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Publications

- **Genetically modified α-amylase inhibitor peas are not specifically allergenic in mice.** *Rui-Yun Lee*, Daniela Reiner*, Gerhard Dekan, Andy Moore, T.J.V. Higgins, Michelle M. Epstein, PLoS ONE, January 2013*

- **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, T J V Higgins. Agric Food Chem., June 2011*

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Abbreviations

2-DE	Two-dimensional electrophoresis
AAI	Alpha-amylase inhibitor
AHR	Airway hyper responsiveness
Ag	Antigen
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
Bt	Bacillus thuringiensis
CD	Cluster of differentiation
ConA	Concanavalin A
Cry	Crystalline protein δ-endotoxin
DC	Dendritic cell
DTT	Dithiothreitol
°C	Degree Celsius
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal calf serum
GMO	Genetically modified organism
GvHD	Graft-versus-Host-Disease
H	Hours
H&E	Hematoxylin and Eosin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
kb	Kilo base(s)
kD	Kilo Dalton
LLP	Lectin-like protein
mAb	Monoclonal antibody

MHC	Major histocompatibility complex
min	Minutes
mg/ mL	Milligram(s)/Milli Liter(s)
mg/ µL	Milligram(s)/Micro Liter(s)
nm	Nanometer
nGM	non GM
OD	Optical density
OVA	Ovalbumin, chicken egg
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohemaglutinin
RBC	Red blood cell
RBL	Rat basophil leukemia
Rpm	Rotation per minute
RT	Room temperature
s	Second(s)
SCID	Severe combined immunodeficiency
SD	Standard deviation
SEM	Standard error of mean
Th	T helper
µg/µL	Microgram(s)/Micro Liter(s)

Summary

Transgenic field peas (*pisum sativum*) expressing the alpha-amylase inhibitor (AAI) protein normally found in common beans (Tendergreen bean) are completely protected from infestation with pea weevils tested in the laboratory, greenhouse and field over several generations, and at many sites for efficacy against the insect pests. Previously, transgenic AAI pea feeding in mice was shown to influence allergic responses to an unrelated, non-crossreactive allergen compared to Pinto bean-fed mice due to different glycosylation *(Prescott et. al.)*. However in their study, they used Pinto bean as control instead of Tendergreen bean which provides the transgene. To determine whether this is a universal finding, we analyzed the immunogenicity and allergenicity of native and transgenic AAI in a mouse model. We observed that both native and transgenic AAIs are immunogenic and allergenic irrespective of the source. These results indicated that AAIs expressed in genetically modified (GM) peas are not more harmful compared with AAI expressed in Tendergreen bean. Different glycosylation does not distinguish between GMO and native proteins. To determine the effect of oral administration of native beans and GM peas, we force fed mice with native and transgenic seed meal then challenged i.n. once with purified AAI. Key features of Th2 inflammation were analyzed and we observed that GMO feeding induces allergy to transgenic protein as seen in *Prescott* study. However, both Tendergreen bean and Pinto bean induced allergic response to AAI which differs from previous study. Mitogen pea lectin expressed in peas has 38 - 54% amino acid structure similarity to AAI. In our experimental mouse model, we investigated whether pea lectin influenced the immune response and our results indicated that both, non GM (nGM) pea and GM pea induced an allergic response to pea lectin. To determine whether AAI pea promoted an adjuvant effect to other antigens, we injected female BALB/c mice on days 0 and 21 with ovalbumin (OVA) i.p. and nebulized them with OVA on days 28 and 29 to initiate allergic asthma and then allowed mice to recover until they were re-exposed to OVA for the induction of a disease exacerbation. We force fed with GM pea (100 mg/ mL), nGM pea, Tendergreen bean, or PBS twice a week for 4 weeks prior to inducing disease or inducing disease exacerbation. We detected no differences in lung and airway inflammation, mucus hypersecretion and

allergen-specific antibody in AAI-pea fed compared to non GM pea-fed mice at the onset of allergic asthma or at the time of disease exacerbation. However, in acute onset disease, pea-fed mice had more severe lung inflammation and elevated mucus hypersecretion compared to Tendergreen bean-fed mice. We observed an increase of OVA-specific antibody production. Taken together, GM pea feeding does not worsen allergic response to OVA. All our experiments were performed with BALB/c mice but to have a better idea of GMO effect in human immunsystem, we created a human SCID mice chimera. PBMCs of legume allergic patient and healthy donor were isolated and engrafted into SCID mice which lack on T and B cells and subsequently force fed with native or transgenic seed meals. Our results showed that PBMCs isolated from healthy individuals or legume allergic patients engrafted into SCIDs developed allergic asthma upon feeding and challenge with GM peas and Tendergreen beans. In our model, we were not able to distinguish between healthy and allergic donor.

Our study indicates that native AAI extracted from beans is immunogenic in mice presented by different route of administration. However, we did not observe differences between native and transgenic AAI after immunization. Our observation does not accord with *Prescott* study. These results have important implications in the context of testing novel proteins in animal models whereas further studies are necessary for the risk assessment.

Zusammenfassung

Mit Hilfe der Gentechnik ist es möglich gezielte Erbinformationen in Nutzpflanzen zu ändern. In der australischen CSIRO Plant Industrie wurde die genetisch veränderte Nutzpflanze (*Pisum sativum*) entwickelt. Wissenschaftliche Ergebnisse zeigten, dass das Protein α-Amylase-Inhibitor (AAI) vorkommend in Bohnen, Pflanzen vor schädlichen Käfern beschützt. Insbesondere in Entwicklungsländern gehört die grüne Erbse zu einer der wichtigsten Nahrungsbestandteile. Durch den jährlichen Befall von Rüsselkäfern auf die Samen, haben australische Wissenschaftler das *AAI* Gen auf Erbsen übertragen, um sie vor Insektenbefall zu schützen. Wissenschaftliche Arbeiten zeigten, dass aufgrund der Unempfindlichkeit gegen Schädlinge, die transgene Erbse einen deutlich höheren Ertrag erbracht hat. Dennoch erweckt der Verzehr von GMO in uns Menschen Unbehagen, da man sich die Frage stellt, ob sie eine auf die Gesundheit schädigende Wirkung haben könnte. Es könnten neue Toxine oder Allergene im Lebensmittel entstehen. *Prescott et. al.* publizierte im Jahr 2005 eine wissenschaftliche Arbeit, die besagt, dass der Verzehr von transgenen Erbsen eine allergische Reaktion bei Mäusen hervorruft bzw. Mäusen mit bereits bestehender allergischer Erkrankung zu Ovalbumin es zu einer Verschlimmerung der Erkrankung kommt. Diese wissenschaftliche Beobachtung wurde durch unterschiedliche Glykosylierung der nativen und transgenen AAI Proteine erklärt. Als Kontrolle wurde Pinto verwendet anstatt Tendergreen Bohne von der das natürliche Protein stammt. Unser Interesse bestand in der Verifizierung dieser Beobachtung. Wir führten Experimente mit reinen AAI Proteinen, extrahiert von der natürlichen Tendergreen und Pinto Bohne sowie von der transgenen Erbse durch. Unsere Studie weist darauf hin, dass bei beiden das natürliche und transgene Protein eine immunologische Reaktion hervorrufen kann, wenn man es in Mäusen intraperitoneal injiziert. Die Mäuse weisen aber auch eine allergische asthmatische Reaktion auf, nachdem die Proteine intranasal verabreicht wurden. Um den allergischen Effekt durch orale Konsumierung zu analysieren, wurden Mäusen Samenbrei von Tendergreen und Pinto Bohnen sowie naive und transgene Erbsen gefüttert. Wir beobachteten, dass beide Bohnenarten, Tendergreen und Pinto, eine allergische Reaktion hervorrufen kann. Das Füttern von natürlichen und transgenen Erbsen an Mäusen zeigte Unterschiede in der Th2 Immunantwort. Sie haben inflammatorische

Zellen in den Atemwegen und in der Lunge und eine niedrige AAI-spezifische Antikörperproduktion.

Pea Lectin ist ein wichtiger Bestand der Erbsen mit 38-54% Aminosäure strukturähnlichen Aufbau wie AAI Protein. Wir untersuchten die Kreuzreaktion zwischen AAI Protein und Pea Lectin, und fanden heraus, dass es zu Pea Lectin ein wesentlich höherer IgG1 Antikörper produziert wird. Diese Ergebnisse weisen darauf hin, dass nicht unbedingt oder nur zum Teil das AAI Protein eine Rolle in der Immunantwort spielen könnte. Des Weiteren waren wir auch daran interessiert, ob transgene modifizierte Nutzpflanzen eine Verschlimmerung auf bereits bestehende allergische asthmatische Krankheiten haben könnte. Mäuse wurden mit Ovalbumin immunisiert und während der Immunisierungszeit mit Samenbrei gefüttert, um die akute allergische Reaktion zu messen, oder die Tiere wurden nach der letzten Ovalbumin Verabreichung ein Monat Ruhepause gegeben, um sich zu erholen und erst danach wurden sie mit Samenbrei gefüttert (Exacerbation). In der Exacerbation konnten wir gleichermaßen starke Entzündungen in der Lunge und in den Atemwegen beobachten. Hingegen im akuten Fall zeigten Mäuse, die mit GM Erbsen gefüttert wurden, eine schwerere Lungenentzündung und erhöhte Schleimsekretion im Vergleich zu den Mäusen, die mit natürlichen Samen gefüttert wurden. Wir beobachteten einen Anstieg der OVA-spezifischen Antikörper-Produktion.

Um in Erfahrung zu bringen wie GMO auf das menschliche Immunsystem wirkt, führten wir das Human-SCID Mausmodel ein. Wir transplantierten mononukläre Zellen des peripheren Blutes (PBMC) von luminosen allergischen Patienten oder aber von gesunden Spendern in SCID Mäusen, welche keine T- und B- Zellen besitzen. Sie wurden danach zwanghaft mit Samenbrei gefüttert. Human-SCID Mäuse zeigten eine allergische Reaktion, aber die Unterscheidung zwischen gesunden und allergischen SCID-Mäusen war nicht beobachtbar.

Unsere Studie zeigt, dass naives und transgenes AAI Protein bei Mäusen eine allergische Reaktion hervorruft. Allerdings konnten wir keinen Unterschied zwischen naiven und transgenen AAI Protein beobachten. Diese Ergebnisse haben wichtige Auswirkungen im Zusammenhang mit Tests neuer Proteine an Tiermodellen. Dennoch sind für die Risikobewertung weitere Studien notwendig.

1. Introduction

In 1996, there were 1.7 million hectares of biotech crops, which increased 94-fold to 160 million hectares in 2011, making biotech crops one of the fastest adopted crop technology in the history of modern biotechnology[1]. In 2011, 29 countries were counted in which plant biotech crops were used and out of these, 19 were developing and 10 were industrial countries. Gene technology has the potential to alter the food supply, which suggests the possibility of developing less-expensive and healthier foods by increasing volume of staple foods, but importantly by reducing the prevalence of micro-nutrient or under-nutrient population. These factors could play a role in the improvement of health and an aid in feeding the growing world population particularly in developing countries[2]. In addition, it has the potential to reduce chemical pesticide use and increase the productivity of land, thus, protecting the habitat for other species. These topics are becoming more and more attractive, especially in the recent years, when the international community is becoming aware of the importance of food quality and safety and their influence on the health of end consumers. The first genetically modified (GM) crops accepted for food use were tomato and soybean. But prior to commercial production of these genetically modified organisms (GMO), they were evaluated for safety by the United States Food and Drug Administration[3].

In the 1980s, the growth of GMOs began with the development of biotechnology techniques and improvements in plant cell culture and tissue differentiation[3]. The definition of a GMO is an organism in which the genetic material has been changed in a way that does not occur naturally. Alterations are performed either by mating and/or natural recombination (EU Directive 2001/18)[4, 5]. Agricultural biotechnology is able to introduce a complete, well-characterized gene with specific transcriptional regulatory elements. These transgenes can be selected from virtually any biological source and be moved into the genome of another species. Sometimes only a small number of genes are inserted into the genome of the recipient species, which is enough to establish the selected beneficial trait. In contrast, conventional breeding is used to transfer of hundreds of genes from one biological source to another. The identity of all these genes is often unknown and therefore, during the process to

develop beneficial GM plants, there is the possibility that unwanted genes may also appear in the new GM plants[6]. The goal of creating new GM plant varieties is to obtain plants, which are pest and/or disease resistant, more productive or with improved nutritional quality, flavor and shelf life. At present, GM crops are classified in generations depending on the product. The first generation of GM crops refers to seeds that increase production, but the crops themselves are not different from their native seeds. In other words, the appearance, taste, or nutritional value is similar for consumers. However, these GM seeds have specific resistance mechanisms to combat herbicides, pests, diseases or viruses. Examples of the first-generation GM crops are the herbicide-resistant (glyphosate) soybean, insect-resistant maize, and herbicide- and insect- resistant potato. These crops are currently planted on millions of farmland hectares[7]. The second generation of GM plants consists of crops with increased benefits such as increased levels of protein, modified and healthier fats, modified carbohydrates, improved flavor characteristics or increased levels of micronutrients or other phytochemicals (higher nutritional values) for consumers. Some examples of these GM plants are rice with beta-carotene or higher iron and zinc levels; tomato with enhanced levels of carotenoids, flavonoids, and phenolics; maize with increased vitamin C levels; soybean with improved amino acid composition, or potato with enhanced calcium content[8-10]. The third generation of GM plants refers to GM plants which provide food with additional health benefits or have a greater ability to resist abiotic stress such as drought, high temperatures, or saline soils. It also includes “pharmaplants”, which are used as biological production systems for manufacturing high-grade active pharmaceutical ingredients[4, 11].

The introduction of new genes and their expression products, but also the potential effects of the transformation process need to be analyzed for safety. Especially the second generation of GM products, which provides increased benefits for the end-consumer and must be considered more carefully. The use of multiple genes or entire metabolic routes and often the nutritional nature of the genetic modification can likely influence the safety assessment of these products and are becoming more complex. Not only the safety of multiple genes and potential interactions need to be considered, but also the nutritional and toxicological assessment will become more important when metabolic pathways are modified that directly influence the nutritional characteristics of the resulting food products[6, 12].

Testing a new GM crop takes approximately 8 to 12 years and an additional 4 years for testing safety and environment before coming onto the market[13]. Until now, there is no global approval for GMOs; there are only country-specific approval and registration processes. Unlike in the United States where importation of food or feed from less than half of the GM varieties were accepted, the European Union (EU) only allows cultivation of one potato (EH92-527-1) and two corn GM events (MON810 and T25 corn)[13, 14]. The EU established with regulation (EC) No 1829/2003 a legal framework for the labeling of products consisting of/or produced from GMO[15, 16]. Since 2005, the number of GMO applications submitted to the European Food Safety Authority (ESFA) grows constantly. About 40 GMOs have already been authorized by the European Commission[17].

1.1 Guidelines for assessment of the allergenicity of GM crops

GM organisms have to undergo a strict safety assessment before being allowed to enter the market. An important concern about transgenic food products are that the modified proteins may cause allergic reactions in susceptible people. An example is the Brazil nut, if a gene from a Brazil nut would be inserted into soybean or corn could trigger a major attack in people with nut allergies. Brazil nut is a recognized allergenic food therefore the 2S albumin protein was evaluated to determine if it was allergenic by conducting IgE-binding studies with sera from individuals allergic to Brazil nuts. The 2S albumin protein was able to bind IgE from Brazil nut allergic patients. In addition Skin-prick testing with Brazil nut and transgenic soybean extracts was performed and confirmed that the protein is a major allergen[18]. It is important to know the details of the protein of interest to protect people with food allergy against accidental exposure to allergens. This a major aspect for food manufacturers and regulators responsible for food safety[19, 20]. The structure, function, bioavailability, specificity and potential allergenicity of proteins need safety assessments. In general, proteins in the diet are not considered as a significant danger to human health, since nearly all proteins are damaged in the digestive tract by proteases. However, there are certain adverse effects associated with proteins which must be considered and therefore, specific safety evaluation strategies should be carefully analyzed. There are several possible adverse effects associated with proteins, for example acute

toxicity, anti-nutrient effects (e.g. soybean trypsin inhibitors) or effects on the immune system (e.g. lectins) and allergenicity. An example a safety evaluation of an introduced protein was the assessment of the CP4 EPSPS enzyme, which was introduced into soybean to produce a herbicide tolerant crop. CP4 EPSPS protein was shown to be: (a) degraded in simulated digestive fluids; (b) it had no toxicity when administered orally to mice at an acute dose thousands of times higher than potential human exposure of CP4 EPSPS in foods; and (c) was not structurally or functionally related to known protein allergens or toxins based on amino acid sequence homology searches[21]. The potential allergenicity of newly transferred proteins is considered as a main component of the safety assessment of the GMOs[22-24].

A classical approach to assess the allergenic potential of transferred proteins is comparing transgenic proteins with known and major allergenic protein sources[25]. In 1996, the International Food Life Sciences Institute (ILSI) developed a decision tree approach for the assessment of the potential allergenicity of plants produced through agricultural biotechnology, and it was modified by the FAO/WHO[26]. This approach focused on evaluating several important points[20], which include:

1. Source of the novel gene (the source of genetic materials)

   Firstly, it is important to know the source of the donor gene, and whether the source is associated with any known toxic or allergenic history which might possibly be transferred during the transformation process[27, 28]. A widely used gene in the biotechnology is *Bacillus thuringiensis* (Bt), a ubiquitous gram-positive soil bacterium which forms crystalline protein δ-endotoxin (referred to as Cry proteins) inclusions during sporulation and has been used as a biological insecticide for more than 50 years. Cry proteins bind to specific sites in the gut cells of insects and from ion-selective channels in the cell membrane. The cells swell due to influx water which leads to cell lysis and ultimately the death of the insect. It is well known that the insecticidal activity is specific and that the endotoxins are non-toxic to non-target insects, birds and mammals. Examples are Bt corn (Cry1A, Cry1Ac, Cry3Bb1, Cry1F), tomato and potato (Cry3A) and cottonseed oil (Cry1Ac)[29]. The next generation of Bt-protected

plants will contain several cry genes, thereby providing growers with a product that offers a broader spectrum of pest control and reduced susceptibility for insects to develop resistance[30].

2. Sequence homology to known allergens

   The amino-acid sequences of a big number of known allergenic proteins were determined and stored on data-base, which is accessible for the public. Nowadays, special computer programs are able to “align” amino acid sequence of a novel protein to the existing sequences of known allergenic proteins[31, 32]. Primary structure (amino acid sequence) of the novel protein has to be compared with the structures of known allergenic proteins by screening for identical stretches between the novel and allergenic proteins using special computer programs. Many amino acid sequences of allergenic proteins were determined and stored in publicly accessible databases. Two types of sequence similarity are considered. Short identical stretches consisting of contiguous amino acids above a specified number (generally six or eight) may constitute linear epitopes for IgE-antibodies, which are involved in the recognition of allergens[31]. In contrast, non-linear, three-dimensional IgE epitopes containing amino acid residues that are spatially grouped together but separated within the primary structure will not be identified by screening for linear stretches[33]. If sufficient homology exists, then suspicions would be raised concerning the possibility that the novel protein might cross-react with the known allergen and provoke symptoms when ingested by individuals with that particular allergy[6, 32].

3. Resistance of the candidate protein to digestion by pepsin because pepsin-resistant food proteins are more prone to induce systemic, severe symptoms.

   Allergenic proteins have to reach the intestinal tract in a structure that is sufficiently intact to provoke the immune system. Proteins that are resistant to pepsin are more likely to become allergenic than proteins which are rapidly hydrolyzed by digestive proteases. In general, known food allergens exhibit greater proteolytic stability than known non-allergenic food proteins in

simulated gastric and intestinal digestive model[6]. Several potent food allergens are known to be very stable in an *in vitro* pepsin digestion assay. However, some proteins not known to cause significant food allergies are also stable. There is also evidence that some important pepsin-labile allergens become more stable with minor shifts in pH. If a protein is found to be stable in gastrointestinal digestion, it is more likely to interact with the gut-associated immune system and thereby become an allergen. Although the increased stability at moderate stomach pH may help explain the allergenicity of some of these proteins, the use of standard pepsin stability testing at pH 1.2 or 2.0 still has a good demonstrated predictive value[34].

4. Immunoreactivity with serum IgE

   If either the transgenic protein was derived from an allergenic source or was homologous to a known allergen, IgE-binding properties of the protein should be investigated using sera from individuals that have IgE-specific to the source of the gene or to the sequence matching allergens[20, 35, 36]. To determine whether IgE in sera from patients react with the novel protein or extracts of the novel food, RASTs or similar tests for IgE binding could be used. However, if the results are negative or equivocal in the *in vitro* immunoassays, the novel food should be investigated further by using *in vivo* skin prick tests and ultimately confirmed with double-blind, place-controlled food challenges with allergic individuals[37, 38]. An example of IgE sera screening for identifying potential allergens was the methionine-rich 2S albumin from the Brazil nut that had been inserted into transgenic soybeans to improve their amino acid profile of animal feed. Based on this study, the 2S albumin transgenic soy product has never been released on the market[18, 39].

Another important point, which has to be considered, is the food processing of GMOs. There are numerous purposes of GMOs processing in the food industry, of which the most important are thermal denaturation, acidification and fermentation. Especially thermal denaturation can cause significant conformational changes in protein structure. Thus, heating generally reduces the allergenicity of a protein by alteration of conformational epitopes. However, heating can create new epitopes

potentially by unfurling the protein and exposing previously hidden linear epitopes which can create new conformational epitopes[40]. It is not possible to predict if the new conformational epitopes affect the immunogenicity of the transgenic product. In other words, we cannot say whether it will induce sensitization and cause allergy in the population. This may only develop after months of exposure and might only be apparent in large epidemiological studies, years after exposure.

GMO product labeling is an important aspect to protect consumers. Europe producers, for example, have to label a food consisting of or containing GMOs, irrespectively of whether there is DNA or protein of GM origin in the final products[41]. In 1997 the EU established the first labeling regulation (EU Regulation 258/97) for GMOs and GM products for the consumer´s right to know the information of GM ingredients in their daily foods[42]. Since then, more than 40 countries and regions, majority of them are members of the Organization for Economic Cooperation and Development (OECD) followed the EU and introduced the regulations for tracing and/or labeling GM products. Two categories are classified in labeling regulations: voluntary (e.g., Canada, Hong Kong, and South Africa) and mandatory (e.g., Australia, EU, Japan, Brazil and China). Among the countries with mandatory labeling, there are many different aspects among their regulations. In China, the mandatory labeling regulation set zero tolerance as the threshold level of GM ingredients; while in Australia, European Union and Japan, their mandatory labeling regulations have the threshold levels of GM ingredients to certain ranges (from 0.9% to 5%)[43].

1.2 Phaseolus vulgaris – Alpha-amylase-inhibitor

Phaseolus vulgaris L., a common bean is broadly grown and is rich and inexpensive source of proteins (20-25%) and carbohydrates (50-60%) for a huge part of the world´s population, but mainly in developing countries. They are beneficial for health, with low glycemic index (GI)[44]. These common beans contain a family of structurally related seed proteins for example phytohemaglutinin PHA-E and PHA-L or lectin-like proteins such as arcelin and lectin-like protein (LLP). All these proteins belong to the lectin family. Lectins are carbohydrate-binding proteins and bind glycans of

glycoproteins, glycolipids, or polysaccharides with high affinity and can be found in many different species and in different organs and tissues[45]. They can be served as recognition molecules within a cell, between cells, or between organisms because of their binding specificity. Lectins like PHA in beans or Concanavalin A (ConA), soybean agglutinin, pea lectin and favin, found in other plants, are all present at relatively high levels and accumulate in vacuoles in the cotyledons (1% to 8% of total protein) and at lower levels in the embryonic axes of the seeds. These lectins are synthesized during seed development together with other seed storage proteins. During germination and seedling growth, both storage proteins and lectins are broken down to provide amino acids for the growing seedling[46].

PHA occurs in 90% of all bean cultivars and in all wild accessions, whereas arcelin is absent from cultivated beans and occurs only in 10% of the wild accessions. Both PHA and arcelin belong to plant defense proteins that protect seeds against insects. Arcelin are resistance to the bean weevils *Zabrotes subfasciatus* and *Acanthoscelides obtectus*[47, 48]. The common bean has long been known to be toxic to a variety of animals and the toxicity of purified PHA toward mammals and birds. It has been shown that adding PHA to the diet of experimental animals, it causes lesions of the intestinal mucosa and the absorption of nutrients across the intestinal wall. Hence, it increases the bacterial colonization of the small intestine. It has also been shown that PHA is toxic to insects and specifically to bruchid beetles. It has been found that purified PHA inhibits the development of larvae of *Callosobruchus maculates*, the cowpea weevil[49-53]. Interesting, *Gatehouse et al.* showed that impure PHA was more efficient than pure PHA in arresting larval development, indicating that the PHA was probably contaminated with α-amylase inhibitor AAI, another protein in PHA family[54].

AAI is a promising gene for genetic engineering of crops that contain enzymes like protease and amylase which encode inhibitors of mammalian and insect. This protein has been in the human food chain for years since plants contain both types of inhibitors (AAI-1 and AAI-2) as part of their natural defense mechanisms. AAI is a glycoprotein which contains approximately 15% carbohydrate and is encoded by a gene that encodes a LLP or by a closely related gene. It has been shown that 0.2% of the AAI fed to insect *Callosobruchus maculates* larvae were enough to inhibit the

development of these larvae quite strongly probably because they are unable to hydrolyze the starch in these peas[55]. AAI is also known as a vacuolar protein and was shown to be synthesized on the rough endoplasmic reticulum as preprotein with molecular weight M_r 25.000 to 28.000 kDa. However, it rises to M_r 32.000 to 36.000 kDa after removal of the signal peptide and glycosylation at two or more sites and then transported to the protein storage vacuoles by Golgi apparatus where some of the N-linked glycans are modified. The difference between AAI and arcelin or PHA is that AAI is synthesized as a proprotein that undergoes proteolytic maturation which is happened in the protein storage vacuoles where two processes are followed: first, removel of a short carbox terminus and second, proteolytic cleavage at the carboxyl side of Asn[77] resulting in the formation of α and β subunits. The inactive proprotein is possibly converted by proteolytically processing into an active mature protein through the release of a conformational constraint within the proprotein. These processes are a necessary step to become a mature active inhibitor[49, 56-58]. Bean seeds contain at least two different α-amylase inhibitors AAI-1 and AAI-2 which belong to arcelin family and have distinct specificities. It has been shown by Mass spectrometric that for activation of the amylase inhibitors it required proteolytic cleavage which lead to loss of the terminal Asn residue in AAI-1, and in all three proteins, seven or more residues were clipped from the C-termini[59, 60]. AAI-1 and AAI-2 differ significantly in their glycosylation patterns. AAI has been characterized in several varieties of beans like kidney bean or black bean[49, 61, 62]. In most cultured common bean varieties AAI-1 is expressed and inhibits several mammalian α-amylases like porcine pancreatic α-amylase and the larval midgut amylases of the Azuki bean weevil *(Callosobruchus chinensis)* and the cowpea weevil *(C. maculates).* Seeds of certain wild accessions of *P. vulgaris* contain the homologue AAI-2 which shares 78% amino acid identity with AAI-1 which indicates considerable evolutionary divergence of the alleles within the same species. However, they differ in an important region that is part of the site where the enzyme binds the inhibitor. AAI-2 does not inhibit mammalian amylases but does inhibit the midgut α-amylase of Mexican bean weevil *(Zabrotes subfasciatus)*[55, 63, 64]. The major interest in alpha-amylase inhibitor protein is that they can be used to produce insect resistant transgenic plants.

