against pea lectin. These anti-pea lectin antibodies also reacted against cowpea and pea αAIs and with less intensity to chickpea and bean αAIs. Taken together, these results demonstrate that feeding with transgenic and non-transgenic peas generates anti-pea lectin responses, which are cross-reactive with αAI and can be confused with anti-αAI antibodies.

To further evaluate immune responses generated by the consumption of pea and bean seed meals, we did an *in vivo* respiratory tract challenge with αAI to assess whether T cell priming occurred. To measure *in vivo* T cell immune responses, we instilled αAI into the nares of mice following 4 consecutive weeks of bean and pea feeding and measured leucocyte infiltration and mucus hypersecretion in lungs. Feeding beans and peas, whether raw or heat-treated, followed by i.n. αAI induced airway and lung inflammation, while gavage with PBS did not induce inflammation (Fig. 3E–G and Fig. S2). Similarly, all mice fed seed meal developed high levels of mucus secretion following i.n. αAI compared with PBS controls (Fig. 3H and Fig. S2).

Consumption of Pinto and Tendergreen bean seed meals led to the highest number of eosinophils in the airway with increased eosinophil recruitment in heat-treated compared to raw seed meal fed mice (Fig. 3E). In contrast, mice consuming raw transgenic peas had higher airway eosinophils compared to heat-treated peas. Tendergreen bean fed mice generated more extensive allergic lung inflammation than all the other seed meals (Fig. 3F and 3G). Both transgenic αAI and non-transgenic peas generated a severe inflammatory response in lung compared to Pinto bean, transgenic
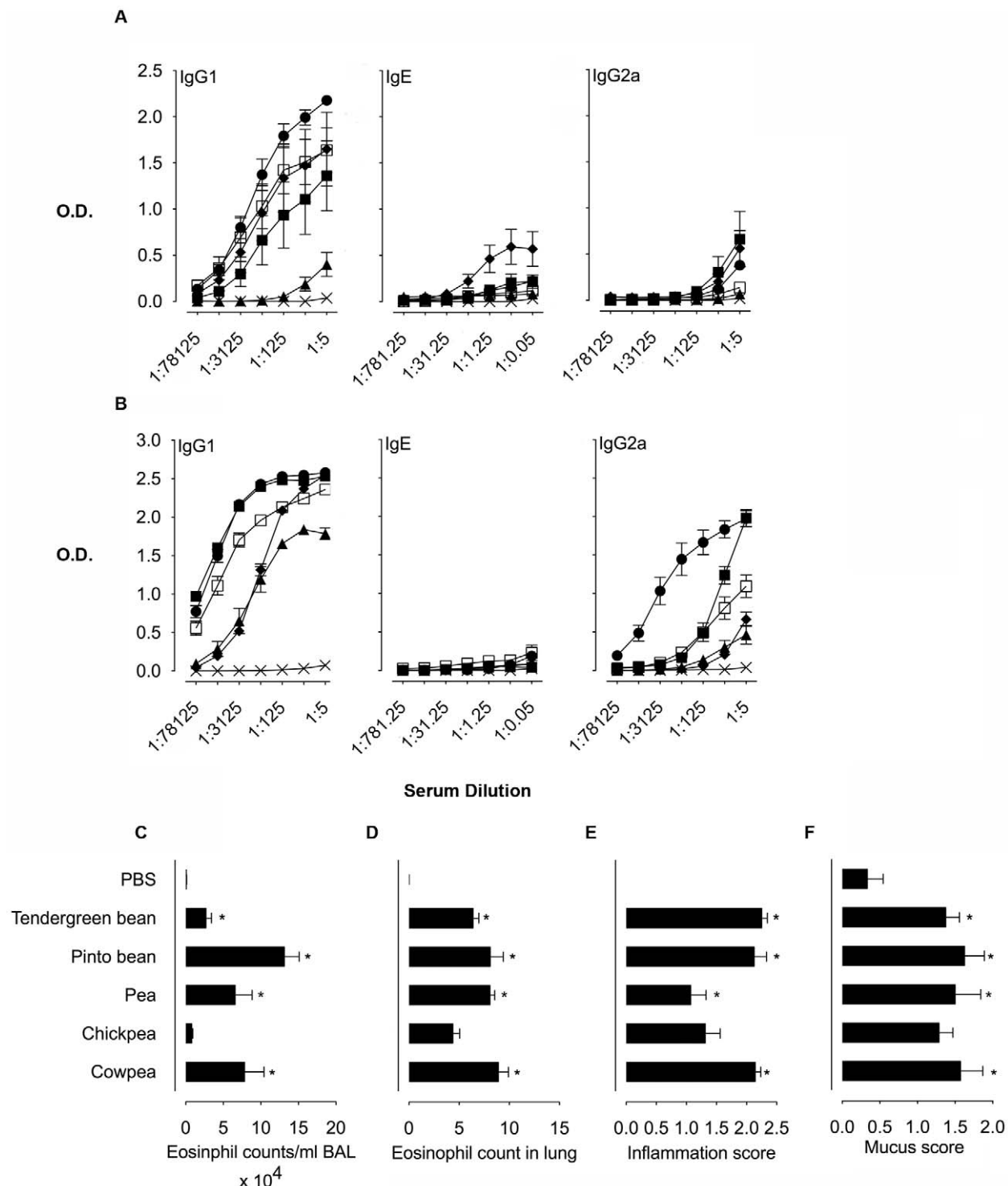
Figure 2. Immune responses to αAIs upon intranasal and intraperitoneal immunization. Serum antibody titres for Anti-αAI IgG1, IgE and IgG2a from A. i.p. αAI immunized mice and B. from i.n. αAI immunized mice. The treatment groups for A and B include PBS only ×, purified αAI proteins from Tendergreen bean ■, Pinto bean □, pea ▲, chickpea ◆, and cowpea ●. Data are expressed as mean OD$_{450\,nm}$ ± SEM; n = 8, duplicate samples. For IgE, dilutions are expressed ×10^3. C. Eosinophil counts in BAL fluid from mice immunized with i.n. αAIs. D. Eosinophil counts in Luna-stained lung sections from mice immunized with i.n. αAIs. E. Inflammation scores of lung sections from mice immunized with i.n. αAIs. F. Mucus scores in PAS-stained lung sections from mice immunized with i.n. αAIs. Data are expressed as means ± SEM; n = 8. For eosinophil counts in BAL and lungs, data were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test. For histological scoring, data were compared with the Mann Whitney test. *p<0.05 for all groups above the PBS controls. These are representative data from 2 experiments.
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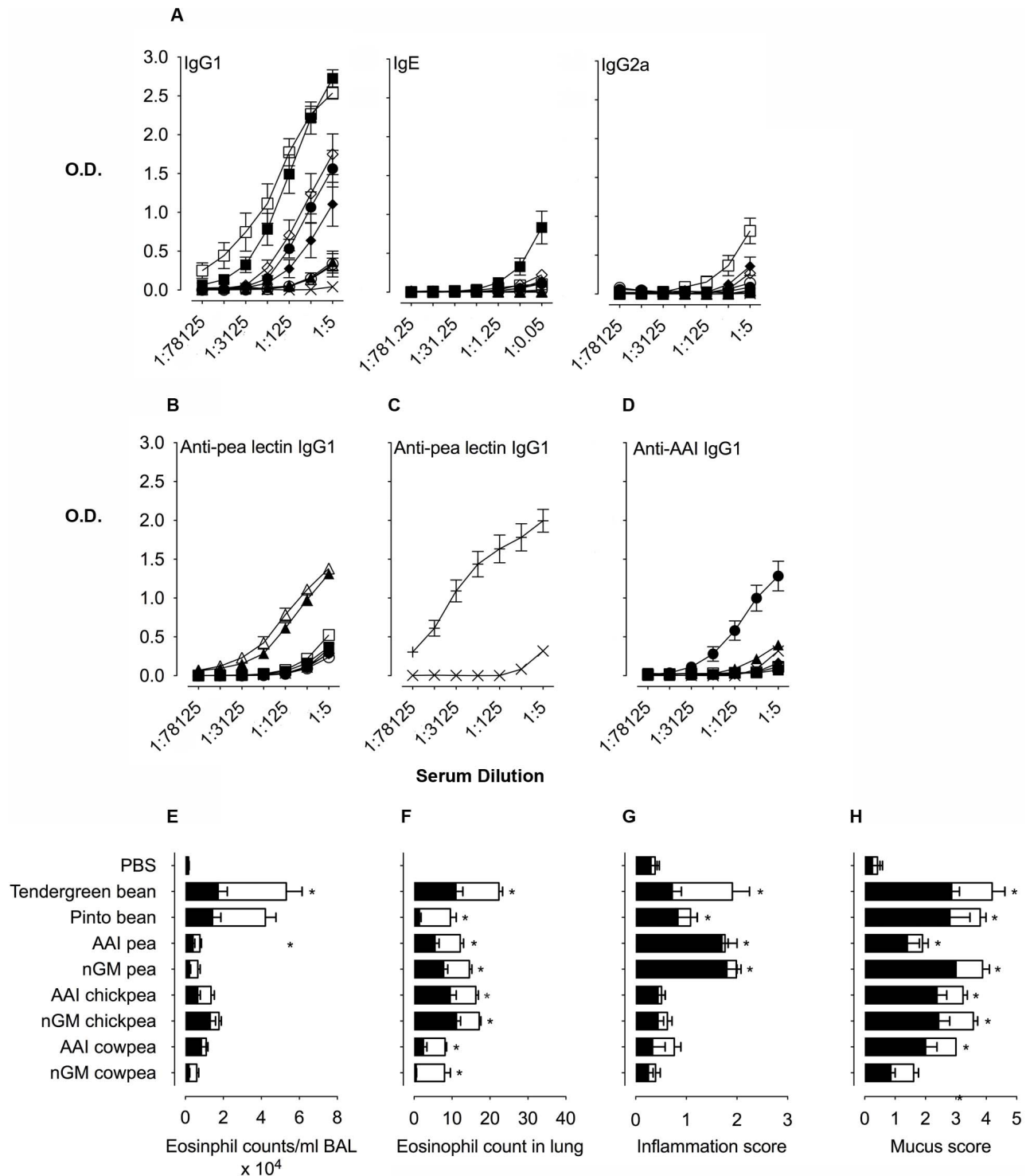