Sequence similarity of genes encoding for lectin in common beans where compared with each other and the data showed that there is 90% similarity between

dlec1 (encode PHA-E) and *dlec2* (PHA-L); then 82% between *AAI-1* (encode AAI-1) and *dlec2* and 78% between cDNA of arcelin 1 and the gene coding for PHA-L. The genes for these three proteins are encoded by a single complex locus in the *P. vulgaris* genome[56]. These data presented the amino acid sequence similarity between AAI, PHA and arcelin showing that these proteins are homologous. However, AAI is unique because it is the only one of the three that undergoes proteolytic maturation.

1.3 Pisum sativum

Pisum sativum belongs to the daily diet because of the rich energy contain which makes it to the most important foods in the world. It is used as both food and stock feed and is an important component in sustainable agriculture. Peas contain the low anti-nutritional factor lectin and moderate trypsin level. Moreover, α-amylase inhibitor which occurs naturally in many food plants is absent in peas. This explains in part why peas are defenseless to insect damage. Peas contain two main groups of proteins, albumins (20-25%) which are water soluble and globulins (55-65%) which are salt soluble proteins. Globulins are composed of two major fractions, vicilin and legumin. Globulins, an important storage proteins provide nitrogen and carbon during seedling development[65]. Albumins play a crucial role in seeds as enzymatic and metabolic proteins, such as lipoxygenase, protease inhibitors, lectins, and pea albumins. Several studies have investigated the susceptibility of pea native proteins hydrolysis *in vitro*, like technological treatments which were applied on pea seed and had shown to affect the pea protein accessibility to solvents and enzymes and protein structure[66, 67]. Other experiments were carried out with pea to determine the protein structure and the resistance of pea proteins in the digestive tract of chickens[61]. Another study was performed *in vivo* with weaned piglets to investigate the biochemistry of digestion of field pea albumins and globulins in the stomach and along the small intestine in these animals[68].

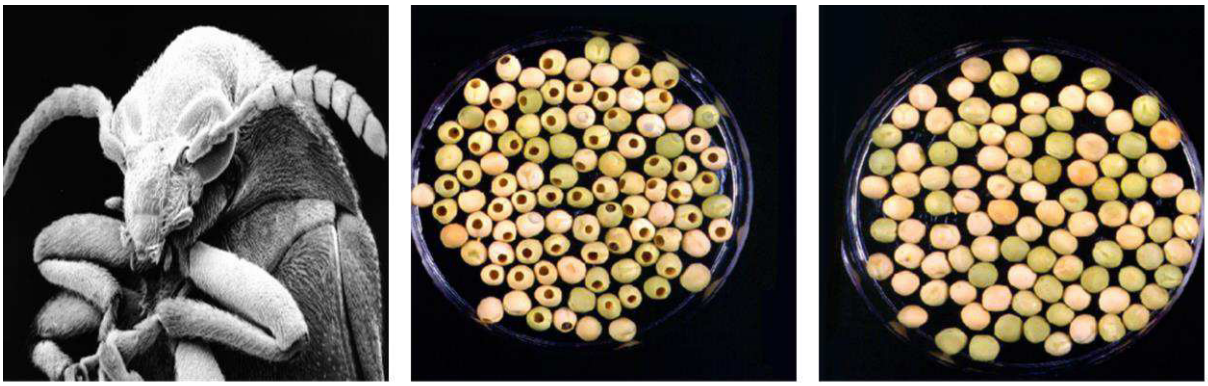
Pea lectin is a dimeric protein and is found in mature seeds and localized within protein bodies along with the major storage proteins legumin and vicilin. Pea seed lectin belongs to a group which includes Concanavalin A (from *Canavalin*

ensiformis – *Jackbean*), lentil lectin (from *Lens culinaris*), and favin (from *Vicia faba*). All four lectins are mitogenic and bind specifically to D-mannose and D-glucose[69-71]. Pea lectin undergoes a number of processing steps; first the co-translational removal of a leader sequence from a pre-pro-form of the protein, post-translational cleavage of the pro-lectin to yield α and β chains and, possibly, removal of 4 amino acid residues at the COOH terminus of the α subunit. Pea lectin can be isolated from pea seeds. A study has shown that the maximum mitogenic activity with low concentration is similar to ConA whereas the inhibition of response is cause by high concentration[72-74]. It has been shown that lectin in kidney bean is highly orally immunogenic. It enhances the formation of anti-ovalbumin IgE in mice. But also pea lectins are orally immunogenic. It has been reported that pea lectin enhances the total immunoglobuline IgG antibody level. Another study has been demonstrated that lectins affect the immune response against OVA. These results suggest that lectins may promote development of food allergy[75-77]. Another interesting study has shown that vicilin and convilin are potential major allergens from pea seeds and frequently associated with lentil allergy in the Spanish population[78].

1.4 Genetically modified organisms

GM pea – AAI pea

The pest of the field pea is the pea weevil *(Bruchus pisorum)* which is distributed worldwide. Pea weevil adults feed on pea pollen, mate and lay eggs on immature pea pods. When the larvae hatch from the eggs, it burrows through the pod wall and into the seed creating a small, dark entry hole which is approximately 0.2 mm in diameter (Figure 1). The larvae develop inside the seed living through cotyledon contents. The fully grown larva prepares a 5-mm exit hole and pupates behind this hole and survives until the following spring. Pea weevils consumed the seed content causing lower germination rates and therefore, the unit price decrease as well which cause an economic loss. At present, this pest is controlled by using chemical insecticides[55, 59, 79]. An attractive candidate who could control the damage created by seed weevils is alpha-amylase inhibitors using biotechnology methods[80].



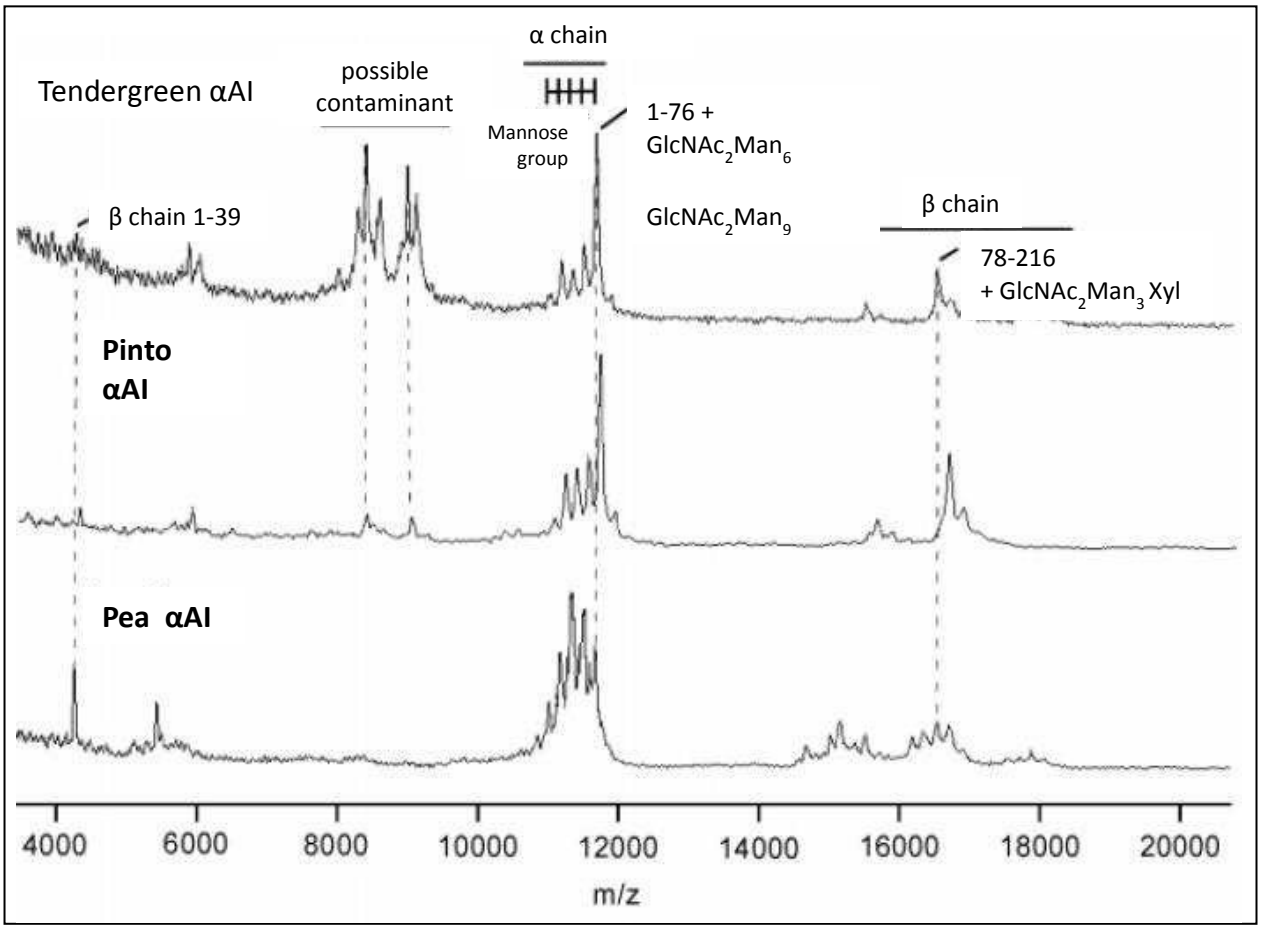
Bruchus pisorum weevil *Pisum sativum* *Transgenic Pisum sativum*

Figure 1. ***The CSIRO-developed GM field peas (right) provided 99.5% protection against pea weevil.*** *Pictures are kindly provided by T.J. Higgins, Division of Plant Industry, CSIRO, Canberra, Australia.*

In 1990, the first successful pea transformation was reported and in the following years robust *Agrobacterium*-mediated gene transfer protocols were developed[49, 81-84]. In Australia, the CSIRO Plant Industry developed GM field peas using a gene from beans. Beans contain the protein AAI which protected them against pea weevils by inhibition the activity of alpha-amylase, an enzyme that helps in digestion of starch. This alpha-amylase inhibitor induces starving of the weevils before they can damage the seed. The advantage for producing insect-resistant transgenic crops is to reduce the use of chemical pesticides and, in that way, also the cost to the farmers and the consumers. In addition, the insecticide loads on the environment can be decreased as well. The insertion of AAI-1 is able to protect against pea bruchid damage and to get rid of the need for both the field and the postharvest chemical pesticide applications. The presence of bean AAI in the developing pea seeds blocks the life cycle of the pea weevils at an early larval stage. The regulation of its expression is done by using seed specific bean PHA (*dlec2*) gene as flanking sequence. The importance of the PHA regulatory DNA sequences is that these sequences also impose a specific time course on the expression of the genes that they regulate[50]. The expression of AAI is only limited to the cotyledon and embryonic axis of the developing seed, for that reason, the infestation of the pea weevil can be observed normally in the transgenic peas until the larva reaches the cotyledons. At this stadium, the larva is exposed to the AAI for the first time and stops

the development of the insect. At this early stage of development only little physical damage was done to the pea seed and only minor weight loss was observed[85].

Proteomic analysis is a common tool to quantify and identify proteins that are mostly differentially expressed between GMOs and the native plants. Several seed storage proteins of garden pea were identified including vicilin, convicilin, legumin, lectin and albumin. However, it was not obvious how the presence of the native bean AAI would have altered the post-translational modification of some of these and reduced the expression of other storage protein. Some proteins included the transgene product were found to be accumulated at significantly changed levels in GM pea. Their proteins were either newly induced or completely suppressed in GM pea. A number of seed storage proteins were significantly over expressed in the GM pea lines[86, 87].



Prescott et. al., 2005 J. Agric. Food Chem.

Figure 2. *MALDI-TOF-MS analysis of purified AAI from transgenic pea and the common beans.*

Animal experiments were performed to examine whether the GM pea AAI caused an immune reaction in mice. In an experiment, mice were fed beans, non-GM peas or GM peas twice a week for 4 weeks. The results showed that mice fed with bean did not cause an immune reaction nor did mice fed with non-GM pea. However, mice fed GM pea showed evidence of an immune response after two weeks and increased at 4 weeks. They observed inflammation in the lungs and increased serum antibody levels in mice. In addition, after eating the GM peas, the GM pea AAI primed the mice to react to other food antigens[88]. These mice also showed significantly elevated serum levels of antigen-specific IgG1 against pea globulins, lectin and vicilin-4. Their results suggest that the altered immunogenicity of the GM pea in mice was not only caused by the transgenic AAI, but was also correlated with elevated levels of food antigens. Furthermore, proteomic analysis presented a number of seed storage proteins that were induced or significantly up-regulated in the AAI1-expressing GM pea[86]. Another study was performed to study GM pea fed to pigs and chickens. Their results have shown that protein digestion was the same in pigs and chickens fed GM or non-GM field peas, but they observed a reduction of starch digestion in animals eating the GM field peas. Therefore, they suggest that the AAI also affected digestion of starch in these animals and may not be suitable for stock feed[89, 90].

To understand why the mice reacted to the GM pea AAI, the CSIRO team analyzed and compared the molecular structure of the bean and pea AAIs using MALDI-TOF-MS analysis and observed small differences in the two proteins (Figure 2). The differences could be most likely caused by different protein processing steps in the two types of legume, including glycosylation. Glycosylation plays an important role in making certain proteins, and can lead to variation in a protein´s structure[91]. The modification of AAI resulted in alterations by glycosylation and the deletion of amino acid residues from both α-chain and β-chain polypeptides of the inhibitor[92].

GM chickpea

The world´s third most important pulse crop in human and animal nutrition, especial in India (75% of the world´s supply) and in the Middle Eastern region is chickpea which represents a valuable source of protein[93, 94]. Chickpea is not only a good source of protein (12.4-31.5%) but also carbohydrate (48.2-67.6%), starch (41-50%) and nutritionally important minerals. But stored chickpeas are likely attacked by bruchid beetles, especially the cowpea weevil, *Callosobruchus maculates* and azuki bean weevil, *C. chinensis*. Therefore, using the same biotechnical method, *Agrobacterium tumefaciens*-mediated transformation of chickpeas with a seed-specific chimeric gene encoding bean AAI-1 was performed using selectable marker gene *nptII*. A successful high level expression of the bean AAI-1 in seeds from transgenic plants could be observed and transgenic chickpea seeds were protected from the weevils. AAI in chickpea seed extracts was shown to be cleaved to the low molecular weight polypeptides observed in bean and was active *in vitro* against porcine alpha-amylase. A study has shown that chickpea germplasm can also protect seeds against weevil damages because of the physical characteristics of the seed coat like altered chemical composition, roughness and thickness. Since these characteristics make the chickpea less desirable for human consumption, especially when consumed raw, transformation of bean AAI seems to be a better solution to protect seeds against insects to avoid undesirable traits[95-98].

GM cowpea

Cowpeas are another important legume, which are cultivated on a total area of over 9.8 million hectares, are nutritious crops that provide the main source of proteins and vitamins to some of the world´s poorest people, particularly in Africa. In 2004, a total production of 3.9 million metric tons of dry cowpeas was harvested but as other legumes, cowpeas are highly susceptible to be attacked by insects such as leaf beetly *Ootheca mutailis S.*, the cowpea bud or flower trips *Megalurothrips sjostediti T.*, the cowpea pod borer *Maruca vitrata F.*, the cowpea aphid *Aphis craccivora K.*, the pod sucking bug *Clavigralla maculates F.* and the cowpea weevil *Callosobruchus maculates F.* Similar to chickpeas, cowpeas contain germplasm. However, the protection against insect pests is poor or absent. Therefore, a gene technology

approach to insert the seed-specific chimeric gene encoding bean AAI to resist against insect is now in consideration and has a high priority[99].

GM corn

There are existing GM crops which are valuable in agronomic productivity and are available on the market including biotech maize which is the second most widespread GM crop after soybean. In 2011, 51 million hectares were cultivated in the EU, and GM corn MON810 is the only cultivated GM for food purposes[1, 100, 101]. GM corn was developed by recombinant DNA technology, which allowed the transfer of genes between unrelated species. *Bacillus thuringiensis* commonly found in soil is a gram-positive bacterium that produces an insecticidal delta-endotoxin and has been used in agriculture as topical pesticide since 1961[102]. During sporulation, *B. thuringiensis* produce crystalline inclusion bodies or proteins (Cry proteins) which are very effective against lepidopteran, dipteran and coleopteran insects; an example are European corn borer *(Ostrinia nubilalis),* the most dreaded insect pest of maize[39, 103, 104]. Most cultivated transgenic maize is MON810 which was produced by the insertion of a DNA sequence that encodes Cry1Ab protein[101, 105-107]. Another Bt maize (MON863) expresses insecticidal Cry protein Cry3Bb1, which is also cultivated. Additionally, Bacillus thuringiensis genes have not only been introduced into corn but in tomatoes, rice or cotton and were field-tested with impressive results against lepidopteran pests[108]. The Cry proteins including Cry1Ab are regarded as harmless to organisms, including birds, mammals or humans probably due to acidified pepsinolysis in the gut and the lack of binding-sites on human gut epithelial cells to Cry protein. The main target of Bt toxins is the midgut of the insect. It stays inactive until they are dissolved by the gut protease. Several studies have tested possible toxicity of Cry proteins, but no significant pathogenicity to mammals, including humans has been observed[104, 109].

1.5 Immunogenicity and Allergenicity

Immunogenicity refers to the capacity of proteins or any other molecules to stimulate the immune system and induce cellular and/or humoral immune responses, including

antigen-specific lymphocyte proliferation/differentiation and antigen-specific antibody production. During infectious diseases, proteins from microorganisms are present in the body of the host, and the proteins are phagocytosed and/ or exposed directly to antigen-presenting cells, and secreted antibodies. On the other hand, immune responses against proteins, such as those from food, drugs, house dust, and tree and grass pollens from non-microbial and nonpathogenic organisms, are active research areas. Additionally, other properties of proteins besides stimulation of the immune system should be considered, such as their absorption, permeation or infiltration into the body across the mucosal epithelium and skin (epidermis)[110].

Allergenicity has basically a similar definition as immunogenicity, but the use of this term is more restricted to cases where the immunological responses to foreign protein lead to the provocation of pathological symptoms, such as allergic disorder. In allergic disorders, immunological responses are categorized generally into four types. The most broadly investigated is type I allergy, which is also referred as the immediate type or the IgE-mediated type[111]. Researchers have divided allergic responses into two distinct steps: sensitization and elicitation. In the first step, sensitization involves an adaptive immune response by antigen stimulation in cells, which induce antigen-specific IgE class antibody production. These antibodies bind to its specific receptors (FcεRI and II) expressed on the surface of mast cells and basophils in various tissues. The second step is termed elicitation, and occurs when sensitized mast cells become activated, degranulate and release mediators such as histamine, prostaglandins, and leukotrienes[110].

1.5.1 Early and late phase of allergenic protein recognition

The characteristic of an immediate hypersensitivity is the release of mediators by the crosslinking of IgE-FcεRI complex on mast cells[112, 113]. This results in symptoms dependent of the target organs of allergy, such as the skin, lungs or gut and are characterized by atopic dermatitis or eczema, asthma or the gut with food allergic reactions[114-116], respectively. Upon contact of allergenic proteins with the immune system, there is uptake of allergen by immature antigen-presenting cells (APC), mainly dendritic cells (DCs)[117]. These APCs are located in the gut, skin and lungs and they capture and internalize foreign antigens, i.e. proteins from the surrounding

milieu. These foreign proteins are processed within specialized proteolytic vesicles to generate peptides[118]. A fraction of these peptides bind with high affinity to major histocompatibility complex (MHC) class II determinants. Mature APCs migrate to regional lymph nodes. The assembled MHC classII/peptide complexes are transported to the cell surface and presented $CD4^+$ T helper (Th2) cells. Presenting the antigen to Th2 cells causes differentiation and release Th2-type cytokines and causes class switching of B cells to enable IgE production. Allergen-specific IgE antibodies bind to high-affinity FcεRI receptors that are expressed on mast cells and basophils. If the same antigen is re-exposed and leads to allergen-specific IgE, which crosslinks and causes degranulation of preformed granules in mast cells resulting in the secretion of histamine and other proteases[116, 119]. This reaction appears within minutes, and it is termed as “early phase” of the allergic reaction. In the early phase, cytokines and chemokines are released and initiate the “late phase”, in which inflammatory cells are recruited and activated[120]. The late-phase response appears during the 6- to 12- hour’s period after allergen exposure and is marked by increased infiltration of eosinophils, neutrophils, basophils, T lymphocytes and macrophages, which releases newly generated lipid-derived mediators such as leukotrienes and cytokines. The cross-linking of FcεRI requires at least two antibody molecules bind to the inducing allergen. Therefore, an allergen must have at least two IgE binding sites (epitopes) with a minimum of roughly 15 amino acid residues long. An example is peanut allergens Ara h 1 and Ara h 3, which have at least 23 and 4 linear epitopes[121], whereas the major soybean allergen Gly m Bd has 16[120]. This late-phase response is probably responsible for the symptoms and chronic signs of allergic diseases[122, 123].

Both mast cells and basophils have receptors with high affinity for IgE. They are secretory cells that play crucial roles in both immediate allergic reactions and in inflammatory responses[124, 125]. Granulocyte basophils are rare circulating cells with less than 1% of the white cell population, while mature mast cells are found in tissues and in regions located at interfaces with the external environment, such as respiratory tract, skin and mucosal surfaces[126]. After crosslinking of IgE-bound FcεRI by allergens, mast cells and basophils release potent chemical mediators including histamine, serotonin and beta-hexosaminidase, which develop major symptoms of allergic diseases, such as vasodilation, mucus vasodilation and bronchoconstriction. To study IgE-FcεRI interaction and the regulation of secretion in mast cells the rat cell

line RBL-2H3 assay has been established, because these cells have similar functions as primary mast cells and normal basophils[127, 128]. RBL-2H3 cell line was cloned from leukaemia cells isolated from rats after injection subcutaneously with the chemical carcinogen β-chlorethylamine[129]. RBL-2H3 cells are used by many study groups in a broad range of applications, such as degranulation studies, investigation of many mast cell stabilizers or physical-chemical properties of the FcεRI. Like mast cells and basophils, RBL-2H3 cells express FcεRI and the cross-linking of IgE with FcεRI by multivalent allergens release a range of mediators that evoke a potent immune allergic response[44, 130-132]. Therefore, RBL-2H3 cells can be considered as an important tool for *in vitro* studies, since a huge number of monoclonal cells can be rapidly obtained by simple cell culture techniques.

MHC classII/peptide complexes are recognized by T helper cells (Th). This recognition is mediated by the clonotypic T-cell antigen receptor, which delivers stimulatory intracellular signals to the T cells. These signals activate immature Th cells (Th0) and lead to differentiation into mature effector Th cells. Th cells can be subdivided into two major types Th1 or Th2[133-136] (Figure 3). These mature T cells provide help to B cells by secreting immunoglobulin in two ways. Either it is mediated by cell/cell contact which stimulates accessory molecules on the B-cells surface, or the release of cytokine activates and binds specific cytokine receptors on the surface of the B cells which increase the strength and the quality of the antibody response. Typically Th2 cytokines are IL-4, -5, -6, -9, -10, and -13, which induce primarily a humoral immune response[137]. IL-5 promotes eosinophil growth and differentiation and is synthesized predominantly by Th2 lymphocytes, but in smaller amounts by mast cells and eosinophils. IL-13 mediates release of chemokines and activates macrophages. In addition, IL-13 differentiates mucus-secreting goblet cells and modifies smooth muscle which contributes to enhanced airway hyperresponsiveness[138, 139]. IL-4, another important Th2 cytokine, promotes isotype switch for the production of IgE by B cells[140]. IL-4 and IL-5 play an important role in the pathogenesis of IgE-mediated allergenicity. Other cytokines may play roles in the Th2 immune response and they include IL-6 which inhibits Th1 differentiation and promotes Th2 and Th17 differentiation[141-143], and IL-9 which contributes the proliferation of mast cells and T cells[144]. IL-9 may also play a role in enhancing the effects of other Th2 cytokines, such as IL-4 mediated production of IgE[145]. IL-10 has

anti-inflammatory and immunomodulatory properties[146]. Th1 cells are involved primarily in cell-mediated immune responses against microbes and produce IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF-α/β), that together downregulate IgE synthesis[147-149].

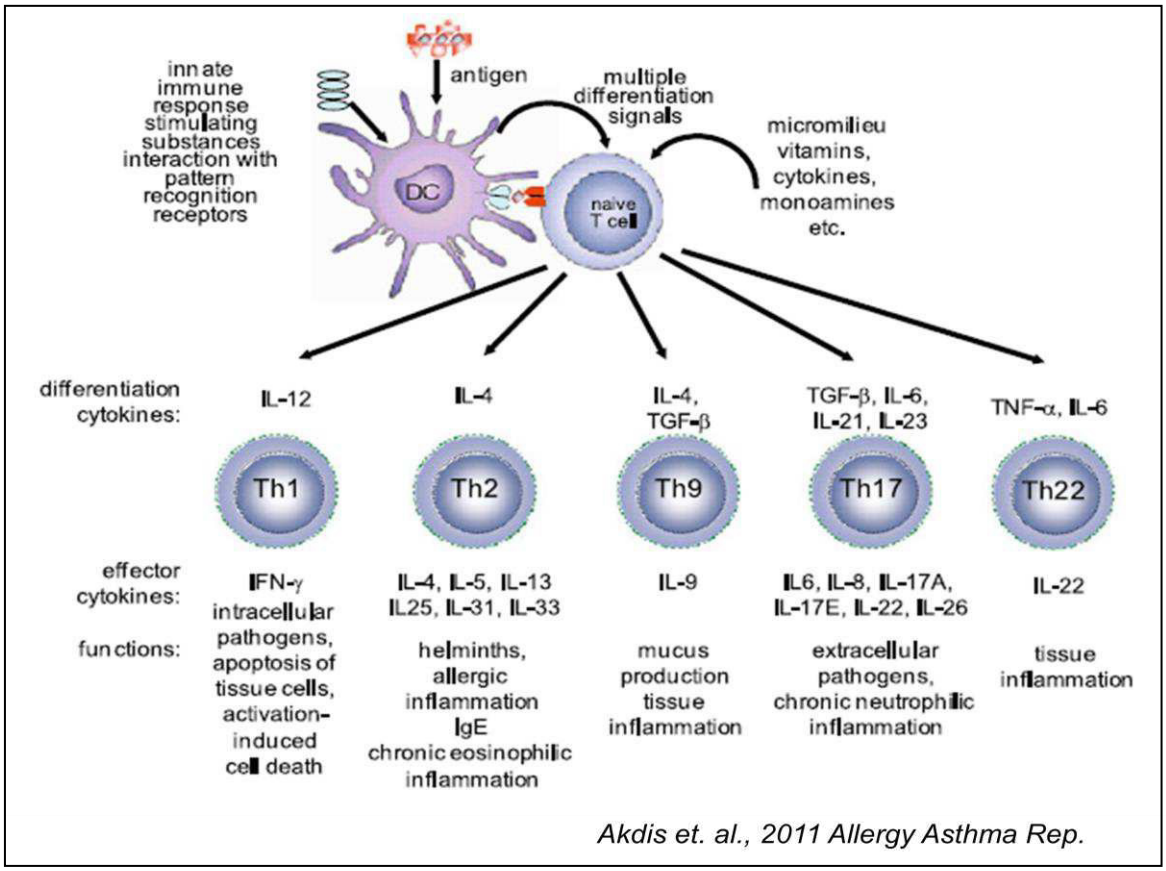


Figure 3. ***Differentiation of naïve T cells.*** *Naïve T cells can differentiate into Th1, Th2, Th9, Th17, and Th22 types of T cells. Dependent on the cytokines secretion, responses to chemokines and interactions with other cell, these T cell subsets can induce different types of inflammatory responses.*

$CD4^+$ T-cells can be sub-divided into Th1 and Th2 cells and additionally into Th17, Th22, Th9 and regulatory T (Treg) cells (Figure 4). Th17 cells are a pro-inflammatory subset of T helper cells, which are characterized by production of IL-17A, IL-17F and IL-22 and may contribute to neutrophilic, steroid-resistant severe asthma and to enhance Th2- mediated airway inflammation[150-152]. An increase of IL-17A has been found in the serum, sputum and bronchoalveolar lavage fluids (BAL) of patients with asthma. In addition, it has been identified several genes in patients with asthma which are key factors for Th17 cell differentiation. Although, Th17 cells seem to play an important role in the development of allergic asthma, the mechanisms

controlling their development in the lung are less clear[153-155]. Other studies have also shown that Th17 cells are probably evolved to help in host defense against bacteria and fungi[156-158]. IL-22 has pro-inflammatory and protective properties but the fully knowledge about the dual effect is still limited. Th22 cells co-express CCR6 and skin-homing receptors CCR4 and CCR10, suggesting that Th22 cells have a major function in the skin[159, 160].

Additional mediators such as IL-25, IL-33 and TSLP (thymic stromal lymphopoietin), and CC family chemokines were found to play an important role in initiating allergic inflammation. IL-25, IL-33 and TSLP can be released from epithelia when activated by antigen or by helminth parasitic worms. TSLP cytokine release is associated with asthma, atopic dermatitis and food allergies in patients. Studies in murine system indicated that TSLP is probably an efficient regulator of Th2 associated inflammatory disease[161]. IL-33 is a member of the IL-1 cytokine family and is produced by epithelial cells, fibroblasts, and endothelial cells and can be induced in macrophages. Th2 cells, mast cells, and the newly described natural helper cells (nuocytes) express the receptor ST2 (IL-1RL1), which is recognized by IL-33 cytokine[162, 163]. Recent studies have reported that IL-33/ST2 is playing a role in allergic asthma, shown by the administration of IL-33 to mice, which induces eosinophilia, airway hyperactivity, and goblet cell hyperplasia. Additional studies detected that the administration of neutralizing antibodies against IL-33 or ST2 decreases airway hyperactivity and eosinphilia[164]. IL-25 cytokine induces MHC class II-expressing accessory cells to produce IL-5 and IL-13 and is also secreted by Th2 cells, mast cells, activated macrophages. IL-25-responsive cells probably help in the further development of the $CD4^+$ Th2 cells[165].

1.5.2 Tolerance

When healthy individuals come in contact with foreign proteins, the immune system may alternatively develop immune tolerance, a state in which the exposure to food proteins is tolerated without the induction of an immune response, so called immunological tolerance. The decision to switch the immune response to tolerance is not completely understood. One possibility is that the decision is dependent on the balance between Th1- and Th2-type cytokines production, which is required for IgE

antibody production and allergic sensitization. Other factor which may influence the development of allergic responses is such as Treg cells. Treg cells secrete immunosuppressive cytokines IL-10 and TGF-ß which play an important role during the induction of oral tolerance[166-168]. Oral tolerance is caused by administration of harmless antigen which induces an immune unresponsiveness[169]. Studies have shown that oral tolerance can be induced in murine animals of either a single high dose of antigen or repeated lower doses. A high dose of antigen induces lymphocyte anergy or deletion which can arise through T-cell receptor ligation in the absence of costimulatory signals such as IL-2, or by interactions between costimulatory receptors on T cells (CD28) and counter receptors on APCs (CD80 and CD86)[170]. Low-dose tolerance is mediated by regulatory T cells. These cells can be divided into Th3 cells, Tr1 cells and $CD4^+CD25^+$ cells. Studies have revealed that Th3 cells produce TGF-ß with various amounts of IL-4 and IL-10, Tr1 cells secrete IL-10, and $CD4^+CD25^+$ cells expressed high levels of cytotoxic T lymphocyte antigen 4 and the regulatory cytokine TGF-ß and IL-10. These expressed cytokines are playing an important role in oral tolerance[171-173].

1.5.3 Allergic Asthma

Allergic asthma is a disorder of the conducting airways characterized by Th2- and IgE mediated inflammation, airway hyperresponsiveness (AHR), recruitment of inflammatory leukocytes to the lung or mucus secretion in airway[162, 174, 175]. Several different T cell subsets are thought to influence the allergic asthma immune response dependent on cytokine production[176]. Th2 cells in combination with natural killer T cells and $CD8^+$ T cells are thought to promote eosinophil recruitment whereas, Th1 cells and Th17 cells are associated with severe steroid-resistant asthma, which is often marked by neutrophilic infiltrates. Regulatory T cells and subtypes of yδ T cells are able to down regulate pulmonary immune responses and are potentially important for maintenance of immune homeostasis in the lungs[56, 177]. In recent years, a second Th2 cell subtype, named Th9, has been linked to asthma as a subpopulation that requires both IL-4 and TGF-β (Figure 3). Th9 cells do not express transcription factors such as T-bet, GATA3, RORγt and Foxp3, indicating that they are different from Th1, Th17 and Treg cells[178]. Signaling through IL-2R and CD28 can increase IL-9 by allergen-specific T cells and requires the help of both IL-4 and

IL-10. IL-9 expression increases in response to allergen challenge, but it is also relevant in the recruitment of eosinophils, basophils and mast cells[145, 179].

Eosinophils are thought to be a major effector cell in the pathogenesis of helminthes infections and allergic diseases because it releases various types of cytotoxic granule proteins that induce airway injury[180, 181]. In response to stimuli, eosinophils leave the circulation and traffic to sites of inflammation which is dependent on cytokines, chemokines and adhesion molecules such as IL-4, IL-5 and IL-13. The first step of eosinophil trafficking is the movement of cells from the bone marrow which is mainly regulated by IL-5 and chemokines (RANTES/CCL5 and the eotaxins). IL-4 and IL-13 regulate transmigration of eosinophils from the vascular bed into the tissue compartments, by increase expression of adhesion molecules on the endothelium[182-184]. In the inflamed tissue, eosinophils cause allergic symptoms through release of granule proteins and pro-inflammatory mediators such as major basic protin (MBP), eoinophilic cationic protein (ECP) and eosinophil peroxidase (EPO) which are toxic to the respiratory epithelium[185].

1.5.4 Food Allergy

Food allergies differ from person to person, some may react with an allergenic response but not in others. Disease can manifest as inflammation of the skin (hives), gut, and/or lung, and in the most extreme form, it can result in anaphylactic shock and death. Allergies affects around 3-4% of the population with prevalence among children than adults. Allergic diseases develop to harmless food or to a food component, mostly to a specific protein and can be divided into toxic and non-toxic reactions. Toxic reactions are due to factors natural occurring in a food and will arise in an exposed individual when given in an appropriate dose. Non-toxic food reactions involve only susceptible individuals and are either non-immune-mediated (food intolerance) or immune-mediated (food allergy/hypersensitivitiy)[186-188]. Food intolerance is an adverse physiologic response caused by specific characteristics of the host, such as metabolic disorders (lactase deficiency). Furthermore, food allergy can be divided into immunoglobulin E- (IgE) and non-IgE-mediated reactions. IgE-mediated food allergy referred to as Type I food allergy, cause for the majority of

food-induced allergic reactions and is characterized by occurrence of antigen-specific IgE antibodies in serum that can presence on mast cells and circulating antigen[189, 190].

Most common allergenic foods such as peanuts, tree nuts, fish, milk or eggs may contain up to 20.000 proteins, but only small number of these proteins may be allergenic[191, 192]. People allergic to the same food may cross react to different proteins. To determine immunogenicity or allergenicity of proteins is simple, since injection of proteins directly into the body (intravenously, subcutaneously or intraperitoneally) can cause basically an immunogenic response except for self-proteins. However, it is more complicated to assess for proteins which are ingested as food, because such oral immunogenicity of proteins are affected by a number of factors including enzymatic degradation in the gut, transepithelial transport from gut lumen into body fluids, and immunological tolerance to oral antigens. However, novel proteins despite of whether they are natural or recombinant originated from non-self-organisms, which have never been used and ingested as food; there are no consistent methods to determine their allergenic potential. The best method would be testing a large number of volunteer oral administered or ingested with the new protein. However, sensitization studies would be difficult because of ethical and efficacy reasons. Such novel proteins have to be assessed by comparison to known allergenic proteins include digestibility, sequence similarity to known allergenic proteins, and the *in vitro* and *in vivo* reactivity with IgE antibodies in the serum from patients allergic to the known allergen with structural similarity to the novel protein[110]. There are several allergy diagnostic methods (listed below) that were published in the recent years[193].

Allergy diagnosis methods:

1. *Skin-Prick testing.* It is a commonly used method for clinical diagnosis of suspected food allergy. The production of allergen-specific IgE on mast cells is the evidence after application of allergen extract on skin.
2. *Food-Specific IgE Increase.* IgE antibody titer is measured by many serological tests like enzyme allergosorbent test, ELISA, and immunoblotting, etc.

3. *Oral food challenge Double blind placebo controlled food challenge (DBPCFC).* In DBPCFC, the food is given at different time intervals to patients to whom the person is suspected to be allergenic and symptoms are observed. Duration of test generally needs 4 to 8 hours[193].
4. *Atopy Patch Test.* It is based on the principle of delayed type hypersensitivity reaction IV in which late phase immunological reactions occurs.
5. *Basophil Activation Test Cellular basophil activation test.* The diagnostic test is based on release of histamine and expression of CD63 antigen on outer surface.
6. *Food-Specific IgG Tests*. This method measures the levels of antigen-specific IgG or IgG4 antibodies in serum. Unfortunately, this test has not much diagnostic value for food allergy as there is lack of convincing result[194].

Glycosylation is a reaction in which oligosaccharides such as asparagines (N-linked) or serine/threonine (O-linked) amino acid residues are covalent attached[195]. There are several protein allergens which are glycosylated and the glycoyl group may be responsible for the allergenicity reaction. This is particulary important for transgenic proteins which glycosylation patterns may differ from their native protein, especially when transgenes are expressed at abnormally high levels in tissues from which they are normally absent and influence the physical properties. These are for example the alteration of stability, solubility, hydrophobicity, or electrical charge. These changes may affect the stability and uptake of a protein and therefore, alter its antigenic and allergenic potential. Glycosylation can also directly affect the immunogenic properties of proteins in many ways such as alter the B-cell epitopes present on the surface of a protein[120, 196]. It is found that glycosylated epitopes are able to produce B-cell epitopes, with a noteworthy quantity of the IgE which binds to glycosylated epitopes [197].

Heat treatment of food is a common process and may play an important role such as in food production, processing, conservation, storage, sterilization and final preparation to improve the taste, appearance or smell. However, the heating process can change the allergenicity of the protein[198]. A number of food proteins are denatured by cooking, and this denaturation includes the destruction of their three-dimensional structure. For that reason, certain epitopes show a decrease ability to

bind immunoglobulin E (IgE) antibodies and therefore, reduced allergenic potential. However, some allergens may become more allergenic by heating by creating new epitopes, for example during the Maillard reaction or enhanced protease activity[199].

Mediterranean and also several Asian populations like Indian people are at risk of experiencing allergic reactions to lentil, chickpeas or peas. Pea allergy is quite rare and therefore, there is only little information known on pea allergens. Two allergens were identified in pea protein namely Pis s1and Pis s2. Pis s1 protein has a molecular weight of 44 kDa and belongs to vicilin like protein. Studies have shown that the sequence identity between Pis s1 and Len c1 (important allergen found in lentil) is high with approximately 90% identity. Whereas, Pis s2 identified as conviclin has a structural similarity with the vicilin family. Pis s2 seems to be a major pea allergen[194]. Another study has shown that patients with a history anaphylaxis to pea allergens have also peanut-related symptoms. They observed that this symptoms were broken out because of the cross reactivity between pea and peanut proteins. There is a homology of 60 to 65% in amino acid sequences among vicilin-like proteins in pea and peanut (Ara h 1). Interestingly, they observed that IgE binding to Ara h 1 could be inhibited by pea vicilin but IgE binding to pea vicilin could not be inhibited by Ara h 1 suggests that pea acts as the sensitizing agent in these patients, and therefore, reflected in the course of symptoms development in these patients[78].

1.6 Rodent animal models for testing protein allergenicity

Safety assessment of newly developed GM crops are required before introduce onto the market. One essential consideration is food allergy. The novel product needs to be determined whether the novel gene which is inserted into plants has the potential to cause allergic sensitization. There are several allergenicity tests available. However, it is necessary to use animal models to test for allergenicity which can be added and supported the existing methods and may provide a more sufficient assessment of the allergenic potential of novel proteins in GM crops[35, 200]. An ideal animal model should resemble human allergic disease and most relevant, it should be able to distinguish between foods known to be allergenic and those thought not to be allergenic. IgE antibody is most strongly associated with allergic reactions,

therefore, the presence of food-specific IgE antibody is often used as an indicator of food allergies both in humans and animals[201].

In the last few years, several animal species, including rats, mice, fish, pigs and broilers, were used to investigate allergenicity either from natural or GM foods[202-208]. Each animal and/or model has been used to study a number of variables like the route of sensitization (including i.p., dermal, i.g. or subcutaneous) or the application of antigen (either as whole food, crude food extract or purified -food allergens). These models have different advantages and disadvantages and are providing important information. However, there are no currently validated models which are accepted to predict the allergenic potential of specific proteins in humans since there are still several open questions[209, 210], such as the correct endpoint for an animal model or what constitutes a positive allergic response in animal models. Furthermore, other questions like, what is the most appropriate design or which animal would provide the best choice to mimic human clinical signs or which dose with/without adjuvant should be used[211]. Among all the approaches using animals for testing food allergy, murine models are the most favorite animals for testing allergenicity due their small size or their short breeding cycle. But most important their immunology has been characterized very well[212].

Brown Norway (BN) rats are one of the most attractive strains for testing food allergy since BN rats have strong anti-IgE responses. A study has shown that BN rats gavage fed daily with a defined dose of ovalbumin (OVA) without adjuvant over a period of 42 days results in an increased of OVA-specific-IgG and -IgE. These results indicate that BN rats provide a suitable model to study oral sensitization to food proteins as well as immune-mediated effects on oral challenge with food proteins[213-216]. Other laboratories have studied food allergy using BALB/c mice. They sensitized by gavaged and challenged BALB/c mice without adjuvant daily with rice to evaluate allergenicity of rice and observed that this strain had similar development of immune response including high IgE production compared to BN rats[217]. Therefore, they concluded that BALB/c mice may provide an appropriate model for the identification of potentially allergenic proteins using gavage dosing[35, 218, 219]. *Lehrer and co* investigated the allergenicity of major food allergen extracts in different strains of mice[220]. Major food allergens, such as those found in peanuts, cashew, walnuts, and

shrimps stimulate significant IgE antibody responses. In contrast, there was a lack of minimal IgE antibody production in mice immunized with non-allergenic foods such as rice, beef and chicken. The results suggest that mice may respond in the same way as man in terms of elevated IgE antibody production to foods delivered through the oral route[220].

Because GM crops are becoming more and more important, scientific groups are studing newly developed GM crops in animal models. However, due to different responses of animals to specific proteins, it is not clear whether animal models are useful in predicting potential allergenicity of transgenic proteins e.g. *Cry* 1C in humans. Feeding *Cry* 1C to BN rats for 42 days did not induce antigen-specific IgE, eosinophilia and mast cells in blood compared to high allergen peanut agglutinin or OVA[221]. In contrast, another study showed that GM pea gavage fed to BALB/c mice increased of antigen-specific IgE and inflammatory cell infiltrates in lung[88]. Consideration of animal models, routes of sensitization, different transgenic proteins is important for the development of appropriate animal models for predicting food allergenicity.

1.7 Severe combined immune deficiency mice

It is difficult to fully understand the human physiological responses to food allergens from data derived from animals. A potential approach, therefore, is to evaluate human allergic responses to food proteins in a mouse-human chimera (i.e. humanized mice) such as in severe combined immunodeficient (SCID) mice[222-224] to generate Human-SCID (Hu-SCID) chimera. Engraftment with human hematopoietic tissues into CB17 SCID mice began over 20 years ago[225]. These mice lack on B and T cells because of a mutation in the gene encoding the DNA repair enzyme protein kinase, DNA-activated, catalytic polypeptide (Prkdc^{scid}, hereafter referred to as *scid*). CB17 mice, however, have a strong innate immune system, which allows low level of human hematopoietic cell engraftment[226-228]. Therefore, other humanized mice were developed to overcome this problem[229-231]. *Rag1*^{null} and *Rag2*^{null} mutations prevent the development of mature lymphocytes, or *B2m*^{null} and *Prf1*^{null} mutations which can strongly prevent development and functional activity of mouse NK cells[232, 233].

Mature human lymphocytes isolated from peripheral blood (PBL) survive for extended periods of time in SCID mice and develop a spontaneous production of human Ig which consists mainly (> 85%) of the IgG isotype, although some IgM, IgA and IgD can be detected[227]. SCID mice engrafted with PBLs from patients vaccinated against tetanus toxoid develop antigen-specific antibodies against the related antigen. Hu-SCID mice reconstituted intraperitoneal (i.p.) with human peripheral blood mononuclear cells (PBMCs) induce an *in vivo* secondary antibody response, and offer an ability to study human memory cells, avoiding the ethical constraints of recall antigen administration in patients with allergic or inflammatory diseases. Taken together, Hu-SCID model is a unique model to evaluate the role of human T and B cells in an *in vivo* animal system[234-238].

1.8 Aim of this study

In 2005, *Prescott et al.* published a series of experiments demonstrating that alpha amylase inhibitor GM peas were allergenic in mice[88]. The study showed that nGM peas and Pinto beans containing natural alpha-amylase inhibitor protein (AAI) did not induce allergy however, the transgenic peas could induce allergic responses and could amplify responses to other allergens. Our experiments were designed to determine whether feeding of AAI and the control Tendergreen bean could prime for allergic disease using a read out of allergic asthma in mice. Experiments were done to examine whether feeding of AAI peas could prime directly for an allergic response and to determine whether purified proteins from native bean and pea generated similar immune responses. Additionally, we engrafted SCID mice with human PBMCs isolated from legume allergic patients or healthy donors to analyze human immune responses to transgenic AAI.

2. Materials and Methods

2.1 Animals

6-8 week-old, female BALB/c and CB-17 SCID (purchased from Charles River Germany) mice were used. Mice were maintained under specific pathogen-free conditions and a 12 h light-12 h dark cycle with free access to water and standard laboratory food (SSNIFF, Soest, Germany) in the University of Veterinary Medicine. The care and handling of the animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Austrian Ministry of Science.

2.2 Clinical selection of human sera with AAI sensitivity

Heparinized blood was obtained from patients with allergy to legume or non-allergic healthy control subjects. Selection of human sera: Adults (n = 80) with chronic urticaria or unrecognized urticaria symptoms and in some cases respiratory symptoms in response to legumes were compared with healthy controls (n = 2) without allergy. Adult patients showed clinical symptoms after legume exposure. Prick-to-prick testing with fresh legumes presented positive reaction to allergen. After a 4 week diet without legumes, patients were re-exposed to legumes and severe clinical symptoms were observed. Patient's sera were positive (n = 18) for cross-reactive IgE antibodies against AAI from pea and beans. AAI was identified on SDS PAGE gels and could separate allergic patients from healthy controls (n = 2) on immunoblots. The clinical assessment and blots were performed in Central Food Research Institute - Budapest, Hungary by a team led by Dr. Éva Gelenscér.

2.3 Preparation of seed materials

Seed meal was obtained from Tendergreen beans (Phaseolus vulgarise), Pinto beans, non-transgenic peas (Excel pea) and GM peas (Excel blue *Pisum sativum*) were kindly supplied by T.J. Higgins, Division of Plant Industry, CSIRO, Canberra, Australia. Plant seed materials were provided as coarse flour and kept at 4°C. Seed meals were homogenized and sieved through a 40 µm mesh to obtain fine flour and

suspended in PBS before use. In some experiments, fine flour suspended in PBS was heated to 100°C for 25 minutes.

2.4 Purification of alpha-amylase inhibitor protein from common beans and transgenic peas

AAI from Tendergreen beans, Pinto beans and transgenic peas were purified as previously described[88, 239]. Seed meals from the transgenic legumes, Pinto and Tendergreen beans have approximately the same concentration of AAI and are in the range of 2-4% of total seed protein[91]. Briefly, seed meal was extracted with (1%) NaCl solution and followed by a heat treatment (70°C), dialysis and centrifugation. Purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15-25% gradient) and MALDI-TOF mass spectrometry. AAI was kindly provided by T. J. Higgins, Division of Plant Industry, CSIRO, Canberra, Australia.

2.5 Immunization by i.p. injection of AAI and pea lectin

BALB/c mice were randomly divided into five groups (n = 6 - 8). On days 0 and 21, groups of mice were injected i.p. with 10 µg AAI extracted from Tendergreen bean (AAI-bean), Pinto bean (AAI-pinto) or GM pea (AAI-pea) resuspended in 200 µL PBS. Injection (i.p.) of 200 µL Phosphate Buffer saline (PBS) was used as negative control. An additional experiment was performed with pea lectin. We used 10 µg pea lectin extracted from non-transgenic peas and resuspended in 200 µL PBS (Figure 4). Seven days, after last injection, mice were anesthetized with a lethal dose of 0.2 - 0.3 mL of Rompun (Bayer AG) Ketanest (Pfizer). Blood samples were collected by cardiac puncture to measure AAI- and pea lectin-specific IgG1, IgG2a and IgE. Individual serum samples were prepared and stored at – 20°C until analysis.

2.6 Intranasal exposure of AAI and pea lectin to induce allergic asthma

BALB/c mice were randomly divided into five groups (n = 6 – 8). Groups of mice were sensitized intranasal (i.n.) with 50 µg pea lectin or AAI extracted from Tendergreen bean, Pinto bean or GM pea in 50 µL PBS on days 0, 2, 4, 14, 16 and 18. The negative control group received 50 µL PBS. Two days after the last i.n. administration

mice were evaluated for allergic asthma. Blood samples were collected from the tail vein after the fourth i.n. administration and by cardiac puncture after 6 instillations to measure AAI- and pea lectin-specific IgG1, IgG2a and IgE (Figure 8).

2.7 Induction of OVA-induced allergic asthma

BALB/c mice were immunized with 10 µg of OVA (Sigma Chemical Co., St. Louis, MO) i.p. on days 0 and 21. Mice were challenged one week later with 1% OVA in PBS in a Plexiglas chamber by an ultrasonic nebulizer (Aerodyne, Kendall, Neustadt, Germany) for 60 min twice daily on 2 consecutive days (days 28 and 29) to induce acute onset disease. For disease relapse/ exacerbation, mice with acute disease were rested for at least 30 days and then rechallenged with 1% OVA nebulization for 60 minutes twice daily on 2 consecutive days.

2.8 Oral consumption of seed meal from native and transgenic plants

To analyze the effect of oral consumption of GM peas, BALB/c mice were force-fed by gavage with 250 µL of uncooked or cooked (100°C for 25min.) seed meal suspension (100 mg of seed meal / mL PBS) from Tendergreen bean, Pinto bean, nGM peas or GM peas twice or three times a week for 4 consecutive weeks using animal feeding needles. Each mouse received a total of 200 mg seed meal. Two days after last feeding, mice were challenged i.n. with one dose of 50 µg in 50 µL PBS of AAI purified either from Tendergreen bean, Pinto bean or GM pea (Figure 15). To evaluate allergic asthma, bronchoalveolar lavage fluid (BAL) and lungs were harvested 72 h after i.n. challenged for cellular infiltrates and mucus hypersecretion. Blood samples were collected either before i.n. challenged from tail vein or by cardiac puncture after challenged to measure AAI-specific IgG1, IgG2a and IgE.

To evaluate the effect of GMO consumption on the initiation and exacerbation of allergic disease to allergen OVA, BALB/c mice were force fed with Tendergreen bean, Pinto bean, nGM pea or GM pea seed meal either prior to acute disease onset or before inducing disease exacerbation. The protocol for OVA-induced allergic asthma is explained in 2.6. Briefly, experimental mice were sensitized on days 0 and 21 with OVA, rechallenge was performed on days 28 and 29 with 1% OVA by nebulization.

72 h after rechallenge by nebulization with OVA, BAL and lungs were performed. Blood samples were collected by cardiac puncture to obtain sera. AAI-specific and OVA-specific immunoglobulin level were measured (Figure 24, 30).

2.9 Bronchoalveolar lavage fluid

Mice were terminally anesthetized, tracheostomy was performed and a plastic catheter was clamped into the trachea. Lungs were washed 3 times with PBS in a total volume of 1 mL (0.4, 0.3 and 0.3 mL) to collect BAL. 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 cyto-centrifuged preparations (Cytospin-4, Shandon Instruments, UK), stained with the Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). BAL samples were collected in experiments after i.n. exposure of AAI, after oral consumption of seed meals, after last OVA-challenge and in Hu-SCID mouse chimera.

2.10 Lung inflammation and mucus hypersecretion

After BAL, lungs were fixed by immersion in 4% paraformaldehyde and embedded in paraplast. 3 µm lung sections were stained either with hematoxylin and eosin (H&E) for morphological evaluation of cell infiltrates, with LUNA stain for eosinophil enumeration or with periodic acid-Schiff stain (PAS) for mucopolysaccharide intensity in lung.

To analyze the intensity of inflammatory cell infiltrates in lung, 3 µm lung sections were stained with H&E. The grading system to characterize the intensity of the inflammatory infiltrates in lungs was performed as described below[52]. Grading system was as follows:

0 – no inflammatory infiltrates,
1 – inflammatory infiltrates in central airways,
2 – inflammatory infiltrates extending to middle third of lung parenchyma and
3 – inflammatory infiltrates extending to periphery of the lung.

For quantification of eosinophil infiltration in lung, lung sections were stained with Luna stain. Eosinophils were counted on ten random fields (40x magnification) containing alveoli and without major airways/ vessels (which were selected from low power magnification), and the average were counted for each lung.

To evaluate the intensity of mucus secretion by goblet cells in formalin-fixed lung tissue, lung sections containing main stem bronchi were stained with PAS and counter stained with hematoxylin. Mucus secretion was scored as described below[45, 77]. Grading system was as follows:

0 – no mucus producing cells in airways
1 – 0-20%
2 – 21-40%
3 – 41-60%
4 – 61-80%
5 – 81-100% mucus producing cells in airway wall positive for mucus.

2.11 Measurement of protein-specific IgG1, IgE and IgG2a in serum.

Blood was collected by cardiac puncture. Blood samples were centrifuged at 1200 x g for 15 min. to obtain sera. Enzyme-linked immunosorbent assay (ELISA) were used to detect protein-specific AAI-bean-, AAI-pea-, AAI-pinto-, pea lectin- or OVA-specific serum IgG1, IgG2a and IgE. Briefly, Immuno Microsorp 96 well microtiter plates (Nunc Maxisorp) were coated with 10 µg/ mL (0.5 µg/ 50 µL/ well) of AAI, pea lectin or OVA and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 BIO-RAD and blocked with 2% BSA in PBS for 2 h at room temperature (RT). 100 µl sera (1:500 dilution of sera for measurement anti-IgG1 and IgG2, 1:5 sera dilution to measure anti-IgE) diluted in PBS containing 1% BSA were added to the first raw of the plate. Serial dilution (1:20 or 1:10) were performed and incubated overnight at 4°C. The bound of IgG1, IgG2a and IgE antibodies were quantitated colorimetrical using biotinylated anti-mouse IgG1 (1:1000 dilution, Southern Biotech), IgG2a (1:500 dilution, Southern Biotech), IgE (1:200 dilution, BD Pharmingen) and followed by incubation with HRP-streptavidin horseradish peroxidase (1:8000 dilution, Southern Biotech) for 1h at RT, and then incubated in dark at room temperature with 100 µL TMB substrate solution (BD

Pharmingen) in each well for 10 min. After incubation, the dye development was stopped with addition of 100 µL of 0.18 M H2SO4. After each incubation, plates were washed with PBS containing 0.05% Tween 20 BIO-RAD. Results were expressed as optical density (O.D. 450 nm) after subtraction of background. Reciprocal titer was determined after three times subtraction of the background.