Figure 3. Immune responses following consumption of raw or cooked seed meal from αAI pea and bean. A. Serum antibody titres for anti-αAI IgG1, IgE and IgG2a from mice gavaged PBS or seed meals. B. Serum antibody titres for anti-pea lectin IgG1 from mice gavaged PBS or seed meals. C. Serum antibody titres for anti-pea lectin IgG1 from mice immunized i.p. with either PBS or pea lectin +. D. Serum IgG1 antibody titres of mice immunized with i.p. with pea lectin against αAI proteins purified from pea, cowpea, chickpea, Pinto bean and Tendergreen bean. Groups include PBS alone ×, Tendergreen bean ■, Pinto bean □, αAI pea ▲, nGM pea △, αAI chickpea ◆, nGM chickpea ◇, αAI cowpea ● and nGM cowpea ○. IgE dilutions are expressed ×10^3. Data are expressed as mean $OD_{450\ nm}$ ± SEM; n = 8, duplicate samples. Allergic lung inflammation evaluated by E. Eosinophil counts in BAL fluid, F. Eosinophil counts in Luna-stained lung sections, and G. Inflammation scores of lung sections. H. Allergen-induced mucus production is graded using mucus scores in PAS-stained lung sections. Raw (filled bars), cooked (open bars). Data are expressed as means ± SEM; n = 8. For eosinophil counts in BAL and lungs, data were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test. For histological scoring, data were compared with the Mann Whitney test. *$p < 0.05$ for all groups above the PBS controls. These are representative data from 2 experiments.
doi:10.1371/journal.pone.0052972.g003
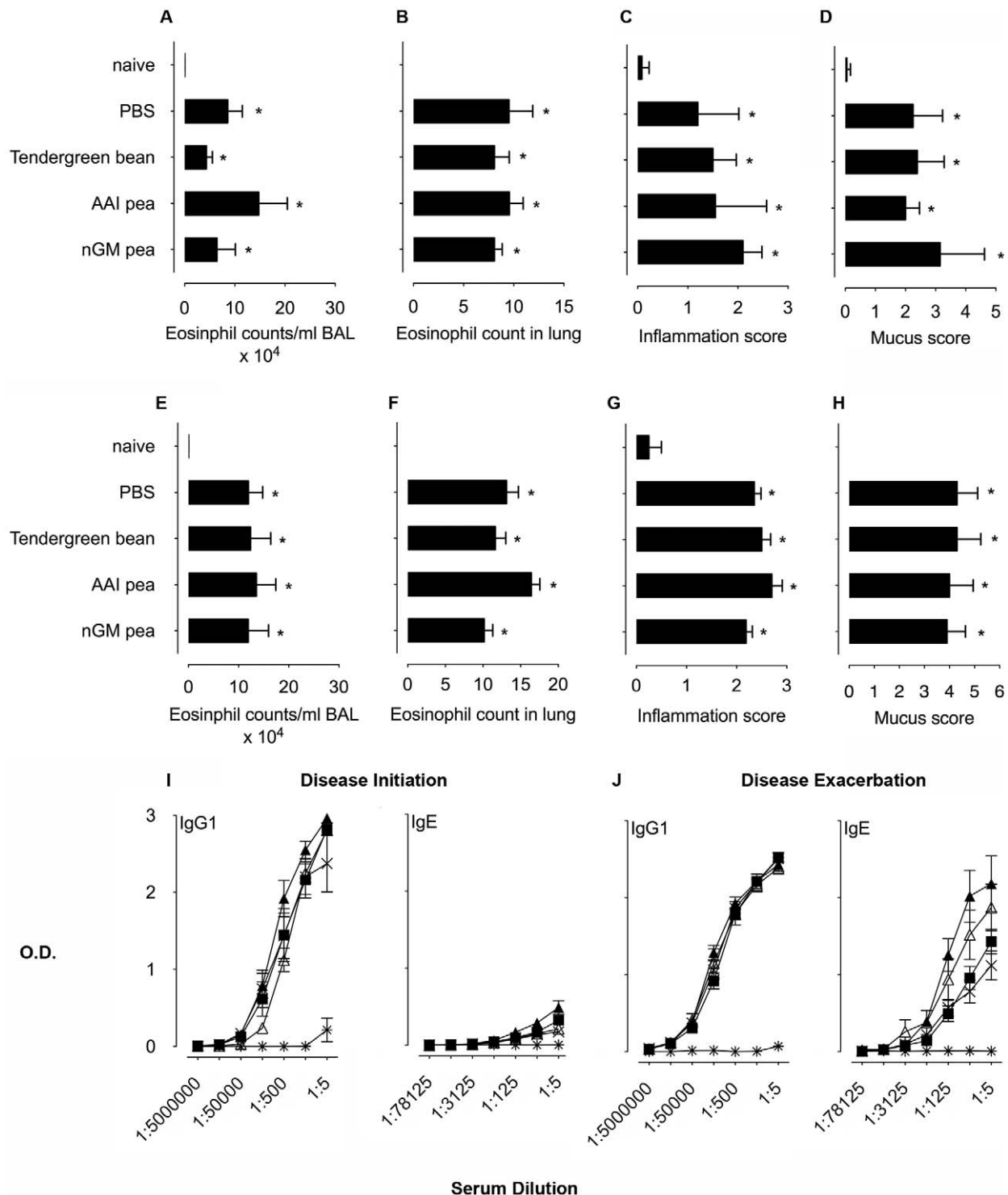
Figure 4. Adjuvant effect of αAI pea and bean consumption. Naïve BALB/c mice were compared with OVA-immunized and challenged mice gavaged with either PBS, or Tendergreen bean, αAI peas, non-transgenic pea seed meal. A. Eosinophil counts in BAL fluid from mice at disease initiation and e. exacerbation. B. Eosinophil counts in Luna-stained lung sections from mice at disease initiation and F. exacerbation. C. Inflammation scores of lung sections from mice at disease initiation and G. exacerbation. D. Mucus scores in PAS-stained lung sections from mice at disease initiation and H. exacerbation. Serum anti-OVA IgG1 and IgE antibody titres for mice at .I. disease initiation or J. disease exacerbation. Groups include naïve mice ✳, PBS alone ×, Tendergreen bean ■, αAI pea ▲ and nGM pea △ gavaged mice. Data are expressed as mean $OD_{450\ nm} \pm$ SEM; n = 8, duplicate samples. For IgE, dilutions are expressed $\times 10^3$. Data are expressed as means $\pm$ SEM; n = 8. For eosinophil counts in BAL and lungs, data were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison test. For histological scoring, data were compared with the Mann Whitney test. *$p<0.05$ for all groups above the PBS controls. These are representative data from 2 experiments.
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and nGM- cowpeas and chickpeas. We did not expect the responses to be higher in mice consuming heat-treated seed meals due to the denaturation of the proteins. However, we observed that some groups had higher eosinophilia in heat-treated compared to raw seed meals. We speculate that there are other components in the seeds that may affect the overall immune response to the seed meals and that these are influenced differentially during heat treatment.