2.12 Cross-reactivity between transgenic, non-transgenic AAI and pea lectin

ELISA was performed to determine cross-reactivity between AAI-bean, AAI-pinto, AAI-pea and pea lectin. The protocol was the same as described above. Briefly, Immuno Microsorp 96 well microtiter plates (Nunc Maxisorp) were coated with 10 µg/ mL (0.5 µg/ 50 µL/ well) of AAI or pea lectin and incubated overnight at 4°C. After blocking with 2% BSA, diluted serum from mice fed with Tendergreen bean seed meal was added to plates coated either with AAI-pinto, AAI-pea or pea lectin to measure the cross reactivity to AAI-bean. The same process was performed with serum of mice fed with other seed meals or immunized with AAI (Table 1 and 2). Results were expressed as optical density (O.D. 450 nm) after subtraction of the background.

Table 1. Cross-reactivity of antibodies in sera of mice immunized with transgenic pea and non-transgenic bean AAI

Sera from mouse immunized with:	Plates coated with (measurement cross-reactivity to):			
	AAI-bean	AAI-pinto	AAI-pea	Pea lectin
AAI-bean		+	+	+
AAI-pinto	+		+	+
AAI-pea	+	+		+

Table 2. Cross-reactivity of antibodies in sera of mice force-fed by gavage with non-transgenic and transgenic seed meals

Sera from mouse gavage fed with:	Plate coated with (measurement cross-reactivity to):			
	AAI-bean	AAI-pinto	AAI-pea	Pea lectin
Tendergreen bean		+	+	+
Pinto bean	+		+	+
GM pea	+	+		+
nGM pea	+	+		+

2.13 ß-Hexosaminidase release

This experiment was performed by Dr. Richard Weiss, Division Allergy and Immunology, University of Salzburg. Briefly, to evaluate biologically active AAI-specific IgE, a rat basophil leukemia cell assay (RBL-assay) was performed. RBL-2H3 cells were plated in 96-well tissue culture plates (4×104/well) and incubated for 24 h at 37°C using 7% CO_2. Passive sensitization was performed by incubation with murine sera raised against the AAI at a dilution of 1:10 for 2 h. To remove unbound antibodies, the cell layer was washed twice in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl2, 1.8mM CaCl2, 0.4 mM NaH2PO4, 5.6mM D-glucose, 12 mM NaHCO3, 10 mM Hepes and 0.1% BSA, pH 7.2). Cross-linking of the Fc4R-bound IgE and subsequent degranulation of RBL cells was induced by adding 100 µL purified AAI (c=1.5 µg/ml) in Tyrode's buffer for 30 min in a humidified atmosphere at 37°C. Supernatants were analyzed for ß-hexosaminidase activity by incubation with 80 µM 4-methylumbelliferyl-N-acetyl- g -D-glucosaminide (Sigma) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37°C. The reaction was stopped by addition of 100 µL glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at λ_{ex}: 360/ λ_{em}:465 nm using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are reported as percentage of total ß-hexosaminidase released after addition of 1% Triton X-100[240].

2.14 Human PBMC isolation

Approximately 7 mL of blood from legume allergic patients and healthy donors were collected in BD Vacutainer® CPT™ Tube with Sodium Heparin. Centrifugation for 20 min at 2850 rpm without break at 18°C tubes were gently inverted 10 times. After centrifugation, mononuclear cells and platelets were observed in a whitish layer under the plasma layer. Approximately half of the plasma was aspirated without disturbing the cell layer. Cells were collected with a pasteur pipette and transferred to a 50 mL size centrifuge tube and tubes were filled up to 30 mL total volume with RPMI (RPMI 1640, GIBCO). Cells were washed twice and each time pellets were resuspended with 3 mL RPMI at 2850 rpm for 5 min with break. To evaluate the cell numbers of each tube, cells were diluted with trypan blue (1:10) and enumerated in a Neubauer hemacytometer. Prepared cells were kept in 4°C until used.

2.15 Reconstitution of SCID mice

CB.17 SCID mice were randomly divided into five groups (n = 5) and reconstituted by i.p. injection of 2.5 x 10^6 PBMCs from legume allergic patients or healthy donors. Two days after engraftment of human cells, mice were force-fed gavaged with 100 mg/ mL of Tendergreen bean, nGM pea or GM pea seed meal twice a week for 4 weeks. Control group received human PBMCs and fed only with PBS or were neither injected with human cells nor fed with seed meals. Two days after last feeding mice were i.n. challenged with one dose of purified AAI extracted either from Tendergreen bean or GM pea and mice were anesthetized 48 h later with a lethal dose of 0.2 - 0.3 ml of Rompun Ketanest. To evaluate allergic asthma phenotypes, bronchoalveolare lavage fluid and lungs were harvested for evaluation. Blood samples were collected by cardiac puncture to measure AAI-specific total IgG, IgG4, IgG1 and IgE.

2.16 Measurement of human-anti-AAI antibody

Blood was collected by cardiac puncture. Blood samples were centrifuged at 1200 x g for 15 min to obtain sera. ELISA was used to detect human protein-specific AAI-bean and AAI-pea total IgG, IgG4, IgG1 and IgE in sera from legume allergic patient or healthy donor and in sera from human-SCID mice chimera. Briefly, Immuno Microsorp 96 well microtiter plates (Nunc Maxisorp) were coated with 10 µg/ mL (0.5 µg/ 50µL/ well) of AAI and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 BIO-RAD and blocked with 2% BSA in PBS for 2 h at RT. 100 µl sera (1:50 dilution of sera for measurement total anti-IgG, IgG4 and IgG1, 1:5 dilution to measure anti-IgE) diluted in PBS containing 1% BSA were added to the first raw of the plate. Serial dilution (1:10) were performed and incubated overnight at 4°C. The bound of total IgG, IgG4, IgG1 and IgE antibodies were quantitated colorimetrical using biotinylated anti-human total IgG (1:2000 dilution, Sigma), IgG4 (1:2000 dilution, Southern Biotech), IgG1 (1:2000 dilution, Southern Biotech), IgE (1:1000 dilution, Southern Biotech). All antibodies were conjugated with Horseradish Peroxidase. On the next day, plates were incubated in dark at room temperature with 100 µL TMB substrate solution (BD Pharmingen) in each well for 10 min. After incubation, the dye development was stopped with addition of 100 µL of 0.18 M H2SO4. Plates were washed after each incubation with

PBS containing 0.05% Tween 20 BIO-RAD. Results were expressed as optical density (O.D. 450 nm) after subtraction of background. Reciprocal titer was determined after three times subtraction of the background.

2.17 Detection of AAI in human sera by two-dimensional electrophoresis and Immune-blot

This experiment was performed by Dr. Éva Gelencsér, Central Food Research Institute in Budapest, Hungary. Briefly, the 2-DE separation was performed with PROTEAN IEF CELL and PROTEAN 3 CELL equipment (BioRad). The first-dimensional separation (isoelectric focusing, IEF) was performed on commercial IPG strip, 7 cm long with pH range 3-10 (BioRad). The strips were rehydrated in 8M urea, 1% CHAPS, 20mM DTT. 50 µg of AAI were applied to the strips. The isoelectric focusing was carried out by linearly increasing voltage from 250V to 4000V. The strips were incubated in equilibrating solution (2% SDS, 6M urea, 1.5M Tris-HCl (pH8.8), glycerol), which contains DTT and iodoacetamide. The second dimension was run for one hour (15% SDS-PAGE). The gels were fixed in 20 % TCA and stained with Commassie blue R-250. After gel electrophoresis the proteins were transferred onto PVDF membrane (Millipore, 0.45 µm). The membrane was blocked with 1% BSA and incubated with the primary antibodies. The bound IgE antibodies were detected with horseradish peroxidase conjugated anti-human IgE secondary antibody. The immune-reactive protein spots patterns were developed with substrate solution (4-chloronaphtol/H_2O_2).

2.18 Statistical analyses

Unpaired student *t* tests were used to evaluate statistical significance between two groups. One-way analysis of variance (ANOVA) followed by Kruskal-Wallis test followed by Dunn´s multiple comparison tests (for cell number and cell percentages in BAL) and by Chi-square test for trend or Fisher´s exact test (for histological scores) was used to evaluate statistical significance between more than two groups. All statistical analyses were performed with GraphPad Instat v.5.0 (GraphPad Software Inc.). *p* values were considered significant at <0.05.

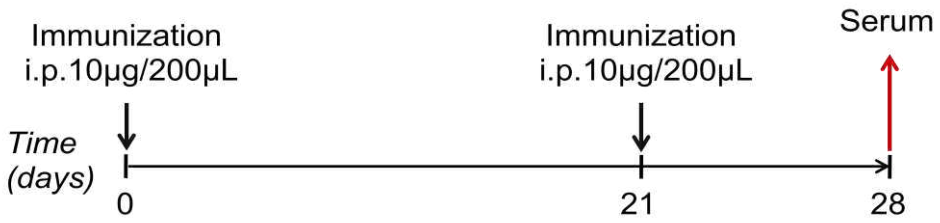
3. Results

3.1 Immunogenicity of native and transgenic AAI

AAIs extracted from native Tendergreen bean induce a higher IgG1 antibody response than AAI extracted from genetically modified peas. To analyze whether purified AAI induces allergic responses in mice, we immunized animals i.p. with purified protein extracted from GM pea, native Tendergreen bean and Pinto bean. Mice were sensitized twice with a three week gap in between. Seven days after last exposure, mice were exsanguinated by cardiac puncture (Figure 3).

	i.p. immunization 10 µg/ 200 µL PBS
Groups:	PBS
	AAI-bean
	AAI-pinto
	AAI-pea

Figure 3. ***Immunization protocol.*** *BALB/c mice were immunized with purified protein (10 µg in 200 µL PBS) extracted from native Tendergreen bean (AAI-bean), Pinto bean (AAI-pinto), GM pea (AAI-pea) or 200 µL PBS on days 0 and 21 by i.p. injection. Seven days after last immunization blood was collected by cardiac puncture and centrifuged to obtain serum.*



Bean allergy is rare and there are only few bean proteins characterized as allergen[241]. Therefore, we sought to determine whether AAI purified from Tendergreen bean and GM pea are immunogenic/allergenic. The presence of AAI-specific IgG1, IgG2a and IgE was measured by ELISA. Immunization with AAI is capable of inducing a Th2 isotype IgG1 antibody response. However, AAI-bean had the strongest effect compared to AAI-pinto and AAI-pea (Figure 4).

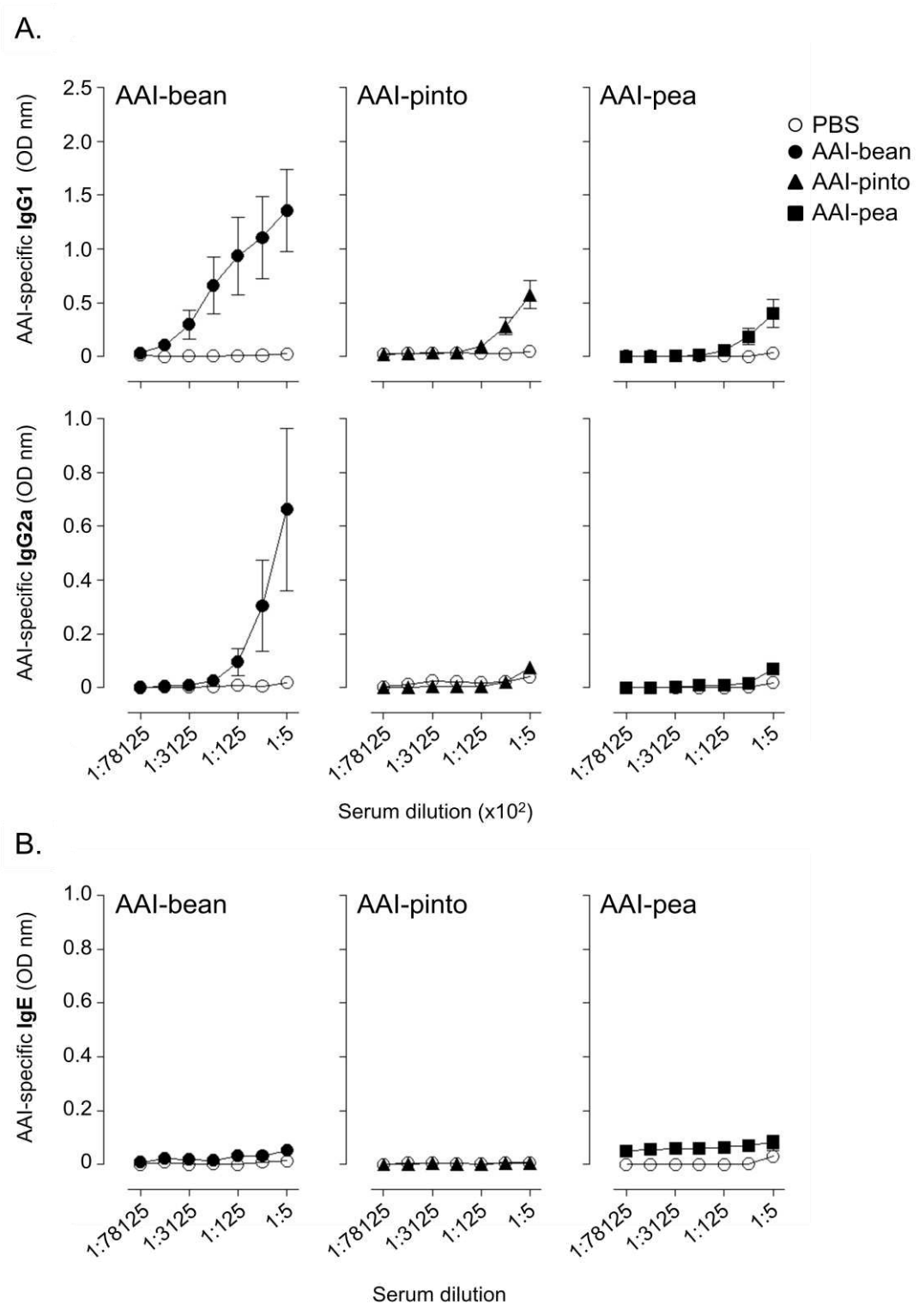


Figure 4. ***AAI-specific IgG1, IgG2a and IgE antibody titers in serum.*** *Groups of mice (n = 5 - 8) received purified protein extracted from native beans (AAI-bean, AAI-pinto) or from GM pea (AAI-pea). Another additional group was injected i.p. with PBS. Seven days after last immunization, bloods from animals were collected by cardiac puncture. Blood samples were centrifuged to obtain serum. 96-well plates were coated either with AAI-bean, AAI-pinto or AAI-pea overnight. Diluted serum was added to the wells. (A.) AA-specific IgG1, IgG2a and (B.) AAI-specific IgE antibody titers. O.D. ± SEM, n = 5 - 8*

These results indicate that AAIs extracted from native or from transgenic legumes are immunogenic (Figure 4). However, AAIs extracted from GM peas are less immunogenic than AAI extracted from native beans. We did not observe statistically significant differences between PBS and AAI and between AAI -bean, -pinto and –pea (Figure 5 and Table 3). A Th1 response was only observed in mice immunized with AAI-bean with an increase level of IgG2a (Figure 4). AAI-specific IgE could not be detected in all groups suggesting, that AAI from native bean or from GM pea does not appear to be allergenic.

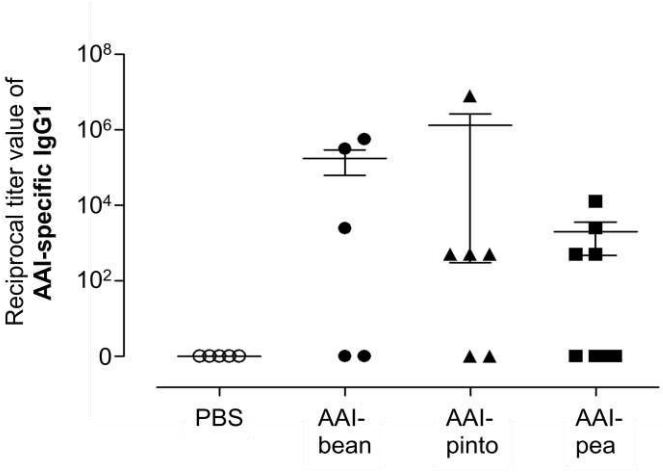
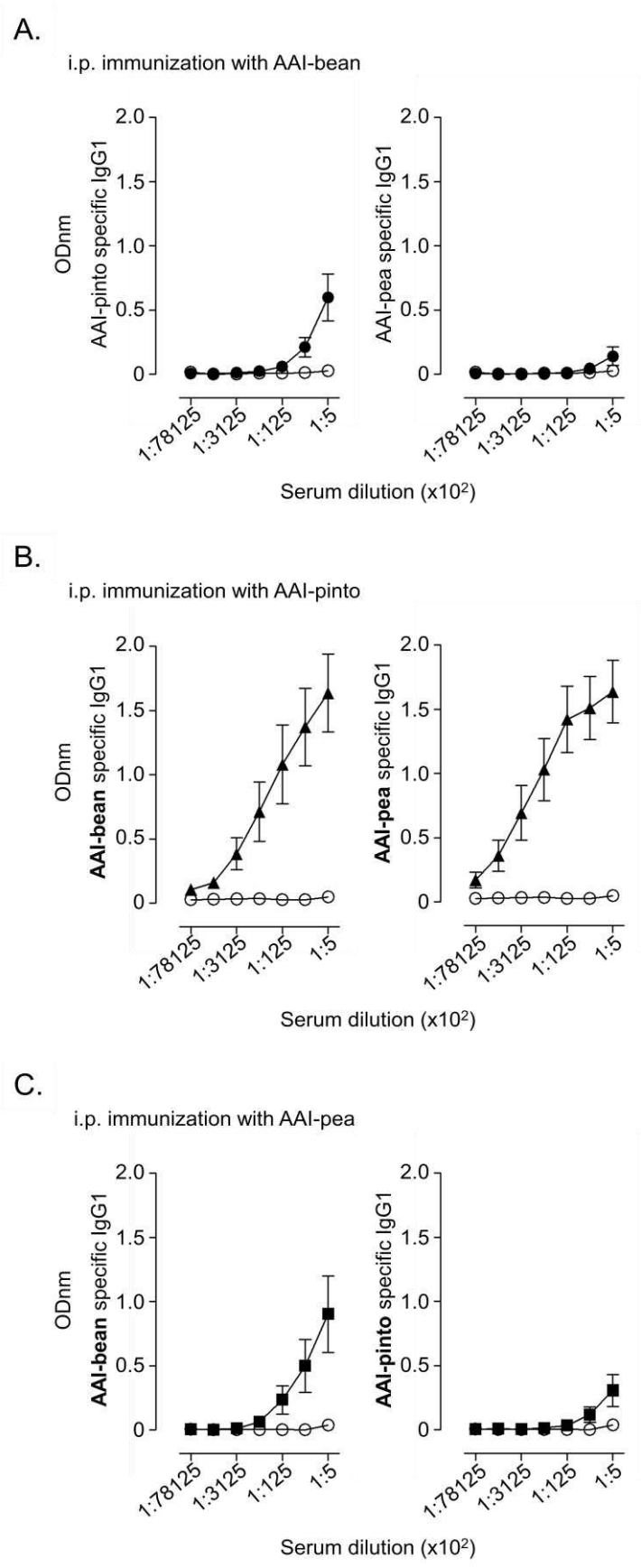


Figure 5. ***Reciprocal endpoint titer of AAI-specific IgG1.*** *To evaluate the differences between groups, reciprocal titers were determined. Data represent mean ± SEM, n = 5 – 8.*

Protein	α-AAI IgG1
PBS	1.000 ± 0.000
AAI-bean	175500 ± 114000
AAI-pinto	1302000 ± 1302000
AAI-pea	200 ± 1529

Table 3. ***Mean reciprocal endpoint titer in serum***

Mice were immunized i.p. on days 0 and 21 with AAI (10 µg/ 200 µL) or PBS (200 µL). Samples were collected one week after the final immunization. The presented data are mean titer ± SEM n = 5 - 8 mice.

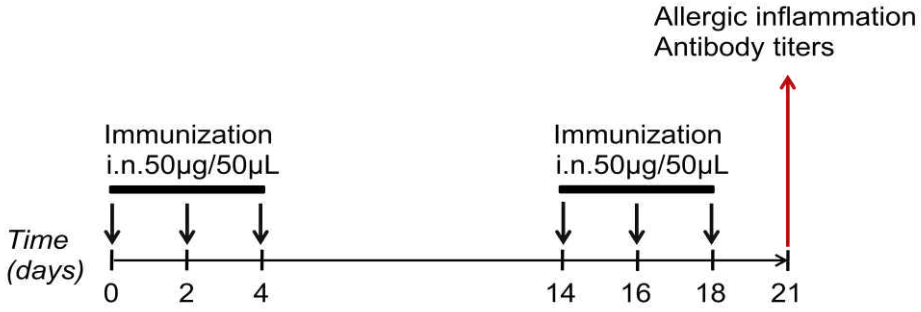


Sera obtained from mice immunized with AAI-pinto revealed a strong AAI-bean and AAI-pea specific IgG1 antibody level. We sought to determine whether there was cross-reactivity between AAIs. We observed that antibodies are cross-reactive with a higher reaction against AAI-bean immunized mice (Figure 6). These data suggests that post-translational glycosylation modifications of AAI in GM peas and beans do not significantly alter immunogenicity between native AAI from beans and transgenic AAI from peas.

Figure 6. ***Cross-reactivity between native AAI-bean, AAI-pinto and transgenic AAI-pea.*** *Mice were immunized i.p. on days 0 and 21 with AAI (10 µg/ 200 µL) or PBS (200 µL). Serum was collected 1 week after the final immunization. To evaluate cross-reactivity, 96 well plates were coated either with AAI-bean, AAI-pinto or AAI-pea (10 µg/ mL) overnight. Diluted serum was added and serial dilution was performed. (A) Mice were immunized with AAI-bean and plates were coated either with AAI-pinto or AAI-pea. (B) Mice were immunized with AAI-pinto and plates were coated either with AAI-bean or AAI-pea. (C) Mice were immunized with AAI-pea and plates were coated either with AAI-bean or AAI-pinto. Mice i.p. injected with PBS (open circle). O.D. ± SEM, n = 5 – 8.*

3.2 Allergenicity potential of AAI purified from GM pea

Intranasal treatment with AAI-pea induced IgG1 antibody response. Our previous results indicated that AAIs are immunogenic. Therefore, we went a step further to investigate the allergenicity of native and novel transgenic AAIs. In our laboratory, we established an experimental model in which 6 doses of i.n. protein induces allergic asthma in mice. Briefly, mice were instilled i.n. with 50 µg/ 50 µL of pure purified protein 3 times a week for two weeks with a week gap in between. Two days after the fourth and sixth instillation, sera were obtained to measure anti-AAI IgG1, IgG2a and IgE (Figure 7).



	i.n. immunization 10 µg/ 200 µL PBS
Groups:	PBS
	AAI-bean
	AAI-pinto
	AAI-pea

Figure 7. ***Experimental protocol: Initiation of allergic asthma with AAI in BALB/c mice.*** *Animals were instilled i.n. with purified protein (50 µg in 50 µL PBS) extracted from native Tendergreen bean (AAI-bean), Pinto bean (AAI-pinto), GM pea (AAI-pea) or 50 µL PBS on days 0, 2, 4, 14, 16 and 18. Forty-eight hours after last instillation, mice were assessed for features of allergic asthma.*

We observed that 4 i.n. instillations induced anti-AAI-specific IgG1 and the antibody level was further increased after sixth i.n. instillation. Similar to our previous results (Figure 4), AAI-bean and AAI-pinto promoted a stronger antibody response compared to transgenic AAI-pea. Anti-AAI-pea IgG1 was undetectable after 4 instillations, but apparent after 6 treatments (Figure 8A).

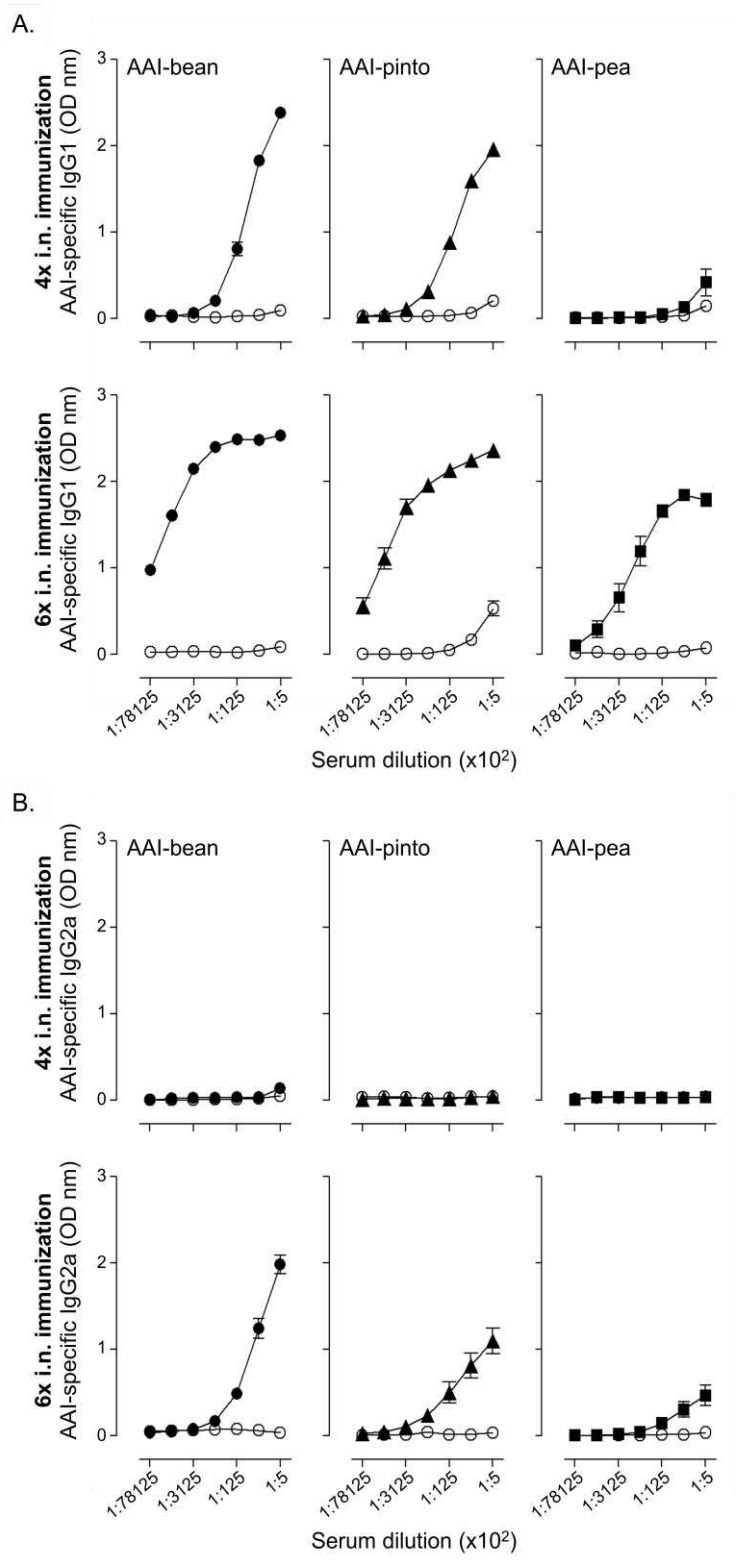


Figure 8. ***AAI-specific IgG1 and IgG2a antibody titers in serum.*** *(A.) AAI-specific IgG1 and (B.) AAI-specific IgG2a antibody production was measured. Groups of mice (n = 5 - 8) were immunized i.n. with purified protein extracted from native beans or from GM pea. Blood samples were collected either after 4 or 6 instillations from tail vein or by cardiac puncture. 96-well plates were coated either with AAI-bean, AAI-pinto or AAI-pea overnight. Serum was titrated in the wells. Mice i.n. instilled with PBS (open circle). O.D. ± SEM, n = 5 - 8.*

Mean of reciprocal endpoint titers were calculated (Table 4) and the calculation showed that 4 i.n. instillations; AAI-bean and –pinto specific IgG1 antibody responses were significantly higher than AAI-pea. These results confirmed again that transgenic AAIs were not more immunogenic than native bean AAI.

Table 4. ***Mean reciprocal endpoint titer in serum***

	α-AAI IgG1		α-AAI IgE
Protein	**4x i.n.**	**6x i.n.**	**6x i.n.**
PBS	1.000 ± 0.0000	1.000 ± 0.0000	1.000 ± 0.0000
AAI-bean	18750 ± 6250*	7813000 ± 0,0000*	1.000 ± 0.0000
AAI-pinto	500000 ± 8183*	7031000 ± 781300*	4.000 ± 3.000
AAI-pea	438.1 ± 305.1#	2013000 ± 1474000*	1.000 ± 0.0000

*Serum samples were obtained either after 4 or 6 i.n. instillations. Data presented in this table are mean reciprocal titer ± SEM; n = 5 - 8, mean ± SEM, Kruskal-Wallis test, *p <0.05 vs PBS, #p <0.05 vs AAI-bean and AAI-pinto.*

AAI-specific IgE antibody response is an important marker for allergic asthma. We detected IgE antibodies in mice immunized with AAI-pinto and a slightly increased titer of AAI-pea and -bean specific IgE (Figure 9). In this experimental mouse model, AAI does not cause a strong increase of IgE, whereas the IgG1 response was very high, indicating that native and transgenic AAI induce a strong Th2 response.

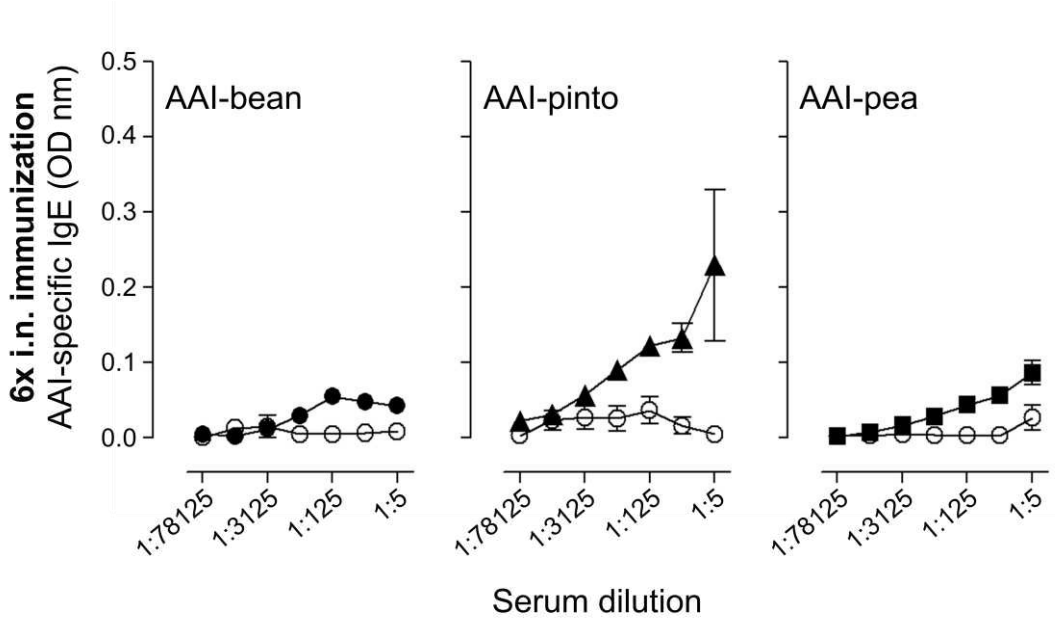


Figure 9. ***AAI-pinto i.n. immunization induced a slightly increase of IgE antibodies in mouse.*** *AAI-specific IgE antibody responses were measured after 6 i.n. instillations. O.D. ± SEM, n = 5 – 8.*

Intranasal immunization with AAI promotes an antigen-specific Th1- type immune response, but with a lower than a Th2- type response with detectable titers only at high AAI doses (after 6 instillations) (Figure 8B). We observed that AAI-bean followed by AAI-pinto had the greatest potential to trigger a Th1 response. These results suggest that due to post-translational glycosylation modifications of native and transgenic AAI, novel AAI-pea has a lower Th1- vs. Th2- type activity.

Furthermore, we analyzed cross-reactivity and as expected, we detected the cross-reactivity between AAI-bean, -pinto and -pea. The antibody response was equal between the groups. Evidence of cross-reactivity between native and transgenic AAI proves that they have homologous epitopes (Figure 10).

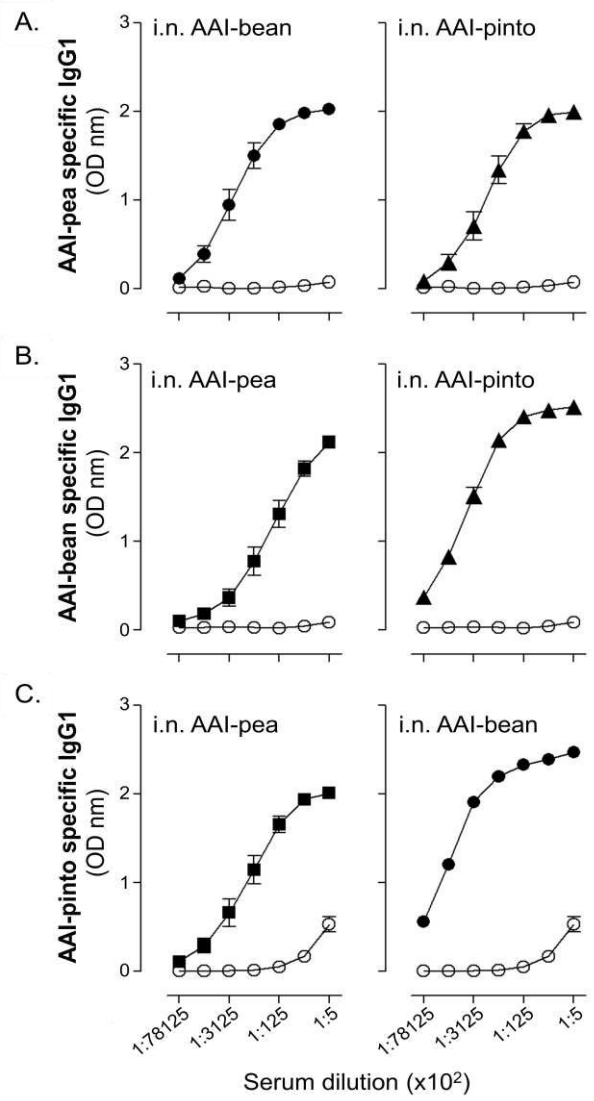


Figure 10. ***Cross-reactivity between native AAI-bean, AAI-pinto and transgenic AAI-pea.*** *Mice were immunized i.n. on days 0, 2, 4, 14, 16 and 18 with AAI (50 µg/ 50 µL) or PBS (50 µL). Serum was collected 48 h after the final immunization. To evaluate the cross-reactivity, 96 well plates were coated either with AAI-bean, AAI-pinto or AAI-pea (10 µg/ mL) overnight. (A) Mice were immunized either with AAI-bean or AAI-pinto and plates were coated with AAI-pea. (B) Mice were immunized with AAI-pea or AAI-pinto and plates were coated with AAI-bean. (C) Mice were immunized with AAI-pea or AAI-bean and plates were coated with AAI-pinto. Mice instilled with PBS (open circle). O.D. ± SEM, n = 5 – 8.*

Both, native and transgenic AAIs are capable of inducing an allergic airway and lung inflammation. Forty-eight hours after last AAI i.n. instillation, total cell numbers in airway were increased 2 to 3-fold compared to mice treated with only PBS, indicating an inflammatory stimulation of the immune system after treatment with AAIs (Figure 11A).

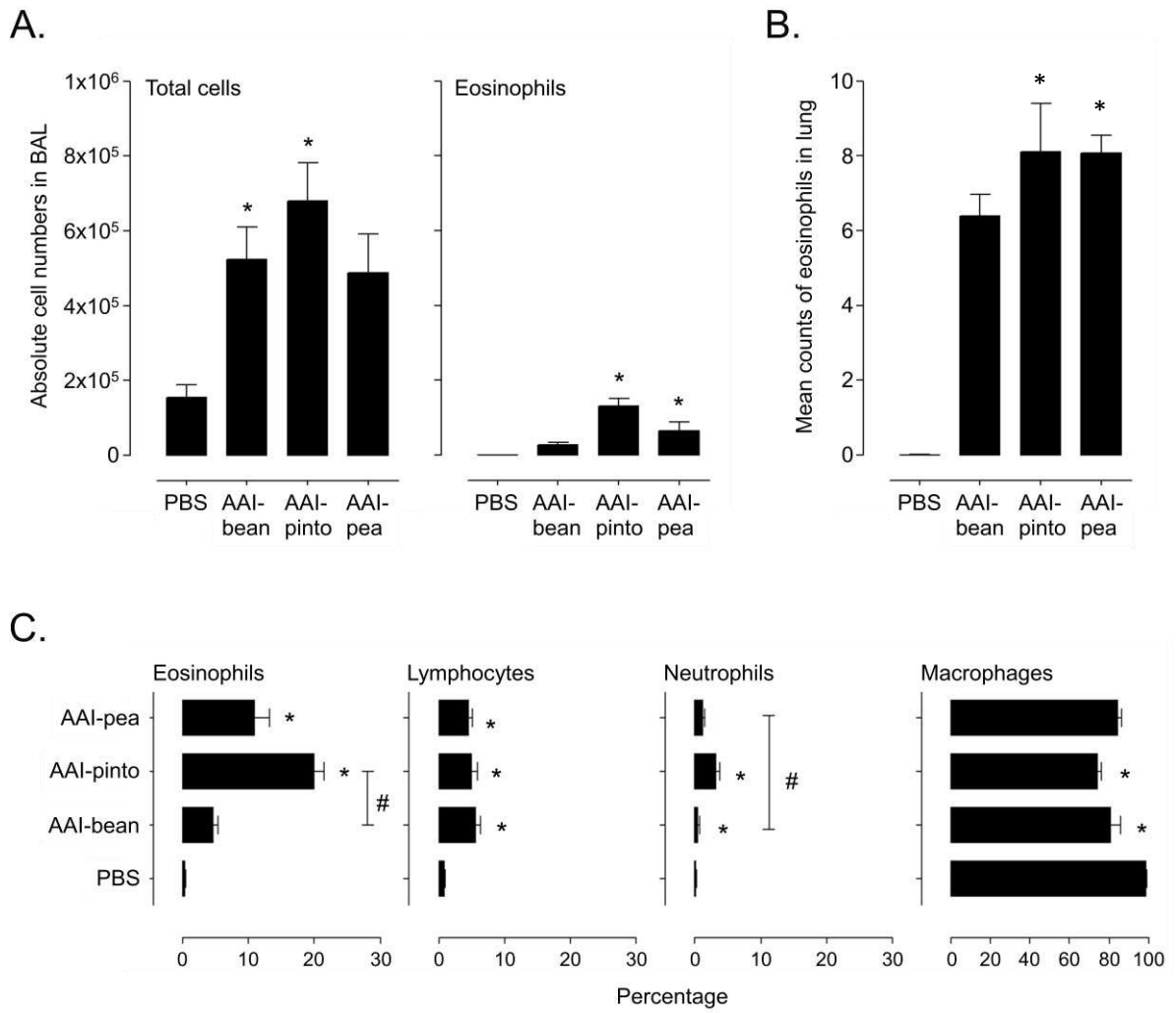


Figure 11. ***Airway and lung inflammation.*** *Groups of BALB/c mice (n = 8) were instilled i.n. six times with AAI extracted from Tendergreen bean, Pinto bean or GM pea. As a negative control, mice (n = 6) were treated with PBS alone. Forty-eight hours after last instillation, BAL fluid and lungs were collected. (A) Total and absolute eosinophil numbers in BAL fluid. (B) Lung sections were stained with Luna stain. For quantification of eosinophil infiltration, eosinophils were counted on ten random fields (40x magnification) containing alveoli and average for each lung were calculated. (C) Differential cell count in BAL fluid is presented in percentage. Mean ± SEM, Kruskal-Wallis test, *p <0.05 vs PBS, #p <0.05*

We detected in airway and lung an augmentation of the number of eosinophils in all groups of mice treated with AAI, but interestingly native AAI-pinto induced the highest number of eosinophils compared to transgenic AAI-pea (Figure 11A-C). This result indicates that the native AAIs stimulate a stronger allergic response than the transgenic protein. Mice instilled with AAI-bean had lower eosinophils than AAI-pinto and AAI-pea. It seems that AAI extracted from different legumes has different allergenicity potential irrespective of whether the source is native or transgenic. The BAL fluid collected from negative control mice which received only PBS was mainly composed of macrophages and few lymphocytes whereas AAI instilled mice had increased of lymphocytes and neutrophils (Figure 11C). Histological analysis of lung tissues recovered 48 h after the last i.n. instillation showed AAI dependent variation in the degree of asthma-like pathology. AAI-pinto and AAI-bean treated mice had the greatest accumulation of cell infiltrates than AAI-pea (Figure 12). The secretion of mucus is an inherent part of the defense of the airways dependent of the stimuli. Transgenic AAI extracted from GM pea discharged little mucus by goblet cells in lung, but native AAI had similar grades of mucus secretion (Figure 13). Intranasal administration of native and transgenic AAI induced airway inflammation associated with eosinophilia and goblet cell hyperplasia.

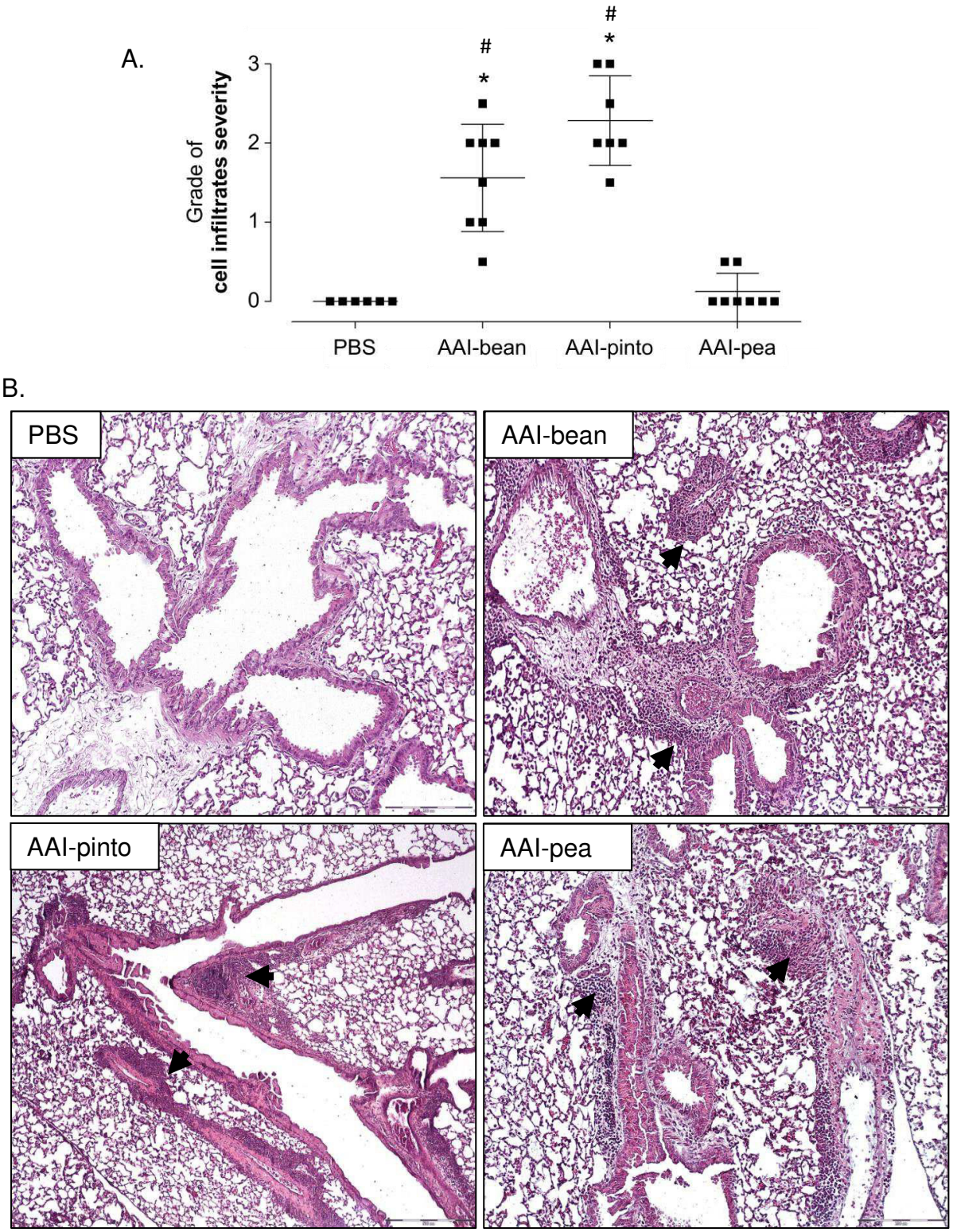
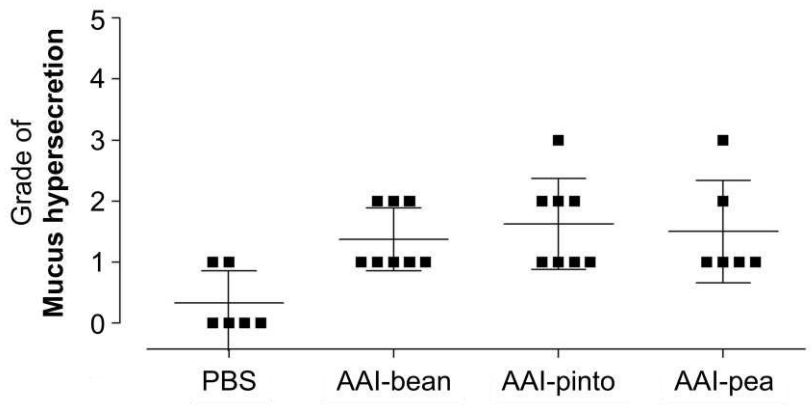


Figure 12. ***Severity of cell infiltrates in lung.*** *Groups of BALB/c mice (n = 8) were instilled i.n. six times with AAI extracted from Tendergreen bean, Pinto bean or GM pea. As negative control, mice (n = 6) were treated with PBS. Accumulation of inflammatory cells in lung was evaluated 48 h after last i.n. instillation. Three µm thick lung sections were stained with H&E. The extent of inflammation into the lungs was scored according to semi-quantitative scoring system. (A) Grade of cell infiltrates severity. (B) Photomicrograph of lung sections (10x magnification). Arrows indicate the areas of cell infiltrates. Mean ± SEM, Kruskal-Wallis test, *p <0.05 vs PBS, #p <0.05 vs AAI-pea*

A.



B.

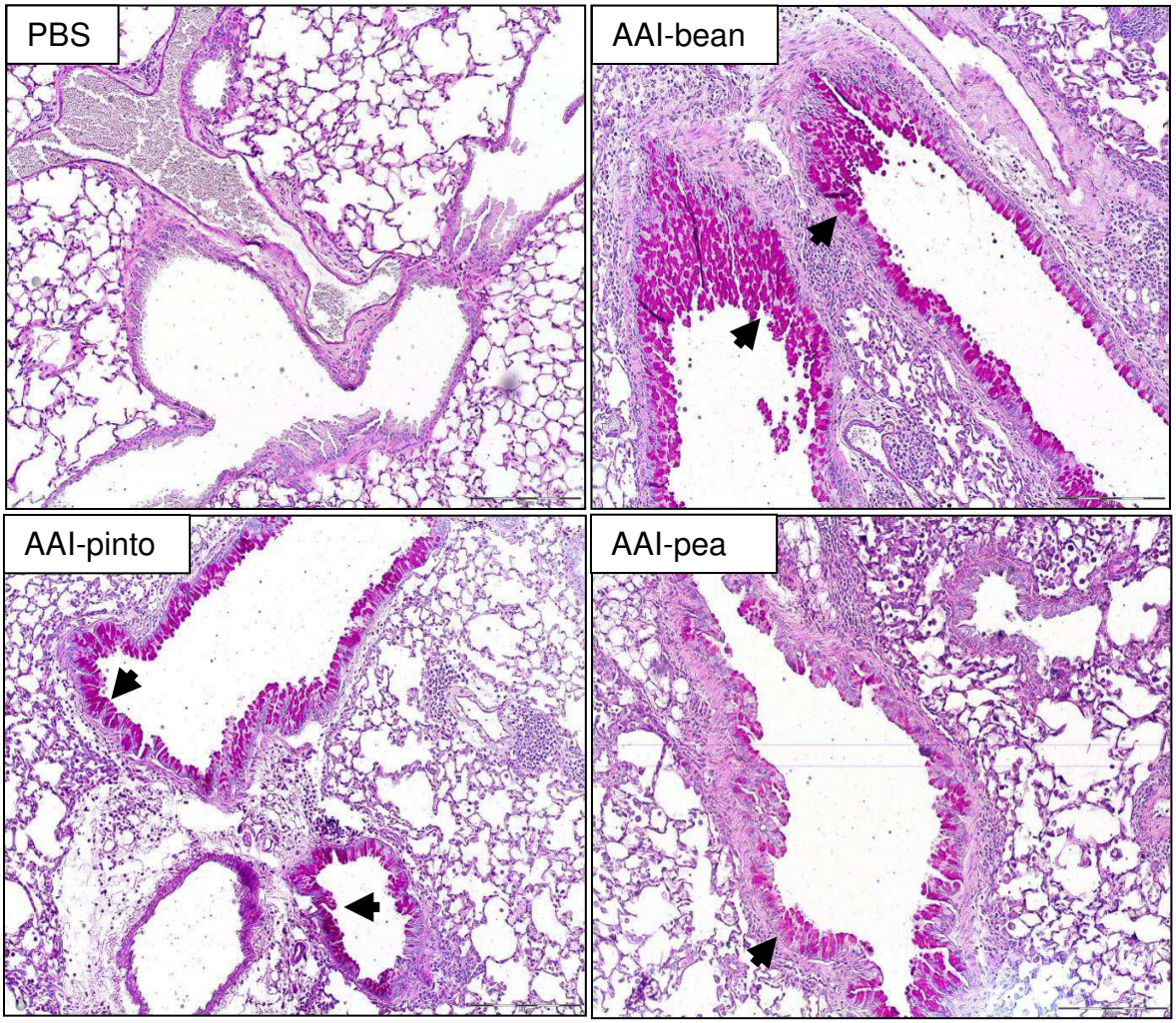


Figure 13. ***Mucus hypersecretion in lung.*** *Groups of BALB/c mice (n = 8) were instilled i.n. six times with AAI extracted from Tendergreen bean, Pinto bean or GM pea. As negative control, mice (n = 6) were treated with PBS. Secretion of mucus by goblet cells in lung was evaluated 48 h after last i.n. instillation. Three µm thick lung sections were stained with periodic-acid Schiff reagent. (A) The quantity of mucus secretion in lungs was scored according to semi-quantitative scoring system. (B) Photomicrograph of lung sections (10x magnification). Arrows indicate mucus secretion in airway. Mean ± SEM, Kruskal-Wallis test.*

3.3 Does feeding of GM peas induce allergy in mice?

Gavage feeding alone of native Tendergreen bean and Pinto bean induces an anti-AAI IgG1 response. To determine whether oral consumption of GM plant can induce an allergic response, BALB/c mice were force-fed by gavage 100 mg/ mL seed meal from Tendergreen bean, Pinto bean, nGM pea or GM pea in 100 µL PBS twice a week for four weeks. Two days after last feeding mice were challenged i.n. with one dose of pure AAI (50 µg/ 50 µL) extracted from Tendergreen bean, Pinto bean or GM pea. Serum was obtained either two days after last feeding from tail vein or 48 h after i.n. challenge by cardiac puncture (Figure 14A, B) to analyze the Ig level.

A.

	Gavage administration of 100 mg/ mL in PBS	i.n. challenged (50 µg/ 50 µL)
Groups:	PBS	PBS
	Tendergreen bean	AAI-bean
	Pinto bean	AAI-pinto
	GM pea	AAI-pea
	nGM pea	AAI-pea

B.

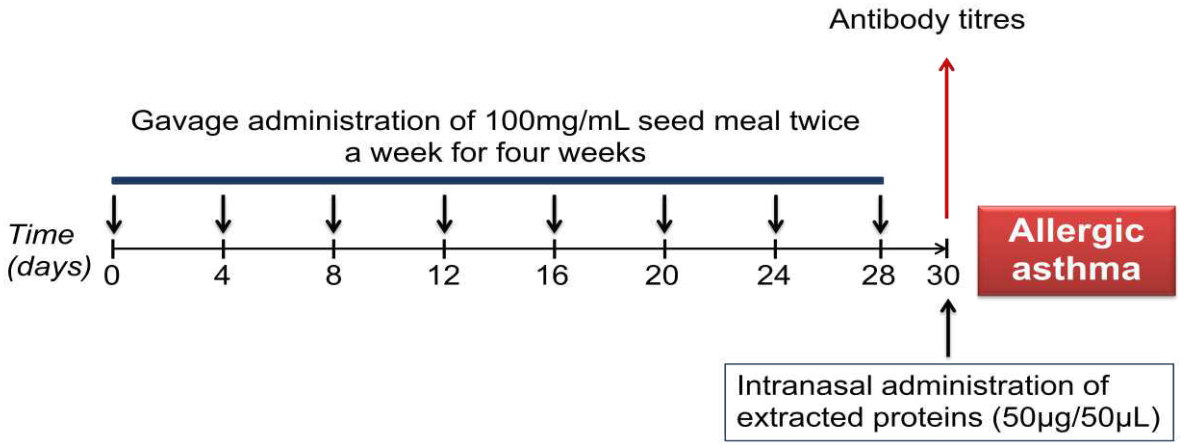


Figure 14. ***Feeding protocol.*** *Groups of mice (n = 6 - 7) were force-fed by gavage administration 100 mg/ mL seed meal twice a week for 4 weeks. Two days after last feeding mice were i.n. challenged with extracted protein (50 µg/ 50 µL). Features of allergic asthma were analyzed 72 h after i.n. challenge. Antibody titers were measured after last feeding and after i.n. administration. As negative control mice were force fed with PBS and challenged with PBS. (A) List of experimental groups. (B) Experimental protocol*

We observed that mice fed with native Tendergreen bean and Pinto bean had an increase of AAI-specific IgG1 antibodies, but it was not detectable in mice fed with nGM pea or with GM pea (Figure 15A, B). This observation was detected after last feeding, and the response did not change when mice were additionally challenged with AAI (Figure 16A, B). I.n. administration of only one dose pure AAI does not harm the animals. In these animals we did not detect AAI-specific antibodies or inflammation in lung (data not shown).