Although adjuvant studies are not routinely used in the assessment of GMOs, the effect of αAI peas on a non-crossreactive protein, OVA was previously tested and shown to enhance OVA-specific immunogenicity [8]. To test the effect of αAI pea feeding on immune responses to OVA, we used a different approach in models of OVA-induced allergic disease. We fed mice with seed meals during OVA sensitization and lung challenge for the onset of allergic disease (Fig. 1D) or fed mice before re-challenging with aerosolized OVA to induce disease exacerbation (Fig. 1E). OVA immunization and aerosol challenge generates an intense allergic response characterized by eosinophilic airway and lung inflammation, mucus hypersecretion and OVA-specific antibody responses [15]. After recuperation, chronic lung inflammatory infiltrates remain and respond to re-exposure to OVA leading to disease exacerbation for the lifetime of the mouse. To test the adjuvant effect of αAI peas, we gavaged mice twice weekly for 4 consecutive weeks with the transgenic αAI and nGM peas, Tendergreen beans or PBS before disease initiation and exacerbation. Naïve mice had healthy lungs and no αAI immune responses (Fig. 4). PBS control mice (OVA immunized and challenged, PBS gavaged), however, illustrate the response to OVA with approximately 30% and 40% eosinophils within the airways for disease initiation and exacerbation, respectively, while neither pea nor bean feeding influenced OVA-induced airway inflammation at either phase of disease (Fig. 4A and 4E). Consumption of peas and beans did not affect the OVA-specific eosinophilic inflammation, mucus secretion or severity of lung inflammation seen on Luna-, H&E- and PAS-stained tissue sections (Fig. 4B–H and Fig. S3 and S4). Antibody responses to OVA were unaffected by feeding αAI pea and bean (Fig. 4I and 4J).

In summary, our results show that there is variation in antibody responses to αAIs, but that there was not an increased antibody response to the αAIs from transgenic legumes compared to the αAIs from beans. αAIs from transgenic legumes and beans have minor differences in post-translational modifications that appear to modify immunogenicity [5]. However, we show here that these differences in immunogenicity did not differentiate αAIs from transgenic legumes with those found in beans. All αAIs induced high IgG1 antibody titres and are thus, immunogenic irrespective of transgenic or non-transgenic source. In feeding experiments, we observed that mice fed transgenic and non-transgenic legumes had immune and allergic responses that were similar to those generated by both Pinto and Tendergreen beans. Furthermore, the responses to the non-transgenic peas were related to a cross-reactive response to pea lectin and the consumption of transgenic, non-transgenic and bean seed meals did not accentuate allergic responses to another non-cross-reactive allergen.

Our results are at odds with the previous study in which mice developed allergic responses to αAI peas but not to beans [8,16]. It is possible that the source of the mice and their normal baseline diets may play a role. The mice used in the Austrian experiments were purchased from Charles River Germany and maintained in a pathogen-free mouse room. The mice used in the Australian studies originated from the Jackson Laboratory and were bred at The John Curtin School of Medical Research by sibling mating for

at least 70 generations in an SPF Unit. These mice were maintained in the Australian Phenomics Facility by inbred sibling mating. The health status of the mice in Austria revealed that there were no pathological or commensal organisms or antibodies detected. These data are not available for the mice used in Australia. There are no data regarding gut microbiota in either mouse house. The diet in Austria was from SSNIFF and the Australian diet was produced by Gordon's Specialty Stock Feeds P/L in New South Wales. The most obvious differences between the two diets are in the sources of the dietary protein (animal vs. plant), fatty acid type, level of soluble fibre and level of vitamin supplementation (Tables S1, S2, S3). While any or all of these dietary differences could influence immune responses, it is unlikely that they could cause a differential response to pea and bean constituents. Another possibility could be that αAI peas and proteins used in the studies differed, but the αAI peas and the non-transgenic controls were from the same batches of seeds produced at CSIRO. Because the previous study showed that only αAI peas caused allergic responses in mice, we were surprised that not only did Tendergreen bean and Pinto bean induce allergic responses, but so did the non-transgenic peas. We discovered that pea lectin antibodies are generated upon consumption of peas and that this antibody crossreacts with αAI.

In conclusion, although our studies show that consumption of both peas and beans leads to immune and allergic responses to αAI and pea lectin in mice, it is still not clear that these immune responses are biologically relevant for humans. In other words, it is not known whether these peas and beans would induce symptomatic allergic responses or indeed be relevant in human disease. These data derive from mice utilizing highly manipulative exposure regimens and therefore, do not provide definitive evidence that αAI peas would be allergenic in humans. Importantly, non-transgenic peas induced similar allergic responses compared to the transgenic peas. The reason for this response is related to cross-reactivity to another protein in peas. The response in this study to αAI in non-transgenic peas and beans is difficult to reconcile with the lack of response in Prescott et al. Moreover, bean allergies in patients are rare. This study emphasizes the importance of repeat experiments in independent laboratories and illustrates that unexpected cross-reactive allergic responses upon consumption of plant products can occur in mice. We recommend that the use of mouse models for testing GMO allergenicity needs to be carefully evaluated on a case-by-case basis.

Supporting Information

Figure S1 Immune responses to αAIs upon i.n. immunization. Representative photomicrographs of lung from mice administered αAIs 6 times over a 3-week period. a. H&E stained lung sections at 10× objectives. b. PAS stained sections at 10× objective. These are representative data for individual mice (n = 8 in 2 experiments). Arrowheads indicate either areas of inflammation or mucus within lung epithelial goblet cells.
(TIF)

Figure S2 Inflammation and mucus secretion following consumption of raw αAI and nGM pea, chickpea and cowpea and Tendergreen and Pinto beans. Representative photomicrographs of lung from mice administered bean, transgenic and non-transgenic peas, chickpeas and cowpeas for 1 month. a. H&E stained lung sections at 10× objectives. b. PAS stained sections at 10× objective. These are representative data for individual mice (n = 8 in 2 experiments). Arrowheads indicate either areas of inflammation or mucus within lung epithelial goblet cells.
(TIF)

Figure S3 Adjuvant effect of consuming raw αAI pea and bean seed meals on acute disease initiation. Representative photomicrographs of lung from naïve BALB/c mice are compared with OVA-immunized and challenged mice gavaged with either PBS, or Tendergreen bean, αAI peas, nGM pea seed meal. a. H&E stained lung sections at 10× objectives. b. PAS stained sections at 10× objective. These are representative data for individual mice (n = 8 in 2 experiments). Arrowheads indicate either areas of inflammation or mucus within lung epithelial goblet cells. (TIF)

Figure S4 Adjuvant effect of consuming αAI pea and bean seed meals on disease exacerbation. Representative photomicrographs of lung from naïve BALB/c mice are compared with OVA-immunized, challenged and then rechallenged mice gavaged with either PBS or Tendergreen bean, αAI peas, nGM pea seed meals. a. H&E stained lung sections at 10× objectives. b. PAS stained sections at 10× objective. Arrowheads indicate either areas of inflammation or mucus within lung epithelial goblet cells. (TIF)

Table S1 Comparison of ingredients between Australian and Austrian diets. (DOCX)

Table S2 Comparison of crude materials between Australian and Austrian diets. (DOCX)

Table S3 Nutrient analysis Australian and Austrian diets. (DOCX)

Author Contributions

Conceived and designed the experiments: ME TJH. Performed the experiments: RL DR AM. Analyzed the data: ME RL DR TJH AM GD. Contributed reagents/materials/analysis tools: TJH. Wrote the paper: ME TJH.

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ARTICLE

Comparison of the α-Amylase Inhibitor-1 from Common Bean (*Phaseolus vulgaris*) Varieties and Transgenic Expression in Other Legumes—Post-Translational Modifications and Immunogenicity

Peter M. Campbell,[*,†] Daniela Reiner,[‡] Andrew E. Moore,[§] Rui-Yun Lee,[‡] Michelle M. Epstein,[‡] and T. J. V. Higgins[§]

[†]CSIRO Ecosystem Sciences, P.O. Box 1700, Canberra, ACT, 2601, Australia

[‡]Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, Medical University of Vienna, Austria

[§]CSIRO Plant Industry, P.O. Box 1600, Canberra, ACT, 2601, Australia

ABSTRACT: The seeds of peas (*Pisum sativum*) and chickpeas (*Cicer arietinum*) expressing a gene for α-amylase inhibitor-1 (αAI) from the common bean (*Phaseolus vulgaris*) are protected from damage by old world bruchids (pea and cowpea weevils). Here, we used electrospray ionization time-of-flight mass spectrometry to compare the post-translational modifications of αAI from transgenic sources with the processed forms of the protein from several bean varieties. All sources showed microheterogeneity with differences in the relative abundance of particular variants due to differences in the frequency of addition of glycans, variable processing of glycans, and differences of C-terminal exopeptidase activity. The structural variation among the transgenics was generally within the range of the bean varieties. Previously, mice showed allergic reactions following ingestion of transgenic pea αAI but not bean αAI. Here, only minor differences were observed following intraperitoneal sensitization. Both of the transgenic pea and bean forms of αAI elicited Th1 and Th2 antibody isotype responses, suggesting that both proteins are immunogenic and could potentially be allergenic.