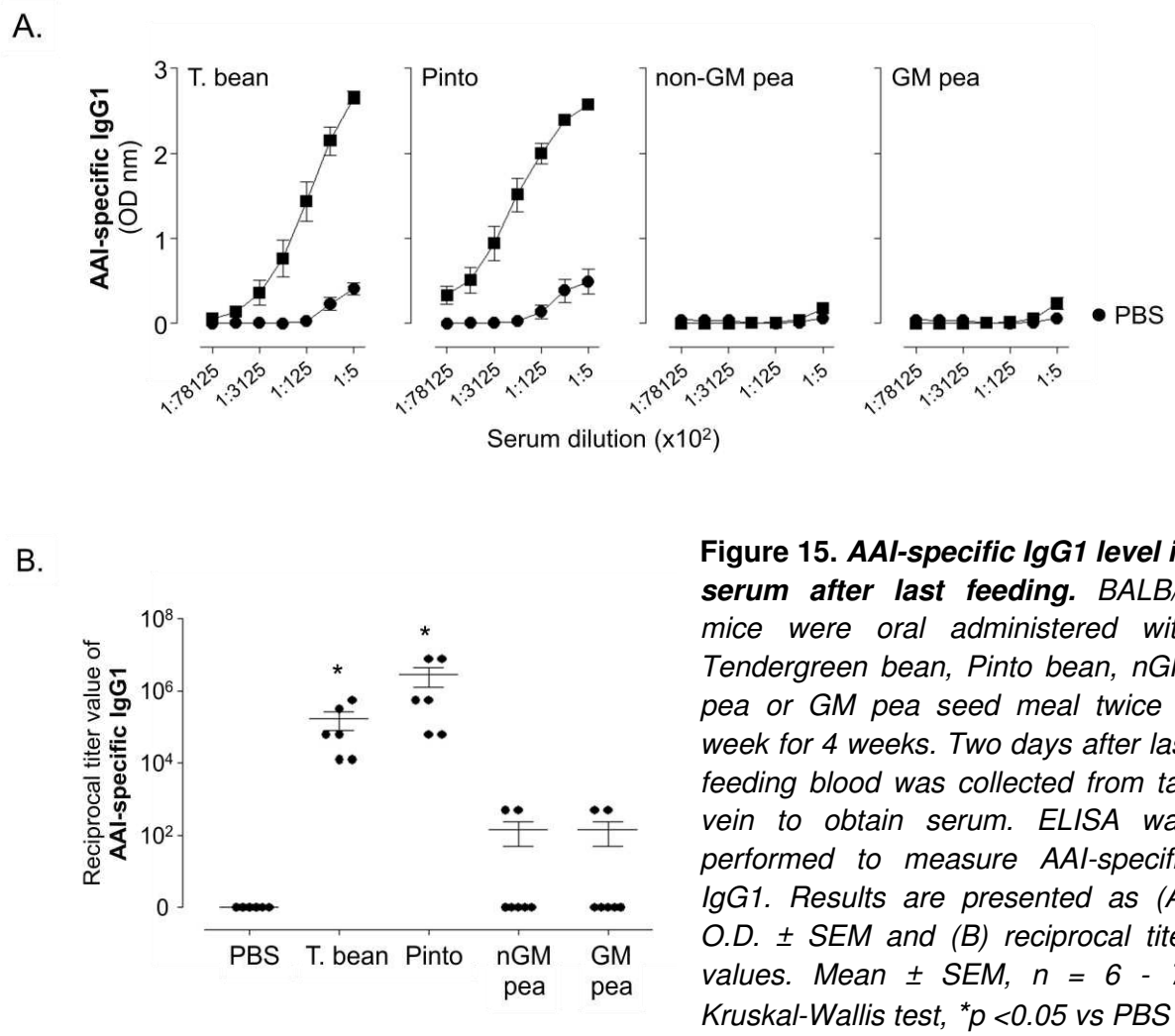


Figure 15. ***AAI-specific IgG1 level in serum after last feeding.*** *BALB/c mice were oral administered with Tendergreen bean, Pinto bean, nGM pea or GM pea seed meal twice a week for 4 weeks. Two days after last feeding blood was collected from tail vein to obtain serum. ELISA was performed to measure AAI-specific IgG1. Results are presented as (A) O.D. ± SEM and (B) reciprocal titer values. Mean ± SEM, n = 6 - 7, Kruskal-Wallis test, *p <0.05 vs PBS*

Interestingly, mice fed with native Tendergreen bean and challenged with AAI-bean induced an anti-AAI IgE response, but not detectable in animals fed with Pinto bean, GM pea or nGM pea (Figure 16A, B). These data suggest that oral consumption of native bean containing native AAI but not the transgenic AAI in GM pea is able to induce an allergic response.

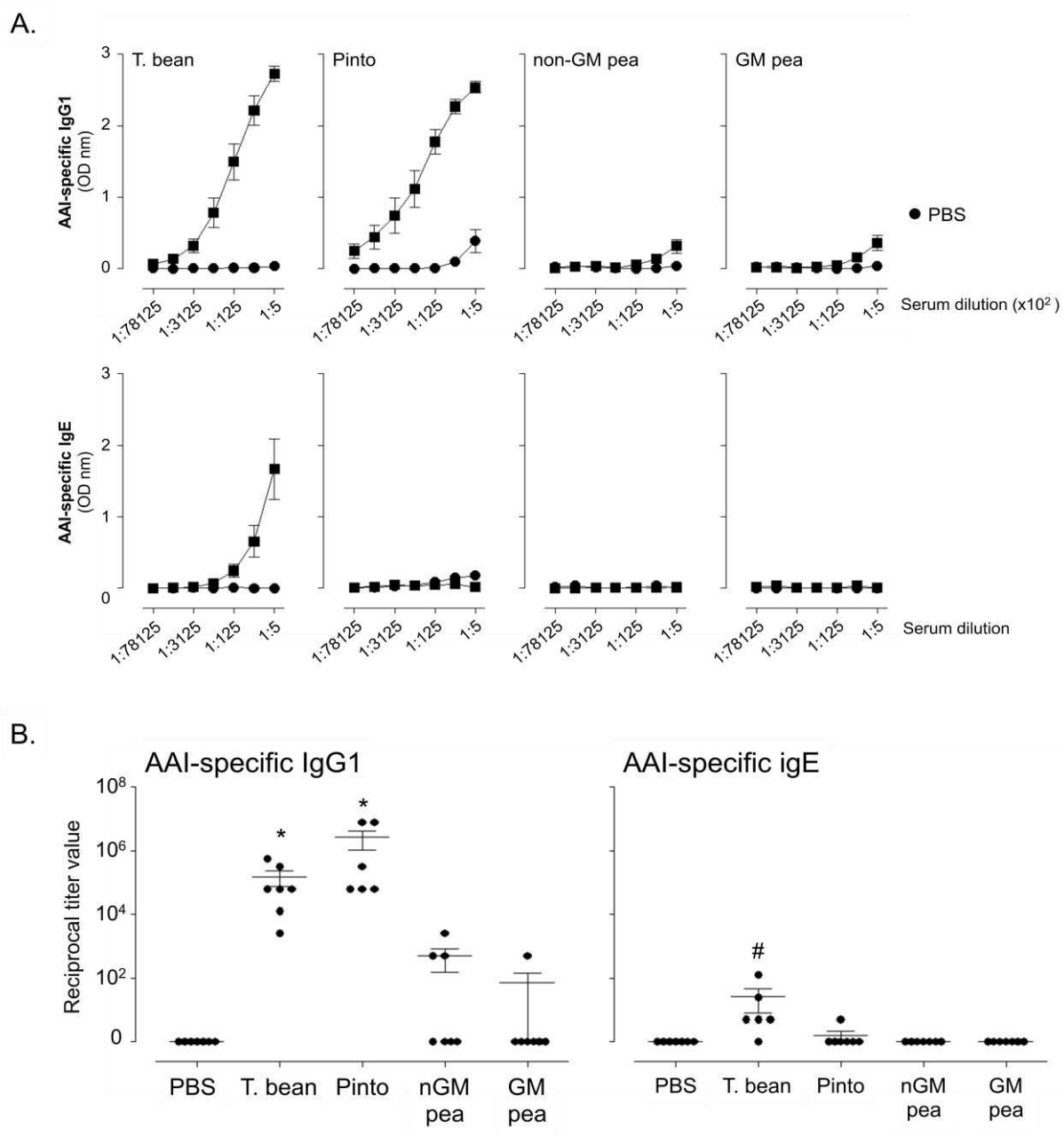


Figure 16. ***AAI-specific IgG1 and IgE levels in serum after oral consumption and i.n. challenged with AAI.*** *BALB/c mice were oral administered with Tendergreen bean, Pinto bean, nGM pea or GM pea seed meal twice a week for 4 weeks. Two days later, mice were challenged with pure AAI extracted from Tendergreen bean, Pinto bean or GM pea and 72 h after i.n. challenged mice were assessed and blood was collected by cardiac puncture. ELISA was performed to measure AAI-specific IgG1 and IgE. Results are presented as (A) O.D. ± SEM and (B) reciprocal titer. Mean ± SEM, n = 6 - 7, Kruskal-Wallis test, *p <0.05 vs PBS; #p <0.05 vs PBS, Pinto bean, nGM pea and GM pea*

Next we assessed IgE functionality with Rat-Basophils-Leukemia cell assay (Figure 17). Basophil histamine release is an *ex vivo* test of the circulating effector cells triggered to release histamine by IgE cross-linking to demonstrate partial reactivity. To evaluate the allergenic activity of AAI, RBL-2H3 cells were passively sensitized with mouse monoclonal IgE against the AAI. Interestingly, we observed that serum from mice fed and challenged with native Tendergreen bean and Pinto bean induced AAI-specific degranulation. In contrast, serum from mice fed and challenged with GM peas did not induce degranulation. This result indicates that oral consumption of only native beans generates functional IgE antibodies.

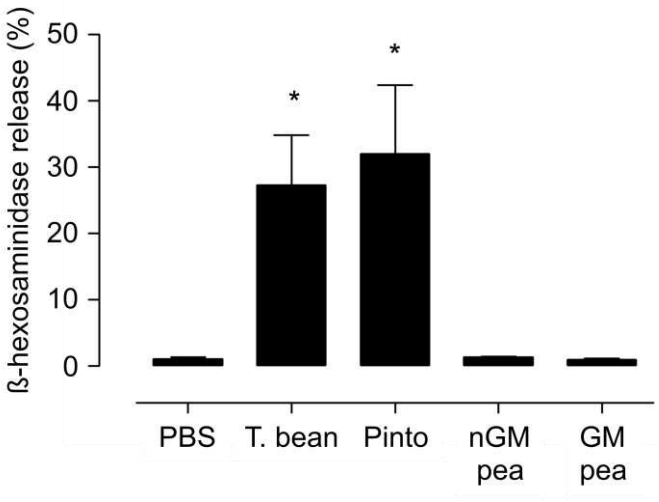


Figure 17. *ß-hexosaminidase release in serum.* *Blood samples were collected by cardiac puncture after protein challenge and centrifuged to obtain serum. RBL-2H3 cells were plated to 96-well plates and incubated with serum (serum dilution 1:10). Sensitized cells were stimulated with antigen (100 ng/ mL). Calculation was performed using the formula: Values are % specific lyses calculated by the formula: (value background)/ (maximum lyses-background) x 100. Results are expressed as percentage of total ß-hexosaminidase released after the addition of 1% Triton X-100 and presented the mean of duplicate determinations. Mean ± SEM, n = 6 - 7, Kruskal-Wallis test, *p <0.05 vs nGM pea and GM pea.*

Oral consumption of native beans induces airway inflammation in mouse model. Allergy response was further characterized by analyzing inflammatory cells in BAL fluid after protein challenge. Mice challenged i.n. after last feeding serve as an *in vivo* readout for T cell priming that occur during feeding process. We detected an increase of eosinophilic cell numbers in animals fed with Tendergreen beans and Pinto beans, whereas feeding with GM peas only a slightly increase of inflammatory cells could be observed (Figure 18). As expected, one dose of i.n. instillation of pure

protein did not mediate inflammation. Tendergreen bean, Pinto bean and most varieties of common beans consist of phytohemagglutinin (PHA), an anti-nutritional factor that induces dietary toxicity in rodents and birds, which could play a role in inducing an increase of inflammatory response. An increase of neutrophils was also detected in mice fed with native beans, but this could be due to the PHA content in beans. GM pea fed mice had the highest macrophage numbers, but this could be due to the low numbers of eosinophils and neutrophils since total BAL cell numbers was equivalent to PBS treated mice (Figure 18).

Furthermore, we analyzed inflammation in lung. Histological scores revealed no significant differences in severity of cell infiltrates between mice fed with native beans or GM peas in lung (Figure 19). However, quantification of eosinophils displayed a significantly higher accumulation of eosinophils in mice fed with seed meals compared with PBS treated group. In addition, animals fed with Tendergreen bean followed by Pinto bean revealed the highest accumulation of inflammatory cells in lung compare to nGM pea or GM pea which match to our previous result observed in airway with one exception: Pinto bean treated group had the lowest counts of eosinophils in lung. The reason for this observation is still not clear. As mentioned previously, Tendergreen bean contain high amount of PHA which could explain the reason of the growth of eosinophils in airway and lung. In the *Prescott* study[88], Pinto bean was used as control because of the low PHA content compared with Tendergreen bean. Another explanation of this result could be due to stronger allergenic provocation of native bean in mice. However, more research is necessary to confirm this argument. We further analyzed the severity of mucus hypersecretion in lung. Histological scores revealed significant increase of mucus secreted by goblet cells in mice fed with different kind of seed meal compare to PBS treated group (Figure 19). With the scoring system, we observed a significant difference between native bean and transgenic peas. Taken together, these results indicate that GM peas do not trigger a Th2 response. The inflammatory response for nGM and GM pea is similar low which proposes that the insertion of the transgenic AAI does not differ the immune response whereas feeding with native Tendergreen bean or Pinto bean can cause an asthmatic Th2 response.

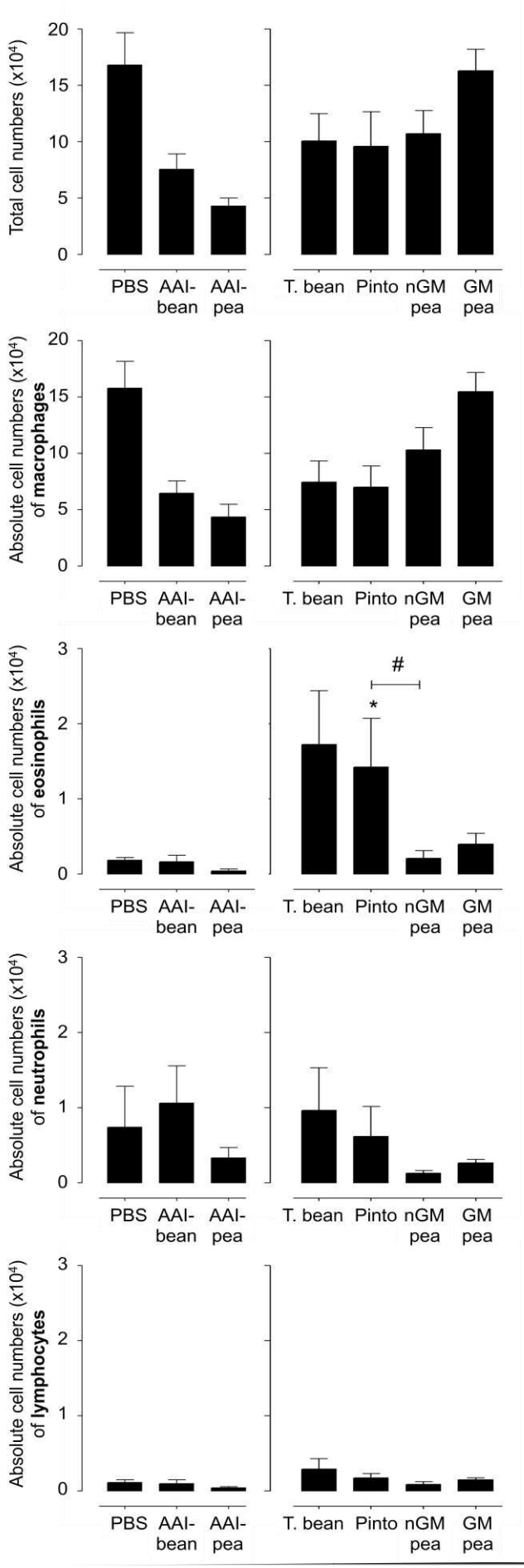


Figure 18. ***Differential cell count in BAL after i.n. challenged.*** *Groups of BALB/c mice were force fed with Tendergreen bean, Pinto bean, nGM pea or GM pea seed meal (100 mg/ mL) twice a week for 4 weeks. Two days after last feeding mice were i.n. challenged with pure AAI (50 µg/ 50 µL) extracted from native bean and GM pea. As negative control, mice were fed with saline. We included two other groups in which mice were only i.n. challenged with one dose of pure protein. Bronchoalveolar lavage fluids were performed 72 h after challenged to collect airway cells. Cytospun cells were stained with Kwik-Diff and approximately 300 cells were counted from each mouse. Graphs present total cell numbers and absolute cell numbers of macrophages, eosinophils, neutrophils and lymphocytes. Mean ± SEM, n = 6 - 7, Kruskal-Wallis test, *p <0.05 vs PBS; Mann Whitney test #p <0.05*

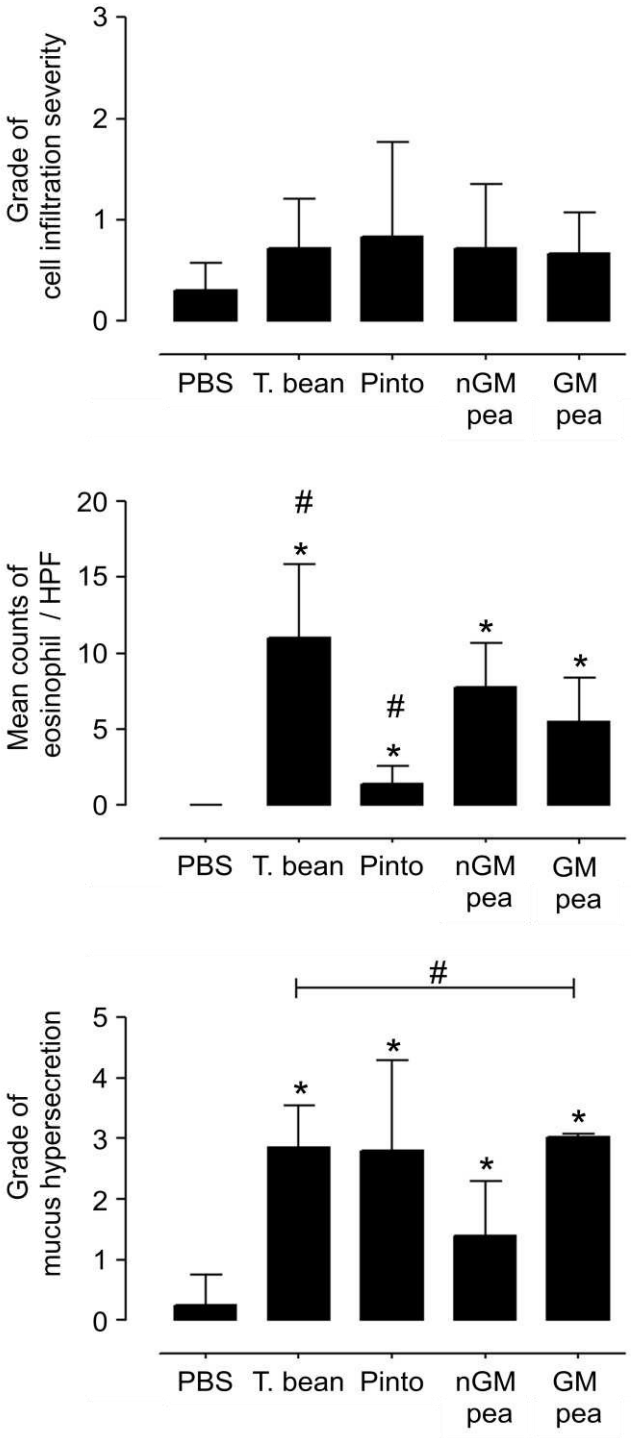


Figure 19. ***Lung inflammation of fed and challenged mice with native or GM legume.*** *Experimental feeding protocol is described in Figure 14 A and B. Lungs were harvested 72 h after i.n. challenged. Three µm frozen paraffin-embedded lung sections were stained either with H&E, Luna staining or periodic-acid Schiff reagent. Histological scored were performed by given grade numbers dependent on the severity of cell infiltrates, accumulation of eosinophils or mucus hypersecretion. Histological scores were given for cell infiltrates from 0 - 3 and for mucus hypersecretion from 0 - 5. Mean ± SD, n = 6 - 7, Kruskal-Wallis test, *p <0.05 vs PBS; Mann Whitney test #p <0.05 vs nGM pea and GM pea. For quantification of eosinophil infiltration, eosinophils were counted on ten random fields (40x magnification) containing alveoli and average for each lung. Mean ± SEM, n = 6 - 7, Kruskal-Wallis test, *p<0.05 vs PBS; Mann Whitney test #p <0.05*

***Heat treatment of native beans and GM peas reduces allergic asthma response*.** Legumes like Tendergreen beans or peas are usually pre-cooked before consumption. Therefore, to resemble the human diet, seed meals were heated for 25 min at 100°C before feeding to the animals. We used the same experimental model as explained in Figure 14. Both cooked and uncooked seed meal were used in our experiment for comparison. We observed that heating of seed meals decreased inflammatory cells in airway irrespective of the origin of the legumes. Native bean and GM pea fed mice had a decrease of eosinophils after heating (Figure 20). However, this observation was not detected in mice fed with nGM pea. Eosinophils were slightly increased in groups fed with cooked nGM pea. In contrast, we observed a significant increase of neutrophils in mice fed with cooked seed meals (Figure 20). These results suggest that cooking of seed meals may lead to alteration in their structure which may result in changes in allergenicity. Percentage of lymphocytes was equal between heat-treated and raw seed meals.

We analyzed the inflammation in lung. Three µm lung sections were stained with H&E and the severity of cell infiltrates were scored by given numbers from 0 – 3 whereas 0 - indicates no inflammation and 3 - severe inflammation in lung. We observed mild inflammatory cell infiltrates in mice fed with cooked and uncooked seed meals (Figure 21). However, the variation within the groups was huge indicated by the error bars. To confirm our observation, inflammatory eosinophils were counted in lung sections stained with Luna. We did not detect differences between treated and untreated groups except Pinto bean fed group. Mice fed with cooked Pinto bean had more inflammatory cells compared with raw Pinto bean fed animals (Figure 21). Histological scores revealed significantly increased of mucus secretion by goblet cells in mice fed with raw seed meals compare to heat treated meals (Figure 21).

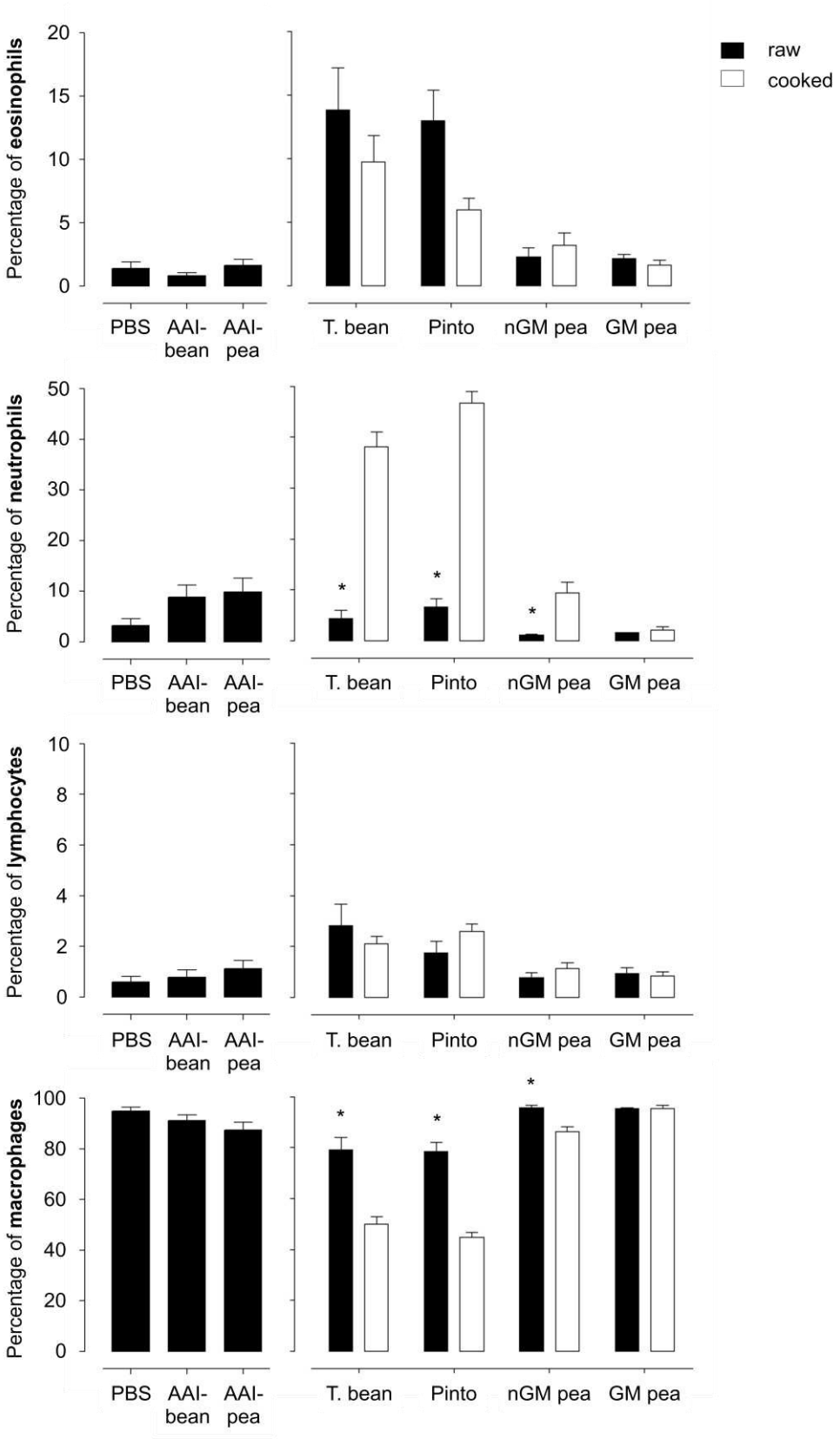


Figure 20. ***Mice fed with cooked seed meals revealed a decrease of eosinophils but an increase of neutrophils.*** *Seed meals were either untreated or preheated at 100°C for 25 min. Groups of BALB/c mice were force fed with Tendergreen bean, Pinto bean, nGM pea or GM pea raw or cooked seed meal (100 mg/ mL) twice a week for 4 weeks. Two days after*

*last feeding mice were i.n. challenged with pure AAI (50 µg/ 50 µL) extracted from native bean or GM pea. As negative control, mice were fed with saline. Two groups of mice were i.n. challenged only with one dose of pure protein. Bronchoalveolar lavage fluids were performed 48 h after challenged to collect the airway cells. Cytospun cells were stained with Kwik-Diff and approximately 300 cells were counted from each mouse. Graphs represent total cell numbers and absolute cell numbers of macrophages, eosinophils, neutrophils and lymphocytes. Mean ± SEM, n = 6 - 8, Mann Whitney test *p <0.05 vs cooked.*

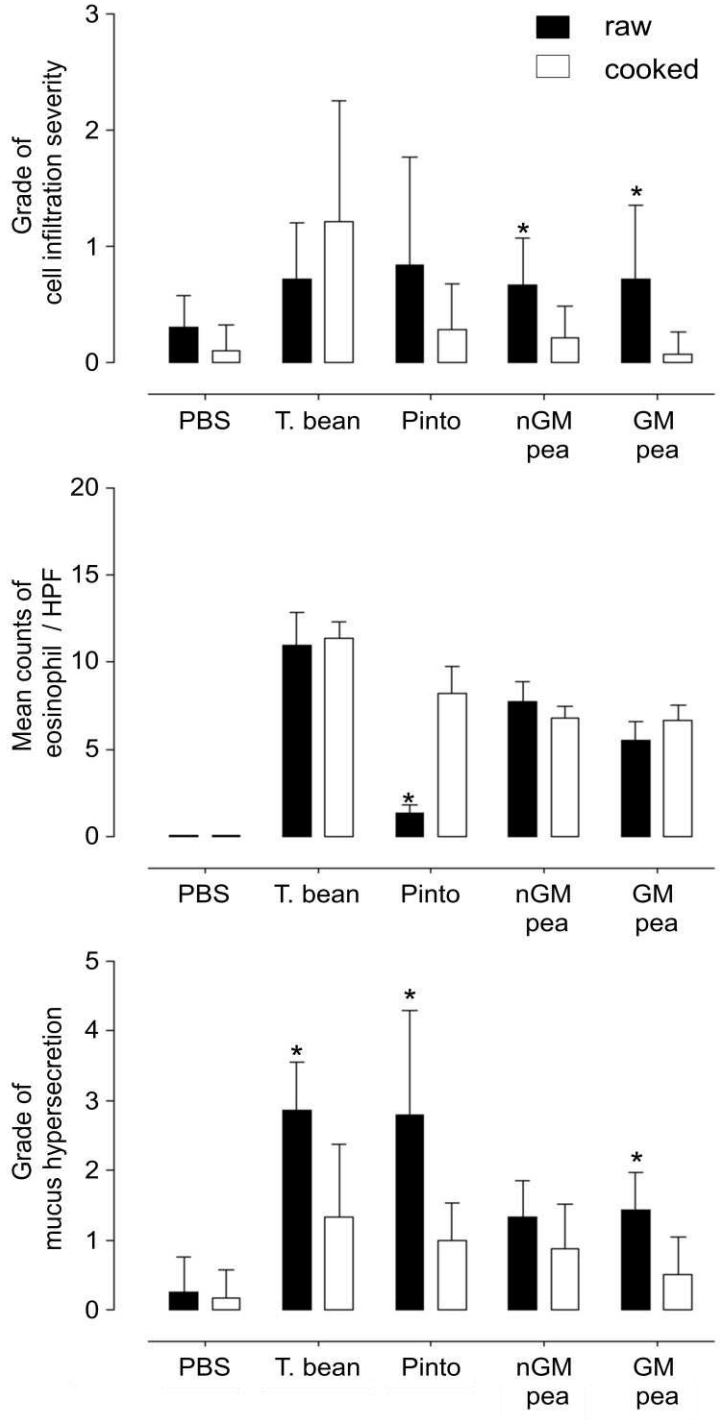


Figure 21. *Lung inflammation in mice fed with raw/preheated native or GM legume.* *Experimental feeding protocol is described in Figure 14 A and B. Lungs were harvested 72 h after i.n. challenged. Three µm frozen paraffin-embedded lung sections were stained either with H&E, Luna staining or periodic-acid Schiff reagent. Histological scores were performed by given grade numbers dependent on the severity of cell infiltrates, accumulation of eosinophils or mucus hypersecretion by goblet cells. Histological scores were given for cell infiltrates from 0 - 3 and for mucus hypersecretion from 0 - 5. Mean ± SD, n = 6 - 8, Mann Whitney test *p<0.05 vs cooked.*

A decrease of antibody response after oral consumption of heat treated seed meals in mice. We observed that feeding of preheated seed meals lead to a decrease of anti-AAI specific IgG1 response detectable in Tendergreen bean and Pinto bean fed groups (Figure 22, Table 5). Non-GM pea and GM pea had low antibody titer regardless of raw or heat treated meals.