KEYWORDS: Transgenic α-amylase inhibitor, post-translational modification of transgenic protein, immunogenicity, glycosylation of transgenic proteins, transgenic pea, transgenic chickpea

■ INTRODUCTION

Pea seeds (*Pisum sativum*) expressing a gene for α-amylase inhibitor-1 (αAI) from the common bean (*Phaseolus vulgaris*) are protected from damage by old world bruchids (pea and cowpea weevils).[1,2] Protection from these major insect pests also makes this an attractive strategy for other susceptible legume crops. This particular transgene was attractive for use in food crops because humans already consume αAI in beans without any known ill effect. However, Prescott et al.[3] found that the matrix-assisted, laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrum of purified, pea-expressed αAI was not identical with the spectrum of αAI derived from the same gene in beans. The differences were assumed to reflect variations in post-translational modifications, but the mass accuracy and resolution were not sufficient for precise interpretation. That finding of nonidentity meant that it was not possible to assume that transgenic αAI would be functionally equivalent to the protein from beans in all respects. Indeed, ingestion of the pea form but not the bean form appeared to induce allergic responses in mice.[3]

The αAI protein from various cultivars of *P. vulgaris* has been characterized extensively.[4−8] It is derived from a prepro-protein by removal of a signal peptide, processing through the ER and Golgi where glycans are added, and then processed (Figure 1) followed by transport to protein storage vacuoles, where it is cleaved at two sites C-terminal to asparagine residues to yield α- and β-chains [9] (Figure 2). The resultant C-terminal Asn residue of the α-chain is then removed by a presumed carboxypeptidase.

The dominant form of the α-chain has both of its glycosylation sites occupied almost exclusively by high mannose glycans, $Man_6GlcNAc_2$ (Man6) and $Man_9GlcNAc_2$ (Man9), resulting in a mass of 11647 Da, while minor forms differ by truncations of one to several mannose residues with only trace amounts of any other glycoforms.[5,8] The dominant form of the β-chain has one of its two potential glycosylation sites occupied with $Man_3XylGlcNAc_2$ (MMX), resulting in a mass of 16526, but mass spectra show minor forms with higher and lower masses. MMX accounted for about 70% of the glycan from the β-chain, while various high mannose and fucosylated glycans accounted for the balance.[5] For two *P. vulgaris* cultivars and one of *Phaseolus coccineus* (Scarlet Runner Bean), the degree of glycosylation at the first site was 70−95%, predominantly by MMX.[8] The second site was 50 or 70% occupied in two cultivars, predominantly by MMXF, but one cultivar lacked the glycosylation site due to an amino acid substitution.

Here, we used higher resolution mass spectrometry than Prescott et al.[3] to show that the structural differences between pea and bean forms of αAI can be attributed to subtle differences in glycan and carboxypeptidase processing of both the α- and the β-chains. The immunogenicity of pea- and bean-expressed αAIs was compared by intraperitoneal immunization of mice. The structural comparisons were extended to include forms of αAI from other *P. vulgaris* cultivars and another transgenic *P. sativum* cultivar as well as
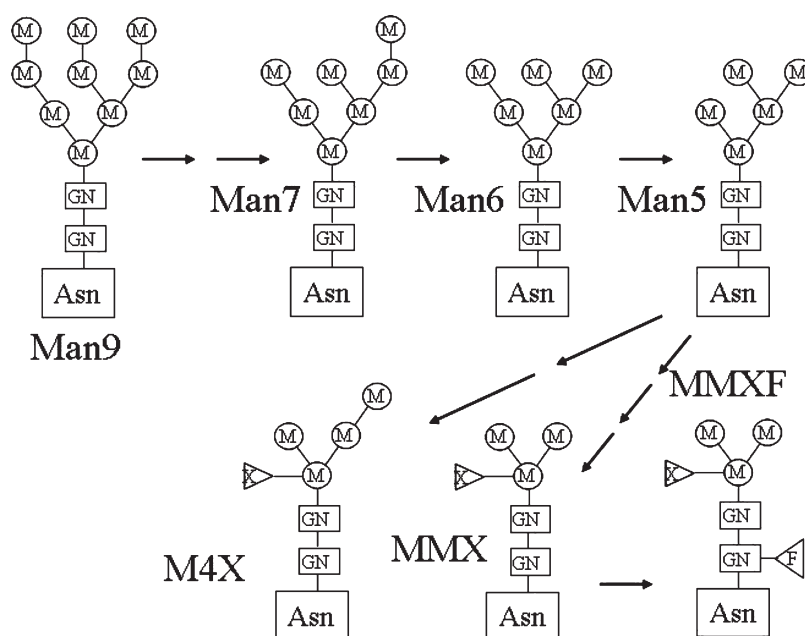
Figure 1. Simplified glycan processing in plants. *N*-Glycosylation of proteins in plants and mammals starts in the endoplasmic reticulum by the transfer of Glc$_3$Man$_9$GlcNAc$_2$ to an appropriate asparagine residue. With transport through the endoplasmic reticulum, the Golgi apparatus, and the vacuole, sugars are removed converting the precursor to high mannose-type *N*-glycans ranging from Man$_9$GlcNAc$_2$ (Man9) to Man$_5$GlcNAc$_2$ (Man5). Further trimming of mannose (M) and the addition of fucose (F) and/or xylose (X) residues produce some of the plant-specific glycans not found in mammals (M4X, MMX, and MMXF). GN indicates *N*-acetyl glycosamine. Adapted from Gomord and Faye[23] with abbreviations according to the proglycan system (www.proglycan.com).

in chickpeas (*Cicer arietinum*), another legume, to survey the range of variability of processing.

■ MATERIALS AND METHODS

Isolation of α-Amylase Inhibitors. The transformation of field peas and chickpeas for expression in their seeds of the αAI gene from the common bean (*P. vulgaris*, cv. Tendergreen) has been described previously.[1,10,11] The field pea varieties, Excell and Laura, and the chickpea cultivar are readily available in Australia, the Tendergreen bean was kindly provided by Maarten Chrispeels, University of California (San Diego, CA), and other varieties of common bean (Pinto, Cannellini, and Red Kidney) were purchased from an Australian health food shop.

αAIs from the seeds of the various beans and transgenic legumes were purified as previously described.[12] Briefly, seed meal was extracted with a NaCl solution (1%) followed by a heat treatment (70 °C), dialysis, and centrifugation. The inhibitors were enriched by anion exchange (DEAE-Sepharose CL-6B, Pharmacia) and gel filtration (Sephacryl S-200, Pharmacia) chromatography. Active fractions were determined by inhibition of porcine pancreatic α-amylase (Ceralpha: α-Amylase Assay Kit, Megazyme International, Ireland), and the most pure fractions were determined by inspection of Coomassie-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) gels. Finally, the appropriate pooled fractions were dialyzed against water, lyophilized, and stored at 4 °C. Aliquots (3 μg) from the various preparations were analyzed by SDS-PAGE on 10% NuPAGE gels (Invitrogen) using MES running buffer according to the manufacturer's instructions and staining with Coomassie (Figure 3). The relative amounts of protein in bands within lanes were estimated by densitometry using an ImageScanner with ImagequantTL software (GE Healthcare).
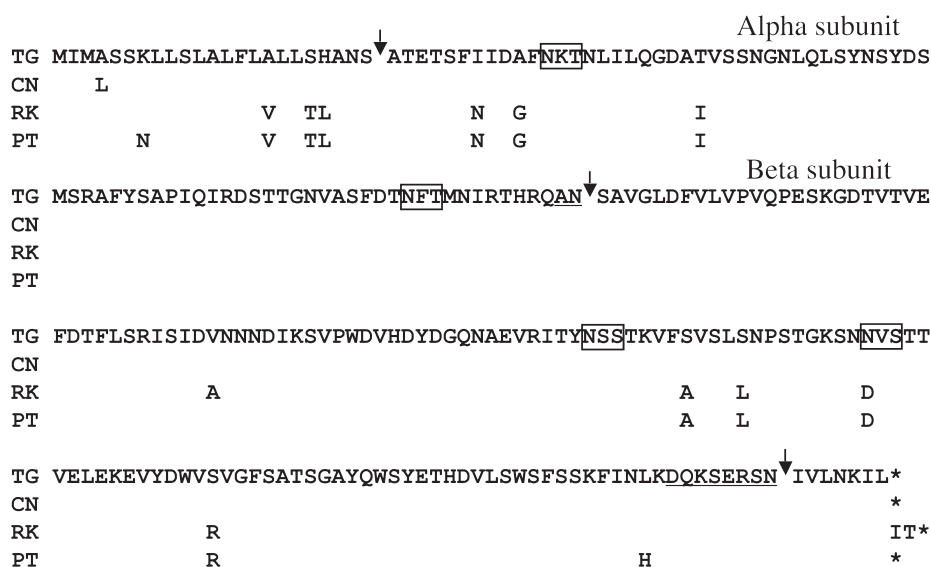
Identification of Protein Bands from SDS-PAGE. Bands from SDS-PAGE were analyzed by in-gel tryptic digestion with C18 reversed phase separation and tandem mass spectrometry of the resultant peptides using an Agilent 1100 capillary LC-ion trap MSD instrument

and searching of protein sequence databases with the mass spectral data as previous described.[13,14] The Viridaeplantae subset of the NCBInr protein sequence database (21/1/2011) includes sequences of αAI and other proteins from the seeds of legumes. Protein identifications were accepted with the stringent "autovalidation" settings of the Agilent SpectrumMill software as previously described.[13,14]

Deglycosylation of α-Amylase Inhibitors. Aliquots of purified αAIs were chemically deglycosylated with trifluoromethanesulfonic acid and 10% anisole scavenger using reagents and methods supplied in the GlycoProfile IV kit from Sigma. The method was essentially as used with αAI by Yamaguchi[4] and was reviewed more generally by Edge.[15] The deglycosylated proteins were recovered with a variant of TCA/acetone precipitation (2D Clean-Up Kit, GE Bioscience).

Chromatography and Mass Spectrometry of Intact α-Amylase Inhibitors. Purified αAI protein, with or without deglycosylation, was dissolved in 0.01% trifluoroacetic acid to approximately 1 mg/mL and analyzed with an Agilent 1100 series LC/MSD TOF system. A well-plate autosampler injected 10 μL of each sample to a Waters Symmetry300 C4, 5 μm, 4.6 mm × 50 mm column. The column was eluted with a constant 0.6 mL/min. Washing with 0.1% formic acid in water (2 min) was followed by a linear gradient to 100% acetonitrile/ 0.1% formic acid over 10 min and then held for 5 min at 100% acetonitrile. Finally, the column was reequilibrated with 0.1% formic acid in water for 5 min prior to the next injection. Material eluting from the column was monitored by UV absorption (220 nm) and the mass spectrometer. In each separation, the column elution profile by UV absorption closely followed the profile of total ion counts.

Electrospray and mass spectrometer instrument settings and the use of an internal mass standard were all as recommended by the manufacturer.[16] Mass spectra from across each chromatographic peak were averaged and deconvoluted using Protein Confirmation Software supplied with the instrument.[16] The software also calculates areas for peaks in the deconvoluted spectra. Peaks in each spectrum were interpreted as matching particular polypeptide chains with or without common plant glycans attached. For clarity, experimental masses are not

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                                                    ↓                          Alpha subunit
TG  MIMASSKLLSLALFLALLSHANS ATETSFIIDAF NKT NLILQGDATVSSNGNLQLSYNSYDS
CN      L
RK                    V   TL            N   G              I
PT              N     V   TL            N   G              I
                                                       ↓    Beta subunit
TG  MSRAFYSAPIQIRDSTTGNVASFDT NFT MNIRTHRQAN SAVGLDFVLVPVQPESKGDTVTVE
CN
RK
PT

TG  FDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITY NSS TKVFSVSLSNPSTGKSN NVS TT
CN
RK              A                                A   L         D
PT              A                                A   L         D
                                                            ↓
TG  VELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLK DQKSERSN IVLNKIL*
CN                                                              *
RK              R                                            IT*
PT              R                                   H        *
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Figure 2. α-Amylase inhibitors from *P. vulgaris*. The amino acid sequence shown is the translation of the DNA sequence from the gene in the Tendergreen (TG, P02873) variety (and is the sequence that was used for the transformation of the other legumes). Residues that differ in other bean varieties are indicated for Cannellini beans (CN, EF087992), Red Kidney beans (RK, EF087993), and Pinto beans (PT, AY603476). Arrows indicate the cleavage sites that produce the α- and β-chains of the mature protein. Sequons for N-linked glycosylation are boxed but note that the second glycosylation site in the β-chain is lost due to an Asn to Asp substitution in two of the bean varieties. Underlined residues are removed from the C termini of some of the α- and β-chains as indicated in Figures 3–5.
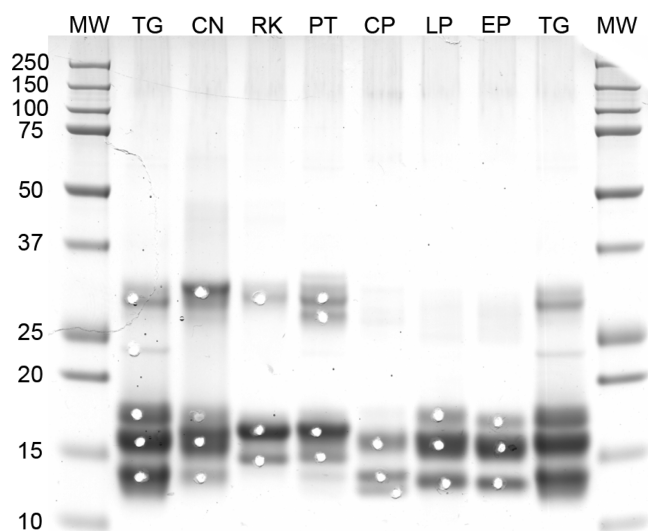
Figure 3. SDS-PAGE of αAI preparations from bean varieties and transgenic legumes. Left to right are molecular mass marker proteins (MW, kDa), αAI from bean varieties, Tendergreen (TG), Cannellini (CN), Red Kidney (RK), and Pinto (PT), transgenic chickpeas (CP), and pea varieties, Laura (LP) and Excell (EP), and then, Tendergreen bean αAI and marker proteins are repeated. Clear circles are visible where gel plugs were excised for identification of the proteins in the bands.

shown for most of the mass spectral peaks in the various figures. However, in each case, they were within 30 ppm or less than 0.5 Da of the theoretical masses of the molecules assigned to them.

Mice. Four to six week old female BALB/c mice were purchased from Charles River Germany and maintained in the Veterinary Medicine University of Vienna animal facility. Mice were provided food and water ad libitum. All experimental protocols were performed in compliance with the appropriate Austrian laws and University guidelines. The

protocol for the experiments was approved directly by the Austrian Ministry of Science (68205/12-II/108/209).

Immunogenicity of α-Amylase Inhibitors in Mice. Groups of five mice were immunized intraperitoneally (ip) on days 0 and 21 with 200 μL of phosphate-buffered saline (PBS) or 10 μg of αAI from transgenic Excell pea or Tendergreen bean dissolved in PBS. On day 28, blood was collected by cardiac puncture using a 27G$\times^{3/4}$ needle, and after centrifugation, sera were stored at −20 °C. Sera for the measurement of αAI-specific antibodies were tested by standard sandwich enzyme-linked immunosorbent assay (ELISA). For the measurement of antigen-specific IgG1, IgG2a, and IgE, ELISA plates were coated with a solution of dissolved Tendergreen bean or transgenic pea αAI at 10 μg/mL overnight at 4 °C. The plates were washed and blocked with 2% BSA in PBS with 0.05% Tween 20 for 2 h at room temperature. Dilutions of serum from each individual mouse were then incubated in duplicate wells for 24 h at 4 °C. After they were washed, plates were incubated for 2 h at 4 °C with biotinylated monoclonal antibodies to detect IgG1, IgG2a (Southern Biotechnology Associates Inc., Birmingham, AL), or IgE (Becton Dickinson Biosciences, NJ), followed by incubation with streptavidin horseradish peroxidase (Becton Dickinson Bioscience) for 1 h at room temperature. Plates were washed and incubated with a TMB-OptEIATM substrate solution (100 μL) (Becton Dickinson Bioscience) in each well. Following 10 min of incubation in the dark at room temperature, dye development was stopped with the addition of 100 μL of 0.18 M H_2SO_4, and the optical density was measured at 450 nm. Each ELISA was repeated at least twice.