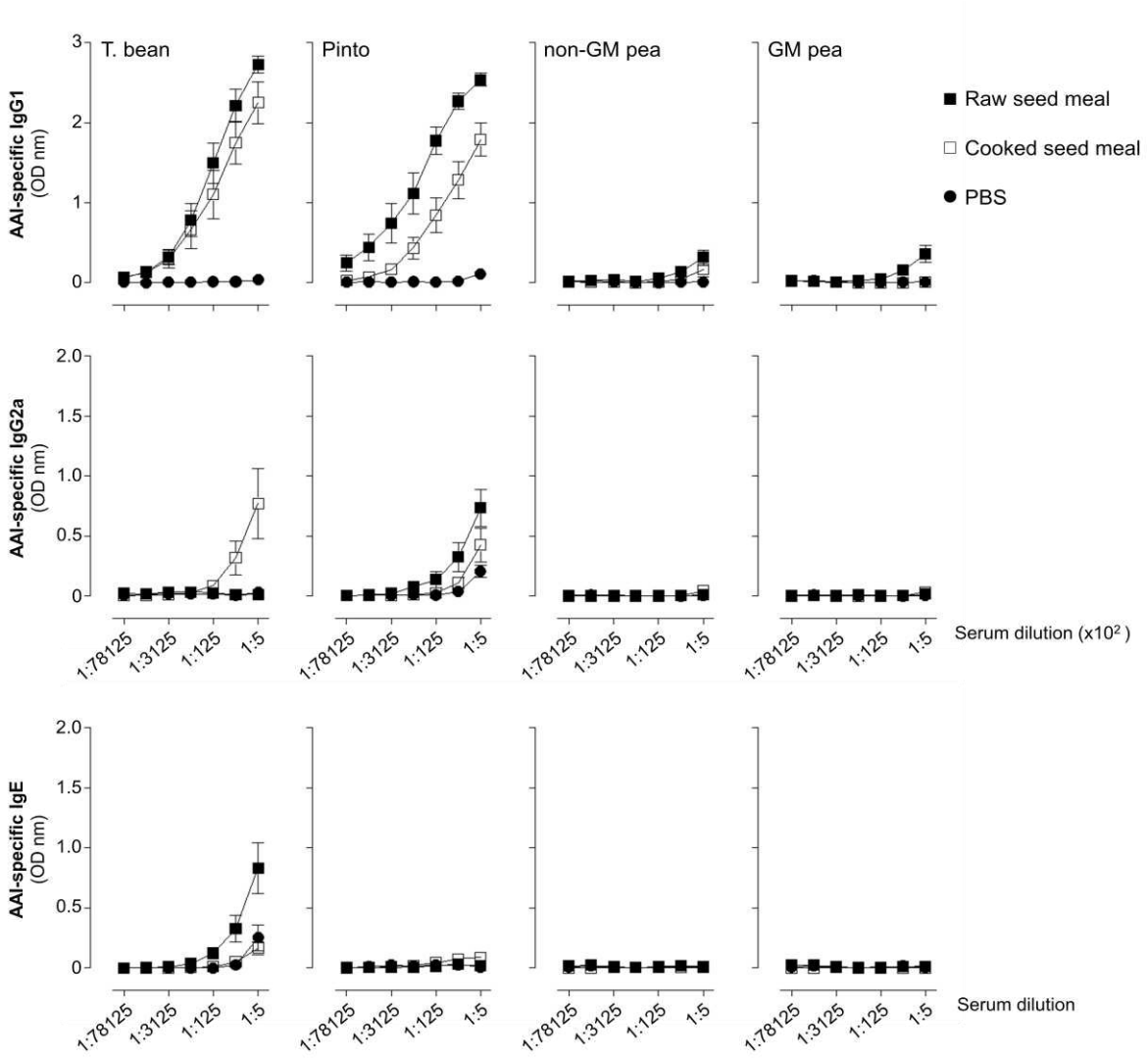


Figure 22. ***AAI-specific IgG1, IgG2a and IgE antibody level in sera.*** *BALB/c mice were oral administered with either raw or cooked Tendergreen bean, Pinto bean, nGM pea or GM pea seed meal twice a week for 4 weeks. Two days later mice were challenged with pure AAI extracted from Tendergreen bean, Pinto bean or GM pea. Forty-eight hours after i.n. challenged mice were assessed and blood was collected by cardiac puncture. ELISA was performed to measure AAI-specific IgG1, IgG2a and IgE. Results are presented as O.D. ± SEM, n = 6 – 8.*

Anti-AAI specific IgE was detectable in mice fed with raw Tendergreen bean but diminished when beans were preheated (Figure 24, Table 5). We did not detect AAI specific IgE titer in groups fed with Pinto bean, nGM pea or GM pea. To determine whether feeding raw or cooked seed meal can induce a Th1 response, anti-AAI specific IgG2a was measured. Surprisingly, cooked Tendergreen beans induced an anti-AAI specific IgG2a antibody response but not raw bean. Whereas, uncooked Pinto beans induced a slightly increase of IgG2a antibody titer but reduced when mice were fed with preheated Pinto beans. Taken together, oral consumption of preheated native and GM legumes induces a decrease of inflammation in airway and lung.

Table 3. ***Mean reciprocal endpoint titer in serum***

	raw	**cooked**	**raw**	**cooked**
Seed meal	**α-AAI IgG1**	**α-AAI IgG1**	**α-AAI IgE**	**α-AAI IgE**
PBS	1.002 ± 0.002	1.002 ± 0.002	1.003 ± 0.003	1.003 ± 0.003
T. Bean	153900 ± 78690	167900 ± 101900	**27.67 ± 19.78***	**1.667 ± 0.667***
Pinto	**2688000 ± 1621000***	**89690 ± 49130***	0.035 ± 0.013	0.011 ± 0.004
nGM pea	500.6 ± 500,6	313.4 ± 312.4	1.014 ± 0.014	1.014 ± 0.014
GM pea	72.29 ± 71.29	1.013 ± 0.012	1.014 ± 0.014	1.013 ± 0.013

Serum samples were obtained after i.n. challenged with pure AAI. Data represent in this table are mean reciprocal titer ± SEM. n = 6 - 8, Mann Whitney test **p<0.05 vs cooked.*

3.4 Effect of GMO consumption on the initiation and exacerbation of allergic diseases to other allergens

Oral consumption of GM peas does not alter the initiation of OVA-induced allergic asthma. OVA immunization and challenge model has been extensively used to study allergic asthma. To determine whether intragastric feeding of GM peas influences the allergenicity of other allergens, we utilized allergic asthma models developed in our laboratory using chicken egg white (OVA). We first gavage fed mice twice a week for 4 consecutive weeks with GM peas. As control we fed them with the nGM pea and native Tendergreen bean or only with saline (Figure 23).

A.

	Gavage administration of 100 mg/ mL in PBS
Groups	Naive
	PBS
	Tendergreen bean
	nGM pea
	GM pea

B.

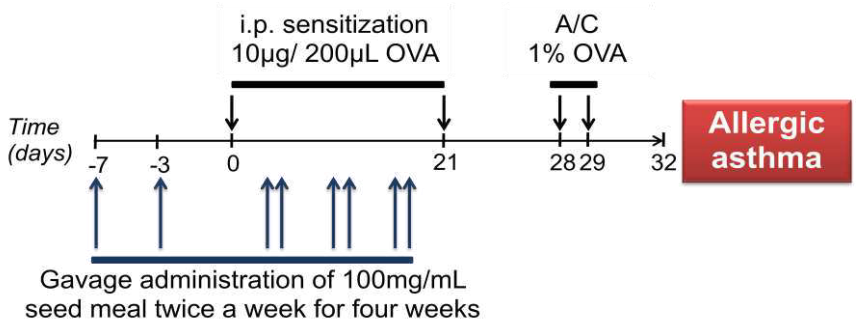


Figure 23. ***Acute model of OVA-induced allergic asthma gavage fed with GM peas.*** *BALB/c mice were sensitized i.p. on days 0 and 21 with 10 µg/ 200 µL OVA. Mice were aerosol challenged one week later twice a day on two consecutive days with 1% OVA. During the sensitization with OVA, groups of mice (n = 5) were force fed by gavage administration of 100 mg/ mL seed meal twice a week for 4 weeks with GM pea and PBS alone, Tendergreen bean and nGM pea. Characteristic features of allergic asthma were analyzed 72 h after aerosol challenge. (A) List of experimental groups and (B) experimental protocol.*

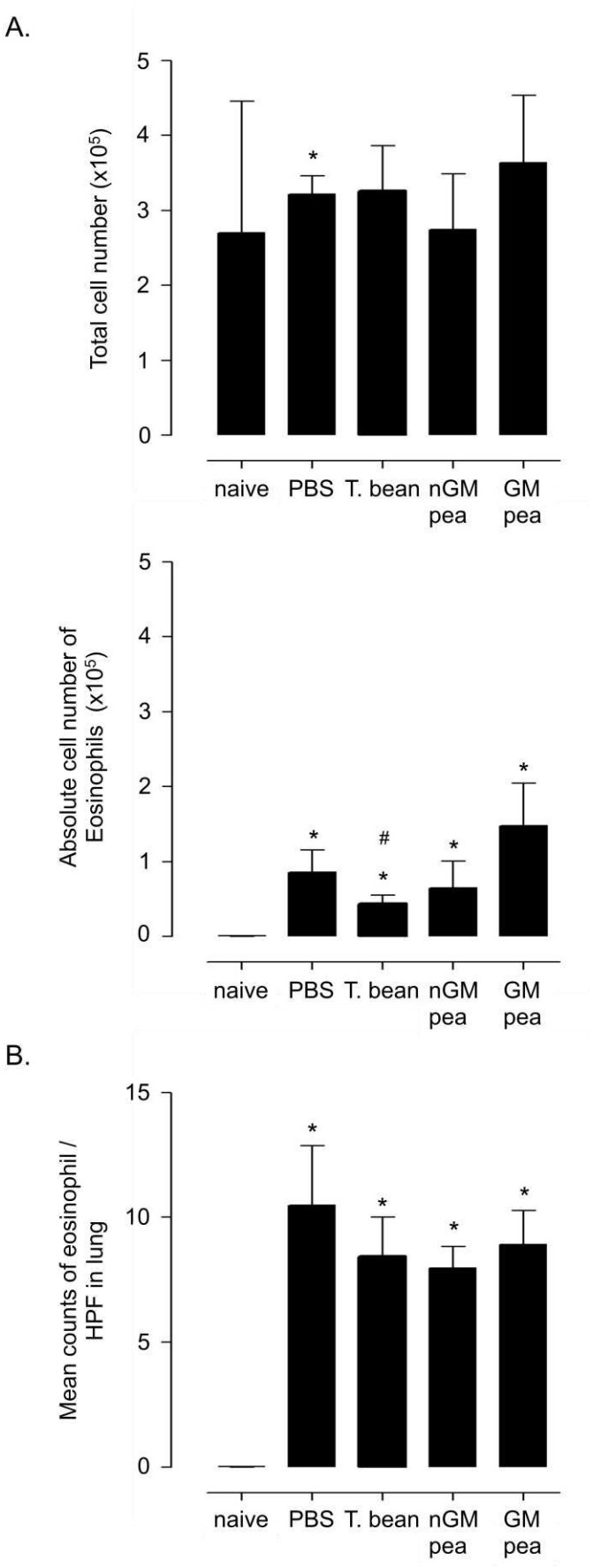


Figure 24. ***Airway and lung inflammation in initiation of OVA-induced allergic disease.*** *Groups of BALB/c mice were force fed with Tendergreen bean, nGM pea, GM pea seed meal (100 mg/ mL) or only saline twice a week for 4 weeks during OVA i.p. sensitization. One group of mice was left untreated (naïve). 72 h after aerosol challenge airway cell composition was evaluated. Cytospun cells were stained with Kwik-Diff and approximately 300 cells were counted from each mouse. 3 µm frozen lung sections were stained with Luna to visualized inflammatory cells. (A) Total cell numbers and absolute cell numbers of eosinophils in the airway. (B) Eosinophil counts per high power field in lung. Mean ± SEM, n = 10, from two independent experiments, Kruskal-Wallis test, *p<0.05 vs naive; Mann Whitney test #p<0.05 vs GM pea*

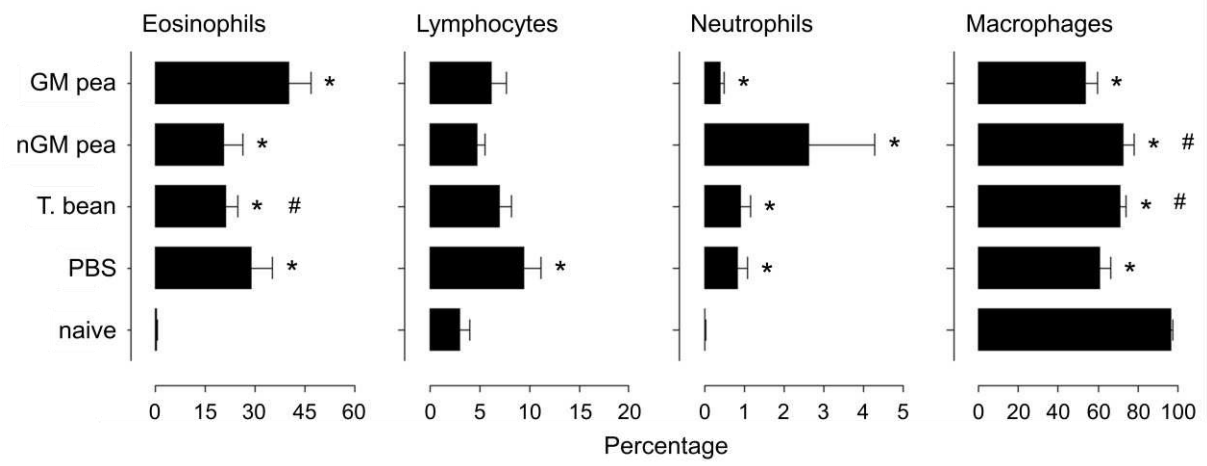


Figure 25. *Differential cell counts in airway.* *Groups of BALB/c mice were force-fed with Tendergreen bean, nGM pea, GM pea seed meal (100 mg/ mL) or only saline twice a week for 4 weeks during OVA i.p. sensitization. One group of mice was left untreated (naïve). 72 hours after aerosol challenge airway cell composition was analyzed. Cytospun cells were stained with Kwik-Diff and approximately 300 cells were counted from each mouse. Data is presented as mean cell counts ± SEM, n = 10, from two independent experiments, Kruskal-Wallis test, *p <0.05 vs naive; Mann Whitney test #p<0.05 vs GM pea*

Mice were immunized with 10 µg/ 200 µL OVA i.p. on days 0 and 21 and aerosol challenged with OVA twice a day for 2 consecutive days. Mice were fed during the sensitization period twice a week for 4 weeks. To analyze the effect of GM pea consumption during existing allergic asthma, we quantified total and differential cell counts in the airways 72 h after aerosol challenge. As expected, mice immunized with OVA had an increase of inflammatory cells in airway and lung compare to naïve mice (Figure 24A, Figure 25). Total cell numbers were significantly increased in PBS treated group compared to the naïve group. Interestingly, more accumulation of inflammatory cells was detected in OVA-immunized mice fed with GM pea (Figure 24A, Figure 25). But this result was not confirmed in lung. Diseased mice fed with native or GM seed meals or PBS had extensive inflammatory cell infiltrates compared with healthy mice. But feeding with native GM legumes did not cause an adjuvant effect (Figure 24B). Eosinophilia was approximately the same between PBS and seed meal-fed groups, whereas neutrophils were increased in nGM-, Tendergreen- and PBS-fed groups compared to naïve (Figure 25). Only OVA-immunized mice treated with PBS induced a small increase in the number of lymphocytes (Figure 25). Upon evaluation of histological sections of lung tissue from mice, we observed. peribronchial and perivascular inflammatory infiltrates in lung tissue. However, GM

AAI feeding did not alter the severity of inflammation in the lung. In contrast, nGM, pea-fed mice developed the highest level of cell infiltration compared with the other groups (Figure 26). Increase of mucus secretion by goblet cells is another important feature of allergic asthma. In our experiments, we observed that OVA-sensitized mice had a high level of mucus secretion (Figure 27), while GM pea-fed groups had less mucus compared with mice fed with native AAI. Interestingly, mice fed with nGM pea had the strongest accumulation of inflammatory cells and mucus secretion, which suggests that there is cross-reactive response occurring upon consumption of peas without AAI. These results demonstrated that feeding AAI peas does not alter the lung inflammation, whereas nGM pea appears to minimally worsen lung inflammation induced by a non-cross-reactive antigen. Additionally, we observed that OVA-specific IgG1 and IgE antibodies were unaffected by feeding AAI peas (Figure 28) thus, indicating that oral consumption of GM peas does not alter B-cell responses to OVA.

The level of GM AAI in peas is about 0.5 - 1% of dry weight as found in Tendergreen bean. In a separate experiment we added an additional 5 µg of AAI (data not shown) to increase the AAI dose in seed meal to determine if an increase of GM AAI can alter the allergenic response. But we did not detect a change in inflammation or antibody production. These results are an additional confirmation that GM AAI peas do not alter allergic asthma in mice. To ascertain that the dose of GM AAI peas are not playing a role in allergenicity, we gavage fed more GM pea seed meal to mice with OVA-induced allergic asthma. We used a similar feeding protocol as described in Figure 23, but increased the feeding from twice a week to three times a week for consecutive 4 weeks. We assessed the mice for disease and observed that a higher consumption of GM peas did not affect the induction of allergic lung inflammation (data not shown).

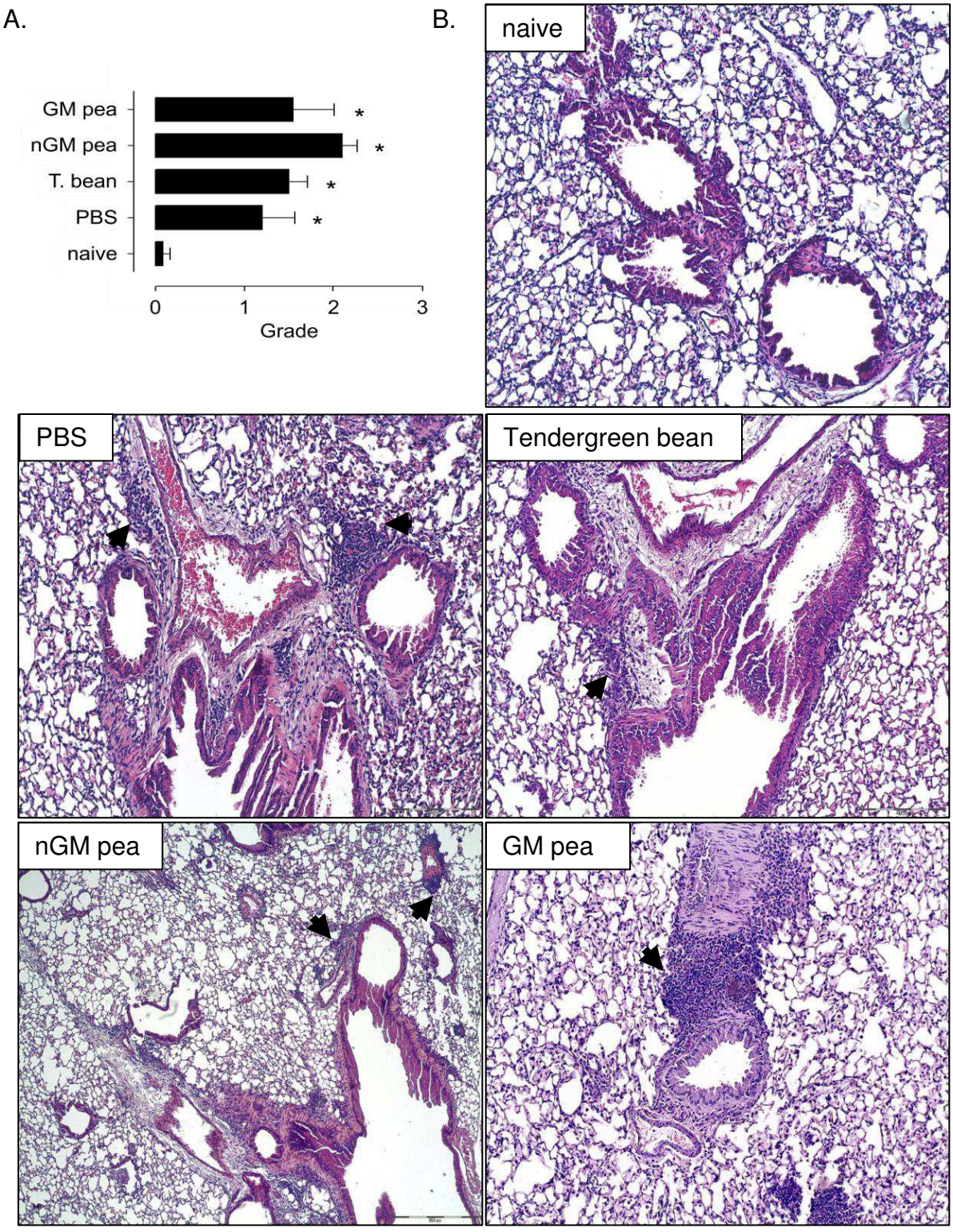


Figure 26. ***Severity of inflammation in mice with OVA-induced allergic asthma fed with GM and nGM seed meal.*** *The experimental design and feeding protocol are summarized in Figure 23. Lungs were harvested 72 h after last aerosol challenge. Three µm frozen paraffin-embedded lung sections were stained with H&E. Histological scores: 0- no cell infiltrates, 1- cell infiltration is spread into the middle part of the lung and 3- describes robust infiltration and reaches the peripheral part of the lung. (A) Grade of intensity of pulmonary infiltrates. (B)*

Photomicrograph of lung sections (10x magnification). Arrows indicate the areas of cell infiltrates. Mean ± SEM, n = 10, from two independent experiments, Mann Whitney test $^{*}p$ <0.05 vs naive.