■ RESULTS

Isolation and Electrophoresis of α-Amylase Inhibitors. The bean αAI gene was transferred previously to two field pea cultivars and chickpeas, where it resulted in the expression of αAI to 2–4% of the total soluble protein in the seeds.[10,11] The various preparations of αAI from peas, chickpeas, and the various beans appeared substantially pure by SDS-PAGE (Figure 3) and similar to each other in their activity as inhibitors. In each case, 100–150 ng of
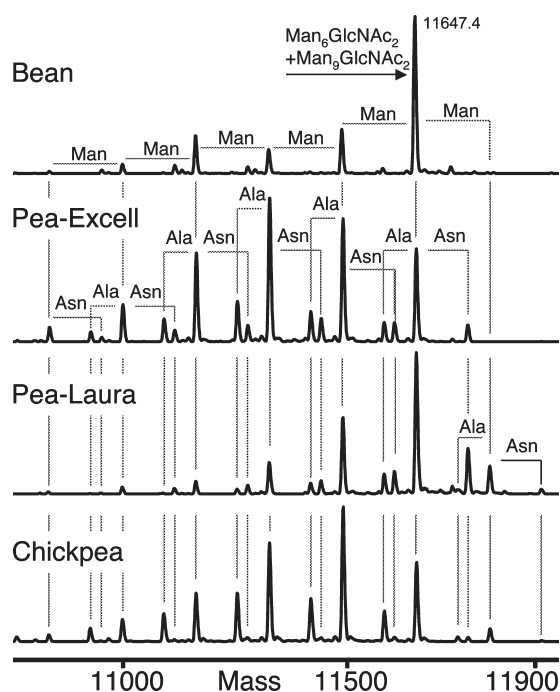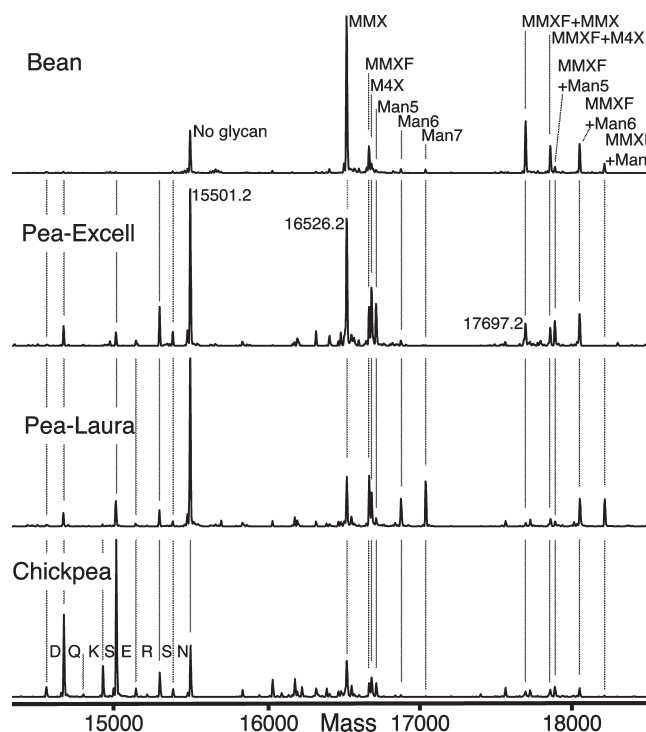
Figure 4. Deconvoluted mass spectra of the α-chain of αAI from *P. vulgaris* cv. Tendergreen (bean) and transgenic peas and chickpeas. The major peak at 11647 Da corresponds to the major form of the α-chain in beans. The major series of mass differences (Man) corresponds precisely to the removal of different numbers of mannose residues from the two high mannose glycans attached to the molecule. Two minor series of mass differences are seen the various transgenics. These are related to the adjacent major glycoform peak by to the removal of an alanine residue (Ala) or the failure to remove an asparagine residue (Asn) from the C terminus of the peptide (Figure 2). Mass spectra for other varieties of *P. vulgaris* are not shown but were virtually identical with the spectrum shown for Tendergreen.

Figure 5. Deconvoluted mass spectra of the β-chain of αAI from *P. vulgaris* cv. Tendergreen and transgenic peas and chickpeas. The major peak at 15501 Da corresponds precisely with the predicted mass of the β-chain without added glycan or other modifications, and the prominent peak at 16526 Da corresponds to the previously demonstrated major glycoform from beans. Peaks in the range up to 17042 are interpreted as indicated by proposing single, alternative glycans, while peaks in the range 17692−18214 are interpreted by proposing glycosylation at both possible sites. In the chickpea β-chain, a series of peaks below 15501 Da show mass separations corresponding to eight residues of the C-terminal sequence of the polypeptide (Figure 2). These truncated forms are also detected to varying degrees in the β-chains from the other legumes. More complex patterns of minor peaks elsewhere in the spectra are consistent with similar C-terminal truncations of the various glycoforms, but for clarity, these have not been annotated. The relative areas of the annotated peaks are shown in Table 1.

αAI preparation caused 50% inhibition of 100 ng of porcine α-amylase (data not shown). Bands from the gel were sampled for identification by mass spectrometry. Across the entire experiment, αAI was identified by 14 distinct tryptic peptides representing 42% coverage of the precursor sequence with a SpectrumMill summed MS/MS search score of 226 where 20 would be sufficient for a confident automatic identification.

The major bands from each sample ran close to each other, between the 10 and the 20 kDa marker proteins, at around 15 kDa (Figure 3). Both subunits of αAI were generally detected in each of these bands. The α-subunit was generally detected only by the peptide, AFYSAPIQIR. The β-subunit was generally detected by 4−9 peptides, generally including the peptide from its N terminus, SAV...ESK. No other protein was detected from these major bands.

The minor bands around 30 kDa also contained peptides from both subunits of αAI. A peptide that spans the site that is cleaved in the mature protein (QANSAV...ESK, Figure 2) was detected from Pinto and Cannellini beans, suggesting that αAI in the 30 kDa bands is not fully processed. The faint band at about 23 kDa from Tendergreen bean yielded two peptides from the β-subunit.

Two contaminating proteins were identified from the minor bands around 30 kDa. Bean phytohemagglutinin (PHA, trEMBL Q8RVX5) together with unprocessed αAI was found in the upper 30 kDa band from Pinto bean. The lower 30 kDa band from Pinto bean was identified as α-amylase inhibitor-like protein (AIL, GenBank BAA86927). PHA and AIL have 50 and 58% amino acid sequence identity, respectively, with the αAI precursor, but they differ from αAI by not being proteolytically processed to form subunits.[17] AIL was not detected from the other beans, and PHA was not detected from Red Kidney bean. The 30 kDa band from Tendergreen bean was mostly unprocessed αAI, and by densitometry on the stained gel, it was about 15% of the total preparation. Comparison of the total ion counts for tryptic peptides suggested that PHA accounted for about 17% of the band or about 2.5% of the Tendergreen bean preparation overall. PHA was much less than 1% of the preparation from Cannellini bean.

Separation of the Subunits of α-Amylase Inhibitors. Purified αAI preparations from peas, chickpeas, and the various beans, with or without deglycosylation, were separated by C4 reversed phase liquid chromatography. This served as a convenient group separation to ensure that the subunits were dissociated from each other and any minor contaminants while being desalted for electrospray ionization and time-of-flight

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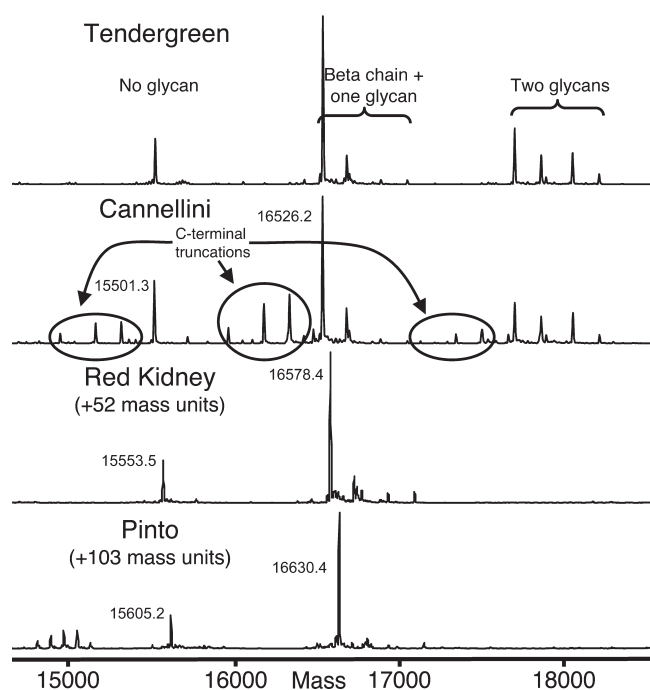
Figure 6. Deconvoluted mass spectra of the β-chain of αAI from *P. vulgaris* varieties. The spectrum for the Tendergreen variety is the same as shown in Figure 5. The amino acid sequence of the β-chain of the Cannellini variety is the same as Tendergreen, while amino acid differences in the Red Kidney and Pinto varieties resulted in mass shifts of +52 and +103, respectively, and the loss of one of the glycosylation sites. Peaks consistent with the removal of residues from the C terminus (like Figure 5) were seen clearly for the Cannellini variety but barely detected for the others. The peaks around 15000 mass units for the Pinto variety appear to be due to a minor contaminant.

mass spectrometry. Generally, three peaks were observed by both UV absorption and a total ion trace from the mass spectrometer. The first contained some low molecular weight contaminants (not shown), the second contained variants of the α-chain of αAI (Figure 4), and the third contained the β-chains (Figures 5 and 6). All samples showed at least some material that eluted in the first peak with acetonitrile concentrations up to 57%. None of the early eluting material was consistent with αAI, and it was not consistent between αAI preparations from different species. The molecular weights of the early eluting material were generally lower than the αAI α-chain. In the case of Tendergreen bean αAI, the mass spectrum of the early eluting material closely resembled the 8–9000 *m/z* region of the MALDI-TOF mass spectrum shown by Prescott et al.[3] and mentioned by Young et al.[7]

The second peak was eluted sharply from all preparations with 59–63% acetonitrile. Averaging and deconvoluting the mass spectra from across this peak showed that various forms of the αAI α-chain coeluted. In the case of Tendergreen bean, Pinto bean, and Excell pea, the spectra aligned precisely with the previously published MALDI-TOF mass spectra[3] but showed much improved resolution and mass accuracy (Figure 4).

The third peak was broader but consistently eluted from all preparations when the solvent gradient exceeded 65% acetonitrile. This peak contained coeluting variants of the β-chain. As above, the spectra for the β-chain variants aligned precisely with the relevant region of the previously published MALDI-TOF

mass spectra[3] (Figures 5 and 6). Furthermore, within each α- or β-chain set, the relative intensities of major and minor peaks were consistent with the corresponding MALDI-TOF spectra. This adds to our confidence that the molecules are ionized in close proportion to their relative abundance within each set of subunit variants. Resolution and mass accuracy were sufficient to allow unambiguous assignment of all of the major peaks in each spectrum.

Mass Spectral Analysis of the α-Subunit of αAI: Transgenics as Compared with Tendergreen Bean. Mass spectra for the α-chain variants from the transgenic sources are shown aligned with the corresponding spectrum for the α-chain from the Tendergreen bean (Figure 4). The same glycoforms were seen for each legume, but they differed in their relative mass spectral intensity. For example, the most abundant glycoform in Excell peas had two fewer mannose residues than the major bean glycoform, whereas the most abundant glycoform in chickpeas had one less mannose residue. On the other hand, the distribution of glycoforms in Laura pea was quite similar to the bean except that it also had a significant amount of a glycoform with one more mannose than the dominant glycoform of the bean. These shifts in the distribution of glycoforms could be due to slight differences in the activity of the enzyme that trims mannose residues in the order Excell pea > chickpea > bean > Laura pea.

Between the peaks corresponding to the glycoforms discussed above were less intense peaks in the mass spectra (Figure 4). For all three transgenic sources, these peaks aligned precisely, but they did not correspond to very minor peaks that can be seen in the bean spectrum. These less intense peaks differed from adjacent glycoform peaks by either +114 or −71 Da. Recalling the known processing of αAI in the bean, the mature α-chain has glycans added and an asparagine residue removed from the C terminus.[7] The +114 peaks are precisely consistent with a subset of molecules retaining the C-terminal Asn residue, while the −71 peaks are consistent with a subset of molecules having both the C-terminal Asn and the next C-terminal residue, alanine, also removed. Thus, it would appear that the presumed carboxypeptidase in the bean is very precise, removing the asparagine residue entirely but not any further residue, while the equivalent enzymes in the transgenics are less precise. In Excell pea, 13 ± 1% of the protein corresponding to any particular glycoform retained the Asn, while 19 ± 2% had both Asn and Ala removed. Laura pea had more protein retaining Asn (24 ± 4%) and less with Ala also removed (10 ± 1%). Chickpea had much less protein retaining Asn (5 ± 2%) and much more with Ala also removed (30 ± 4%). The small standard deviations for all of these data indicated that the extent of C-terminal processing was independent of the extent of processing of the attached glycans.

From the above analysis, deglycosylation would be expected to produce an homogeneous α-chain from the bean, while two further forms (+114 Da, Asn; and −71 Da, Ala) would be produced in the transgenics. Rather than seeing simple peaks following deconvolution, tight envelopes of peaks about 70 mass units wide were seen at approximately the expected masses (not shown). This microheterogeneity was probably due to a complex array of side reactions during deglycosylation involving Ser, Thr, Asp, Cys, Met, and aromatic residues.[15] However, these envelopes of peaks were separated precisely as expected (+114, −71) and with ratios of abundance consistent with the analysis above.

Mass Spectral Analysis of the β-Subunit of αAI: Transgenics as Compared with Tendergreen Bean. Mass spectra for the β-chain variants from the transgenic sources are shown

Table 1. Relative Abundances of Glycoforms and C Terminally Truncated Forms of the α-Amylase Inhibitor β-Chain from Bean Varieties and Various Transgenic Legumes[a]

	no glycan	MMX	MMXF	M4X	Man5	Man6	Man7	one glycan totals	MMXF + MMX	MMXF + M4X	MMXF + Man5	MMXF + Man6	MMXF + Man7	two glycans totals	% C-terminal truncations
bean TG	**11**	42	7	3	1	1	<1	**56**	14	7	2	8	2	**33**	<1
bean CN	**13**	33	9	4	2	2	<1	**51**	9	11	2	12	2	**37**	52
bean RK	**14**	55	9	8	6	5	4	**86**	0	0	0	0	0	**0**	<1
bean PT	**16**	69	2	3	4	1	5	**84**	0	0	0	0	0	**0**	<1
pea Excell	**27**	25	7	12	8	1	<1	**53**	5	3	5	6	<1	**20**	37
pea Laura	**34**	11	11	8	2	7	10	**49**	<1	2	1	7	6	**17**	30
chickpea	**29**	24	8	12	8	<1	<1	**52**	3	5	6	5	<1	**19**	87

[a] The areas of mass spectral peaks annotated in Figures 5 and 6 are expressed as percentages (omitting peaks due to C-terminal truncations). In the right-most column is the percentage of nonglycosylated forms showing truncations of 1−8 residues from the C terminus. Bean varieties are Tendergreen (TG), Cannellini (CN), Red Kidney (RK), and Pinto (PT).

aligned with the corresponding spectrum for the α-chain from the Tendergreen bean (Figure 5). As with the α-chain, it is apparent that the same glycoforms are seen for each, but they differ considerably in their relative abundances.

Considering first the spectrum for the Tendergreen bean form of the β-chain, the major peak at 16526 Da corresponded to the previously described major glycoform.[7] Peaks in the range of 16672 to 17042 Da were consistent with the mass of the polypeptide with alternative, single glycans attached. A peak at 15501 Da corresponded to the mass of the polypeptide without any glycan or other modification, and peaks in the range 17697−18214 Da were consistent with the polypeptide with various combinations of two glycans. The relative abundances of the various glycoforms (Figure 5 and Table 1) were broadly consistent with the identities and relative abundances of glycans that were cleaved, isolated, and identified from the β-chain of αAI from another bean cultivar.[5] Specifically, a major bean glycoform (42% of the total) had one glycan comprising the mannose core with a xylose residue attached (MMX). Glycoforms with single alternative glycans attached were less abundant: The mannose core with both a xylose and a fucose residue attached (MMXF, 7%), a glycan identical to the major form except for one more mannose residue (M4X, 3%), and high mannose glycans (Man5, Man6, and Man7, each about 1%). In total, 56% of the polypeptide had a single glycan attached. The nonglycosylated polypeptide accounted for about 11% of the total. The remaining 33% of the total consisted of polypeptides with two glycans attached. Their masses were consistent with one MMXF glycan in combination with MMX (14%), M4X (7%), Man5 (2%), Man6 (8%), or Man7 (2%). Other peaks consistent with various C-terminal truncations were only barely detected.

The various transgenic β-chains all differed from the Tendergreen bean β-chain first by showing less overall glycosylation and second by showing significant C-terminal truncations (Figure 5 and Table 1). In comparison with bean (ignoring the C-terminally truncated forms for the moment), the Excell pea and Laura pea showed less polypeptide with two glycans (22 and 17%, respectively), about the same with one glycan (60 and 49%), and more with no glycan (18 and 34%). Similarly, chickpea showed less with two glycans (18%), about the same with one glycan (49%) and more with no glycan (27%). In particular, it can be seen that the major MMX glycoform was more prominent than the nonglycosylated form in the spectrum for the bean β-chain, but the converse was observed in the transgenics (Figure 5).

A series of eight peaks around 15000 Da corresponded precisely with the masses predicted by the sequential removal of amino acid residues from the C terminus of the nonglycosylated polypeptide (Figure 5). These truncated forms were most prominent for chickpea. Corresponding peaks were seen for the other transgenics and Cannellini bean but barely detected in the other beans (Figure 6). Comparing the areas of the peaks for the truncated forms of the nonglycosylated β-chains, we calculate that virtually 100% of the Tendergreen bean β-chain was not truncated, 37 and 30% are C terminally truncated to some degree in Excell pea and Laura pea, respectively, 52% is truncated in Cannellini beans, while only 13% was not truncated in chickpea (Table 1). More complex arrays of minor peaks at masses below the singly and doubly glycosylated peaks (Figure 5) were consistent with the same C-terminal truncations applied to the various glycoforms, but for clarity, we have not attempted to annotate these. As was described above for the α-chain, it would appear that presumed carboxypeptidases in the transgenics act less precisely on this peptide than the bean's enzyme. It is possible that the truncations reflect degradation of the expressed protein rather than differences of processing, but the chickpea αAI is just as active as an inhibitor as the other preparations (data not shown). These results are reminiscent of the variable C-terminal trimming of lectins in the seeds of different legumes, which sometimes has structural consequences and sometimes not.[18]

As with the α-chain, chemical deglycosylation resulted in envelopes of peaks at approximately the masses one would predict from the above analysis and at precisely the expected mass separations between those envelopes, and ratios of relative abundance were also consistent (not shown).

Comparison of α-Amylase Inhibitor from *P. vulgaris* Varieties. The αAIs from several *P. vulgaris* varieties were compared first by DNA sequencing and then by mass spectrometry as above. None had identical sequences (Figure 2), but the amino acid sequences of the Tendergreen, Red Kidney, and Pinto αAIs are, respectively, the same as the TAI, DAI, and MAI αAIs.[8] The Cannellini variety had one predicted amino acid difference from the Tendergreen variety, but this is not in the mature protein. The Red Kidney and Pinto varieties shared three amino acid differences from Cannellini and Tendergreen in the α-chain, resulting in a difference of one mass unit, which was clearly detected in the mass spectra. The α-chain mass spectra of the bean varieties (not shown) were virtually identical to the Tendergreen variety (Figure 4) aside from that one mass unit shift and trace levels of glycoforms with one or two additional mannose residues from the major form.

The spectrum of the β-chain of Cannellini bean resembled Tendergreen very closely (Figure 6 and Table 1) except that 52% of the protein showed truncations of up to five C-terminal residues, thereby resembling the various transgenics (Figure 5). The β-chains of Cannellini and Tendergreen had identical amino
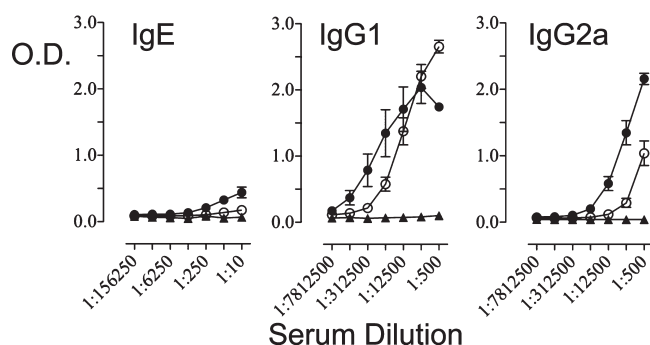
Figure 7. Serum anti-αAI antibody response in mice. BALB/c mice were immunized with 10 μg of Excell pea αAI (○), Tendergreen bean αAI (●), or PBS (▲) ip on days 0 and 21. On day 28, blood was taken, and serum was separated and stored at −20 °C before use. We measured sera for αAI-specific IgG1, IgG2a, and IgE antibodies by standard sandwich ELISA. Data are expressed as the means ± SEMs.

acid sequences, but Red Kidney and Pinto both differed from Tendergreen at five sites (four in common with each other), resulting in mass differences of +52 and +103, respectively (Figure 6). A shared Asn to Asp substitution caused the more C-terminal of the two potential glycosylation sites to be lost in Red Kidney and Pinto beans, and this was reflected in the mass spectra that showed no peaks in the mass range where the other varieties had peaks attributed to the addition of two glycans. However, the relative intensities of peaks attributed to no glycosylation or a single glycan were consistent between the bean varieties (Table 1). For example, the spectrum for the Red Kidney bean β-chain superimposed very well over the Tendergreen spectrum except for the peaks attributed to two glycans after accounting for the shift of 52 mass units (Figure 6).

Immunogenicity of the Transgenic Pea and Tendergreen Bean αAIs. To determine whether there were immunological differences between differentially processed αAIs, mice were immunized intraperitoneally twice with either transgenic pea or Tendergreen bean αAI, and αAI-specific antibody responses were measured (Figure 7). Both forms of αAI induced antibody responses with the titers for IgG1 > IgG2a > IgE. Tendergreen bean αAI generally induced higher titers than the protein expressed in Excell pea, and IgE against the pea αAI was barely detected.

■ DISCUSSION

Previously, the Excell pea and Tendergreen bean forms of αAI were shown to be nonidentical by MALDI-TOF mass spectrometry and also appeared to be nonidentical in a mouse model for food allergy.[3] Here, we further analyzed the molecules using higher resolution mass spectrometry and compared the immunogenicity of the purified proteins in mice. We describe a wider range of post-translational modifications that occur among edible bean varieties, and we examined an additional pea cultivar and another legume, chickpea, transformed with the same gene. None of the modifications were unusual, or at least all of the observed masses were consistent with typical glycosylation in plants and known modifications of αAI. All of the molecular forms found among the transgenics were also found among the varieties of edible beans.

The α-chains of αAI from the beans and transgenic Excell pea showed the same set of glycoforms albeit in differing proportions.

These high mannose glycans are produced by the part of the glycosylation pathway that is common to plants and mammals. It therefore seems unlikely that the tendency to have one or two fewer mannose residues on an otherwise identical molecule would result in a difference of immunogenicity between the transgenic and the native α-subunits. A small proportion of pea αAI had one more amino acid residue or one less at the C terminus. The αAI from transgenic chickpea showed varying proportions of the same set of α-subunit glycoforms as the beans and also showed the more varied C-terminal processing like the Excell peas. While some of the transgenics have a glycoform with one more mannose residue than the major form of αAI in the beans, this was also detected at a trace level in two of the beans.

The variation in the β-chains was more complex. The Excell pea form had (1) relatively more molecules without added glycan, (2) fewer with two glycans, (3) more molecules with the Man5 and M4X glycans although these are minor components, and (4) more molecules with up to eight amino acid residues removed from the C terminus.

A further difference between the beans and the transgenics is that the beans appear to contain variable amounts of unprocessed or incompletely processed αAI precursor seen as bands around 30 kDa by SDS-PAGE (Figure 3).

Mice immunized with isolated αAI proteins from either Tendergreen bean or Excell pea generated Th1 and Th2 isotype antibodies albeit with very low titers of IgE for pea αAI. The Th2 type response (IgG1 and IgE) following intraperitoneal sensitization of BALB/c mice shows that both proteins are immunogenic and potentially allergenic.[19] The bean and pea proteins induced similar immune responses with the bean form minimally more immunogenic, perhaps due to the presence of incompletely processed αAI or about 2.5% PHA. Our data differ from an earlier in vivo study in which αAI from transgenic peas was more immunogenic and allergenic as compared to beans.[3] However, differences between the experimental protocols of the two studies may explain the disparate results. Here, we used isolated Tendergreen bean αAI (Tendergreen being the original source of the gene for peas and chickpeas) and isolated pea αAI for intraperitoneal immunization. In contrast, Prescott et al.[3] initially administered αAIs by the oral route as flours of transgenic pea or Pinto bean. They concluded that the mice were subsequently unresponsive to bean αAI relative to pea αAI. However, the Pinto form of αAI has eight amino acid differences from the Tendergreen αAI that was used for subsequent exposure to bean αAI, including one difference, which removes one of the two glycosylation sites in the β-chain (Figures 2 and 6 and Table 1).

Other animals than mice have been exposed to αAI from transgenic peas. Rats, pigs, and chickens were fed raw, transgenic peas at around 30% or more of their diet in short feeding trials. The only effects on their health could be attributed to dose-dependent reductions of the digestion of starch due to amylase inhibition rather than immunological effects, diarrhea in the case of pigs, and a reduction of weight gain in the case of chickens.[20−22]

We found no evidence for increased immunogenicity of the transgenic αAI, and we note that immunogenicity is not sufficient for allergenicity. Whatever their status as potential allergens, the pure proteins seem to induce similar immune responses, and the ranges of post-translational modifications are similar between the transgenic and the nontransgenic versions. How best to use animal models and other tools to predict the potential allergenicity of novel proteins in transgenic crops is an active area of continuing research and debate.[19] Ongoing work in our

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laboratories will compare the immunological consequences of oral exposure of mice to the transgenic and nontransgenically produced proteins.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: Peter.M.Campbell@csiro.au.

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