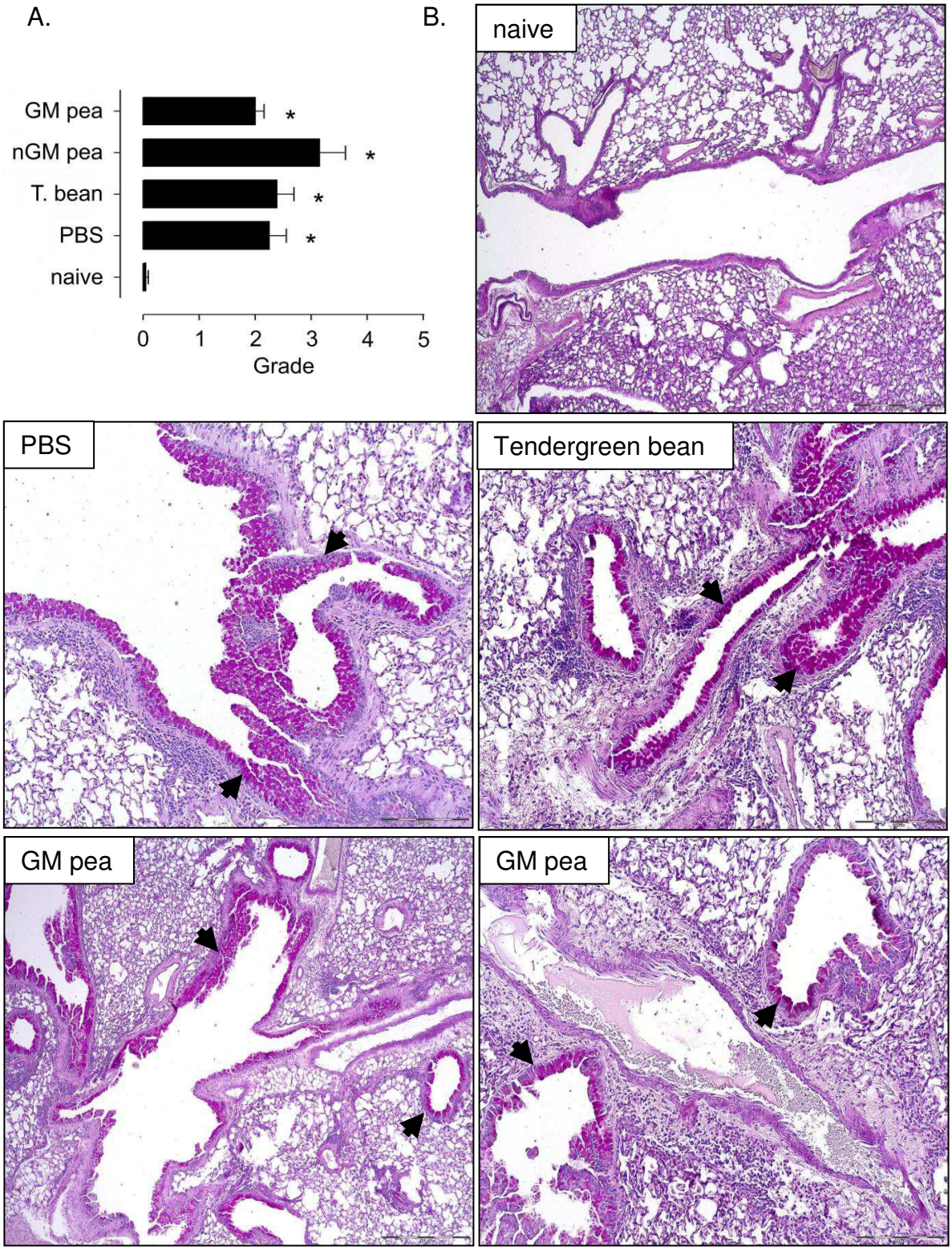


Figure 27. ***Mucus secretion in lungs of mice with OVA-induced allergic asthma fed with GM and nGM seed meal.*** *The experimental design and feeding protocol is summarized in Figure 23. Lungs were harvested 72 h after last aerosol challenge. Three µm frozen paraffin-embedded lung sections were stained with PAS reagent. Goblet cell hyperplasia in the airway epithelium was quantified based on a five-point grading system: 0 → 0% PAS positive cells; 1 → 0 - 20%; 2 → 21 - 40%; 3 → 41 - 60%; 4 → 61 – 80% and 5 → 81 - 100% PAS positive cells. (A) Mucus secretion. (B) Photomicrograph of lung sections (10x magnification). Arrows indicate mucus secretion in airway. Mean ± SD, n = 10, from two independent experiments, Mann Whitney test* $^{*}p < 0.05$ *vs naive.*

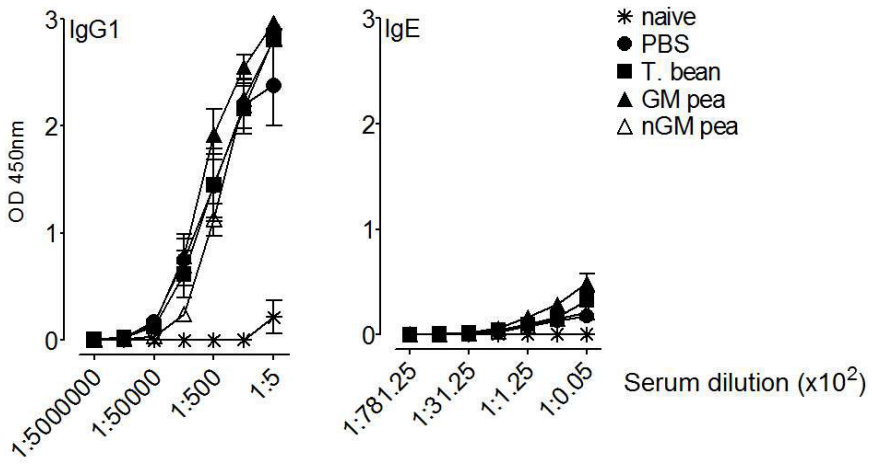


Figure 28. ***OVA-specific IgG1 and IgE antibody titer in serum.*** *The experimental design and feeding protocol are summarized in Figure 23. Serum samples were obtained by cardiac puncture after last aerosol challenge with OVA. ELISA was performed to measure OVA-specific IgG1 and IgE. Results are presented as O.D. ± SEM, n = 5.*

Table 6. ***Mean reciprocal endpoint titer in serum***

	Acute	
Group	**α-OVA IgG1**	**α-OVA IgE**
naive	167.3 ± 166.3	1.000 ± 0.0000
PBS	2120000 ± 1179000*	11.40 ± 5.600
T. bean	2120000 ± 1179000*	36.20 ± 22.75
nGM pea	410000 ± 190000*	10.60 ± 5.879
GM pea	3110000 ± 1160000*	81.00 ± 27.13*

Serum samples were obtained by cardiac puncture after last aerosol challenge with OVA. Data presented in this table are mean reciprocal titers ± SEM. n = 5, Mean ± SEM, Mann Whitney test $^{*}p<0.05$ *vs naive.*

Oral consumption of GM peas does not alter the exacerbation of OVA-induced allergic asthma. We evaluated whether GM pea feeding influences the induction of a disease exacerbation which is commonly referred to as an asthma attack, mice with pre-existing allergic asthma were tested. We injected female BALB/c mice on days 0 and 21 with OVA i.p. and nebulized them with 1% OVA on days 28 and 29 to initiate allergic asthma. Mice were recovered for one month until they were re-exposed to OVA for the induction of a disease exacerbation. We fed mice during disease remission with GM peas (100 mg/ mL), nGM pea, Tendergreen bean, or PBS twice a week for 4 weeks (Figure 29).

A.

	Gavage administration of 100 mg/ mL in PBS
Groups	Naive
	PBS
	Tendergreen bean
	nGM pea
	GM pea

B.

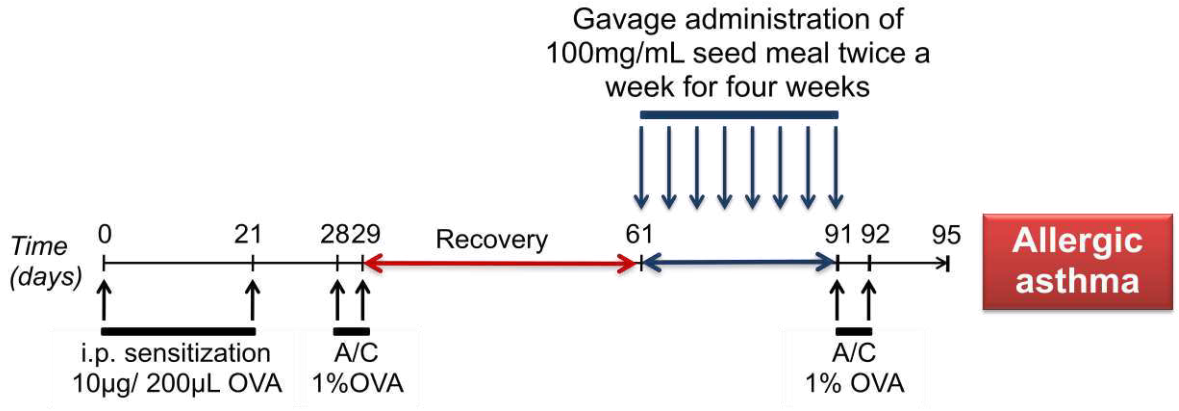


Figure 29. ***Exacerbation model of OVA-induced allergic asthma gavage fed with GM peas.*** *BALB/c mice were sensitized i.p. on days 0 and 21 with 10 µg/ 200 µL OVA. Mice were aerosol challenged 1 week later twice a day on two consecutive days with 1% OVA. After one month recovery mice were force fed by gavage administration of 100 mg/ mL GM pea and as control PBS, Tendergreen bean and nGM pea twice a week for 4 weeks. After last feeding, mice were rechallenged with OVA-aerosol. Features of allergic asthma were analyzed 72 h after rechallenge. (A) List of experimental groups and (B) experimental protocol.*

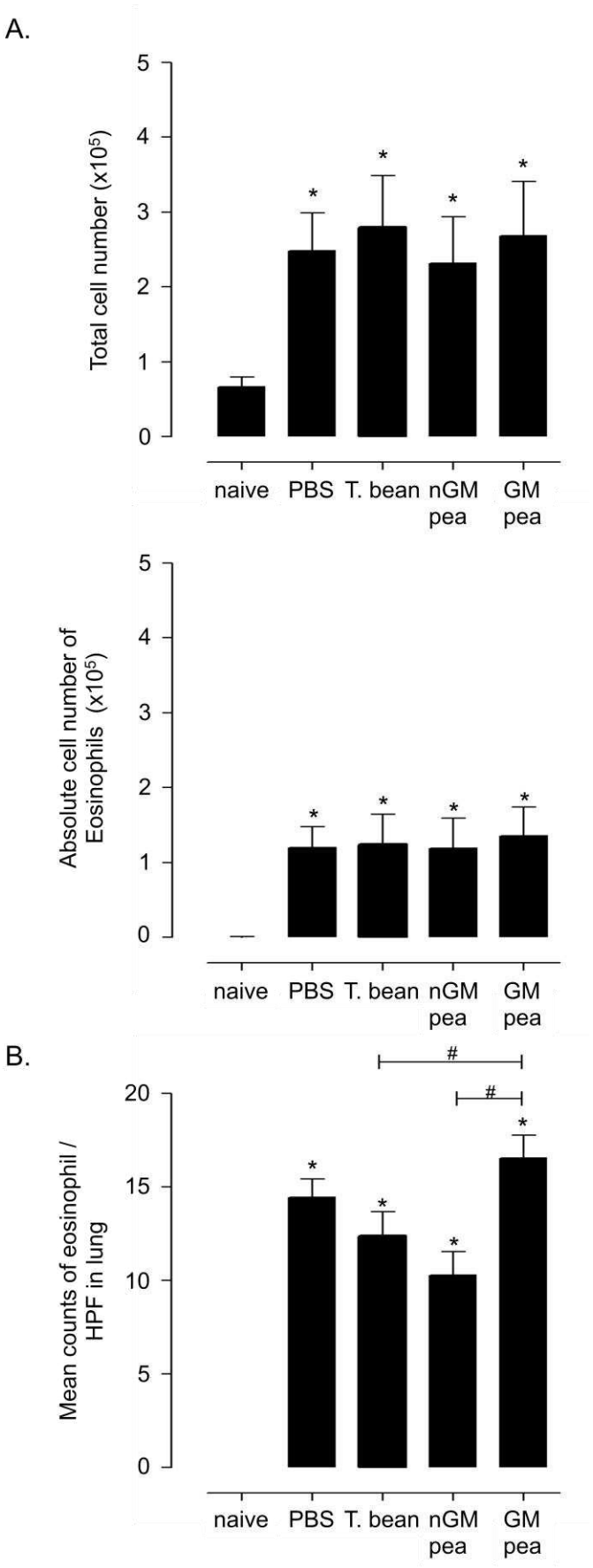


Figure 30. ***Effect of GM AAI pea consumption on airway and lung inflammation in exacerbation of OVA-induced allergic asthma.*** *Groups of BALB/c mice were force fed with Tendergreen bean, nGM pea, GM pea seed meal (100 mg/ mL) or saline twice a week for 4 weeks one month after recovery. One group of mice was left untreated (naïve). Bronchoalveolar lavage fluids were performed 72 h after last aerosol rechallenge to collect the airway cells. Cytospun cells were stained with Kwik-Diff and approximately 300 cells were counted from each mouse. 3 µm frozen lung sections were stained with Luna to visualized inflammatory cells. (A) Total cell numbers and absolute cell numbers of eosinophils. (B) Eosinophil counts per high power field. Mean ± SEM, n = 10, Kruskal-Wallis test, *p <0.05 vs naive; Mann Whitney test #p<0.05*

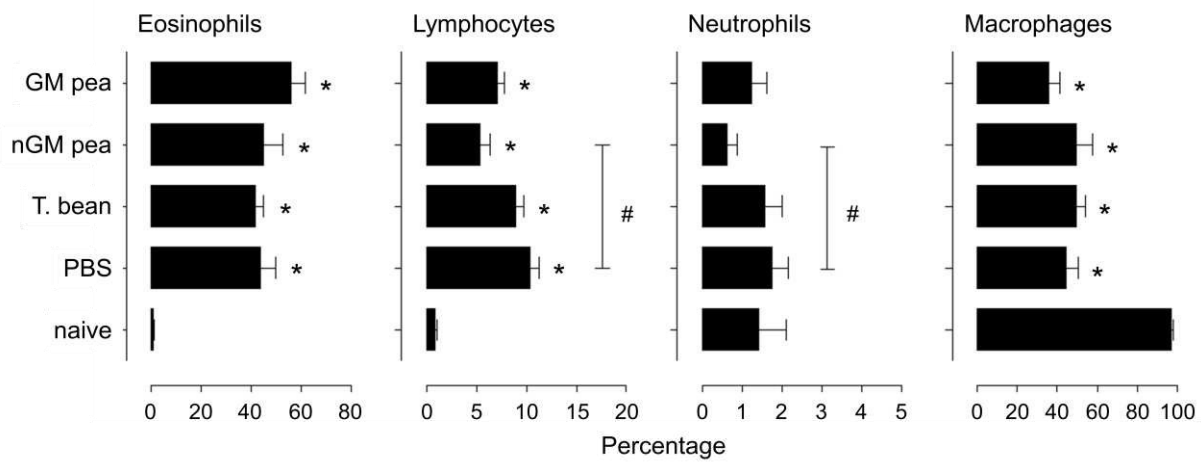
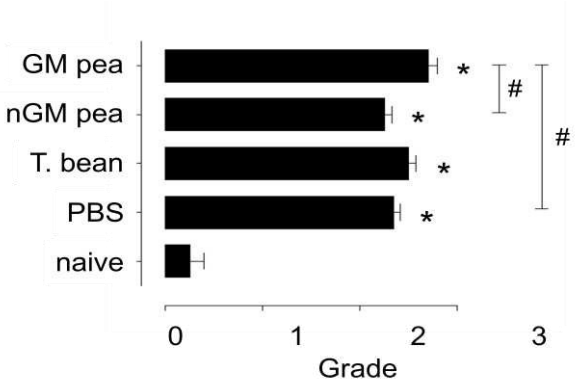


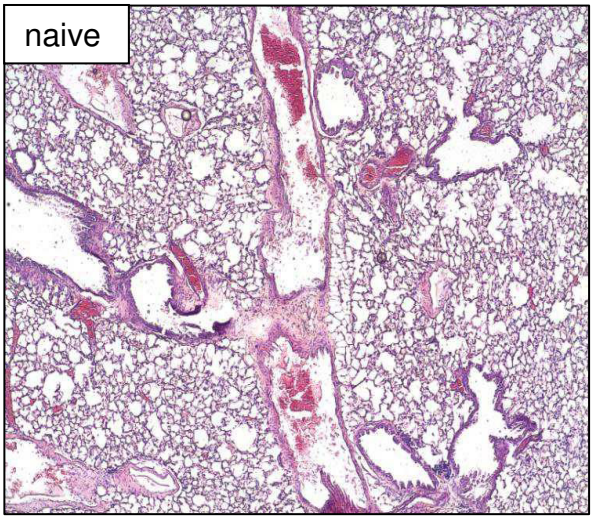
Figure 31. ***Differential cell counts in the airways.*** *Groups of BALB/c mice were force fed with Tendergreen bean, nGM pea, GM pea seed meal (100 mg/ mL) or saline twice a week for 4 weeks, 1 month after recovery. One group of mice was left untreated (naïve). Bronchoalveolar lavage fluids were performed 72 h after last aerosol rechallenge to collect the airway cells. Cytospun cells were stained with Kwik-Diff and approximately 300 cells were counted from each mouse. Mean percentage of eosinophils, lymphocytes, neutrophils and macrophages ± SEM, n = 10, from two independent experiments, Kruskal-Wallis test, *p<0.05 vs naive; Mann Whitney test #p<0.05.*

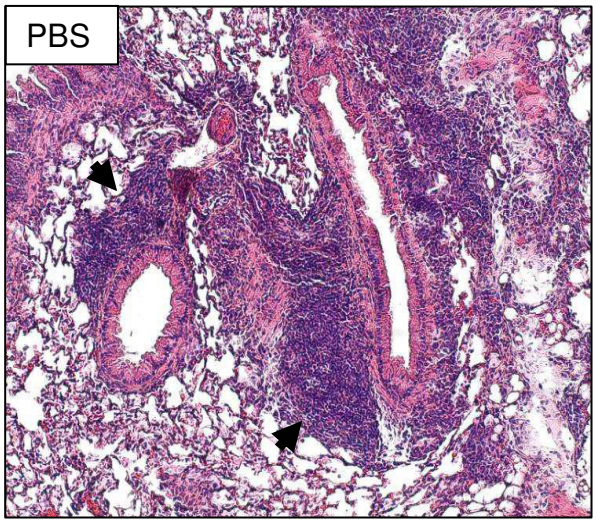
Analysis of airway cells and cell infiltrates in lung showed that pre-existing allergic asthma had increased total cell numbers compared to naïve mice. Feeding of GM pea, Tendergreen bean or nGM pea did not significantly change the cell numbers in airway compared to the PBS treated group (Figure 30A). We did not observe any differences in eosinophil numbers in the airways of mice with OVA-induced allergic asthma compared to bean and GM pea-fed groups. However, eosinophils were higher in exacerbation compared to acute onset disease (Figure 25, 31). Accumulation of inflammatory cells in lung was significantly higher in mice fed with GM pea compared with Tendergreen bean or native pea but not significantly different from the PBS group, which suggests that it is not a GM effect (Figure 30B). As expected, lymphocyte and macrophage percentages differed compared to naïve mice, but the percentage of neutrophils were similar between naïve and fed groups (Figure 31).

A.


GM pea
nGM pea
T. bean
PBS
naive
*
*
*
*
#
#
0
1
2
3
Grade

B.


naive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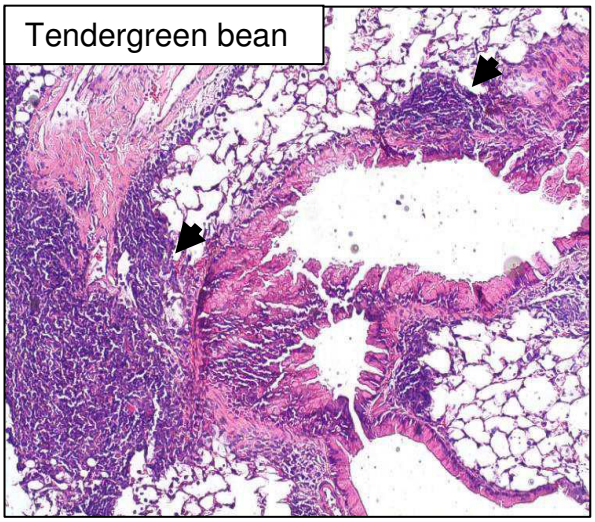
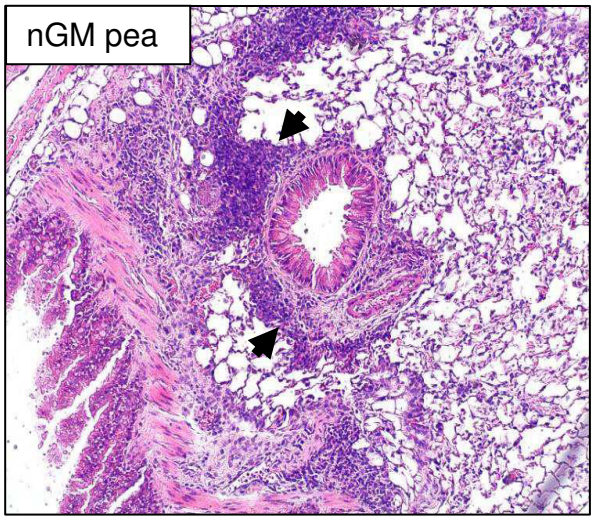

Tendergreen bean


nGM pea

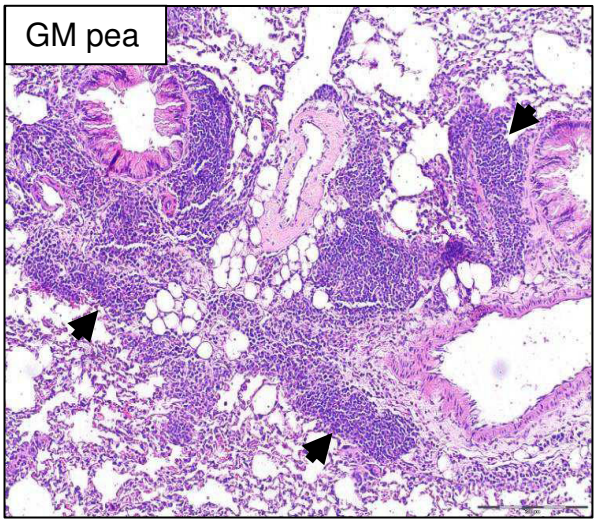

GM pea

Figure 32. ***Severity of inflammation in OVA-induced allergic asthma exacerbation in mice fed with GM and nGM peas.*** *The experimental design and feeding protocol are summarized in Figure 29. Lungs were harvested 72 h after last aerosol challenge. Three µm frozen paraffin-embedded lung sections were stained with H&E. Histological scores: 0- no cell infiltrates, 1- cell infiltrates is spread into the middle part of the lung and grade 3- describes robust infiltration and reaches the peripheral part of the lung. (A) Intensity of lung infiltrates. (B) Photomicrograph of lung histology sections from naïve and mice with allergic asthma fed with Tendergreen bean, GM pea or nGM pea (10x magnification). Arrows indicate the areas of cell infiltrates. Mean ± SEM, n = 5, Kruskal-Wallis test, *p<0.05 vs naive; Mann Whitney test $^{\#}p < 0.05$.*

Tissue inflammation during an exacerbation is more severe than during acute onset disease (Figure 32). We observed more intense accumulation of inflammatory cells in mice fed with GM pea compared with nGM pea or PBS group (Figure 32). But no differences in mucus hypersecretion were detected in lung tissues between native and GM seed meal fed mice (Figure 33). Allergen antibody response was measured in mice sera. We analyzed OVA-specific IgG1 and IgE and found that disease exacerbation leads to higher titer than disease initiation. We observed an overlapping OVA-specific IgG1 curve of disease mice fed with seed meals (Figure 34). These data indicate that GM AAI does not induce a stronger Th2 response compared with native AAI. GM pea fed mice had the highest OVA-specific IgE response followed by nGM pea and Tendergreen bean (Figure 34). However, we did not observe significant different level of IgE antibodies between bean and GM pea (Table 7). To ascertain whether the quantity of GM AAI influences disease exacerbation, we repeated this experiment but added additional 5 µg of purified AAI to each seed meal. The results did not change from those without extra protein inclusion (data not shown). These data show that there is no dose effect of transgenic protein but it is possible that the dose is too low to observe a difference. In summary, we did not observe that GM pea feeding worsened allergic responses to another non-cross-reactive allergen. Disease exacerbation led to higher level of OVA-specific IgE antibody production.

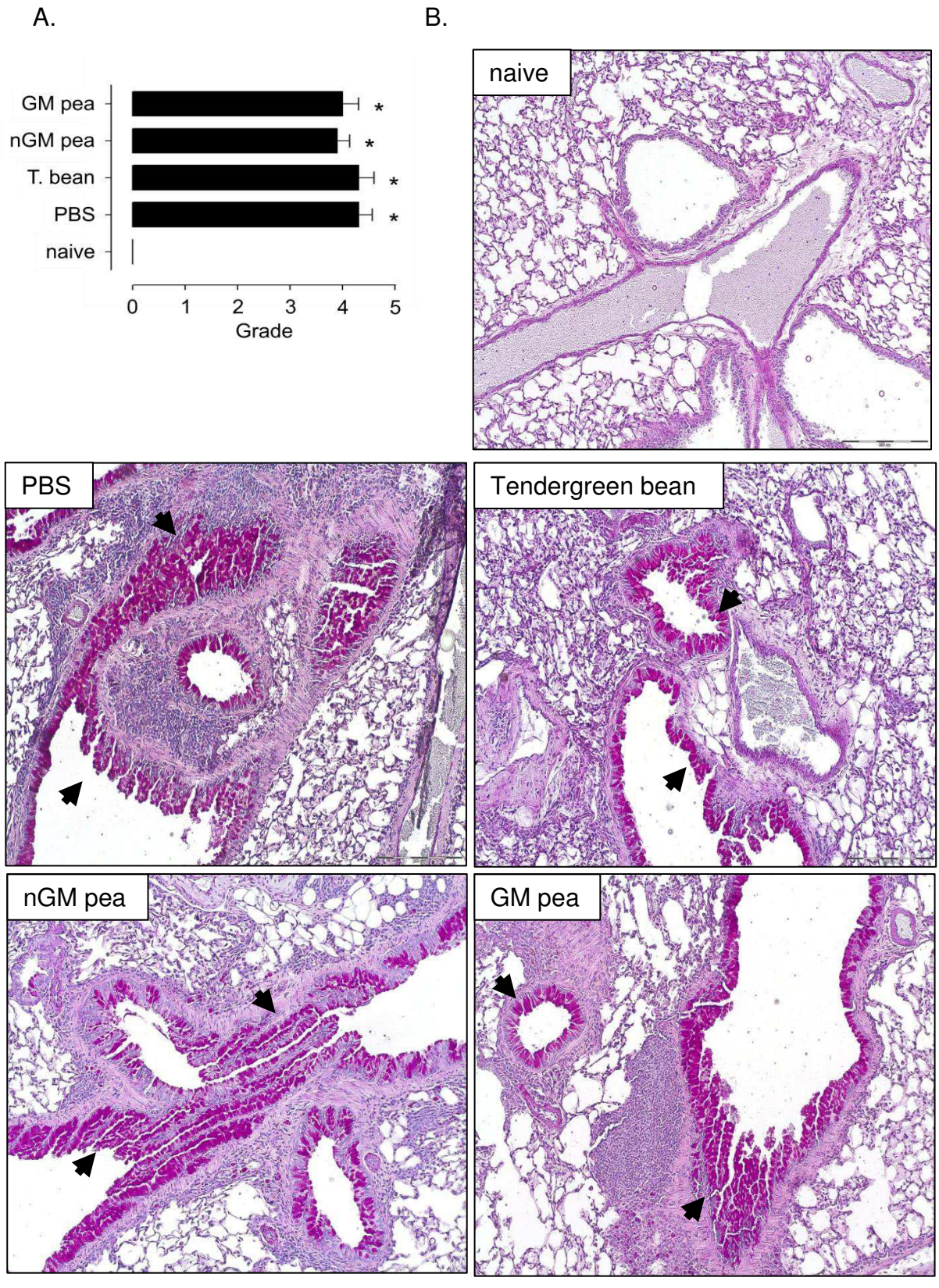


Figure 33. ***Mucus secretion in mice with OVA-induced allergic asthma fed with GM and nGM seed meal.*** *The experimental design and feeding protocol are summarized in Figure 29. Lungs were harvested 72 h after last aerosol challenge. Three µm frozen paraffin-embedded lung sections were stained with periodic-acid Schiff reagent. Goblet cell hyperplasia in the*

*airway epithelium was quantified based on a five-point system: 0 → 0% PAS positive cells; 1 → 0 - 20%; 2 → 21 - 40%; 3 → 41 - 60%; 4 → 61 – 80% and 5 → 81 - 100% PAS positive cells. (A) Grade of mucus secretion. (B) Photomicrograph of lung sections (10x magnification). Arrows indicate mucus secretion in airway. Mean ± SD, n = 10, two independent experiments, Mann Whitney test *p<0.05 vs naive.*

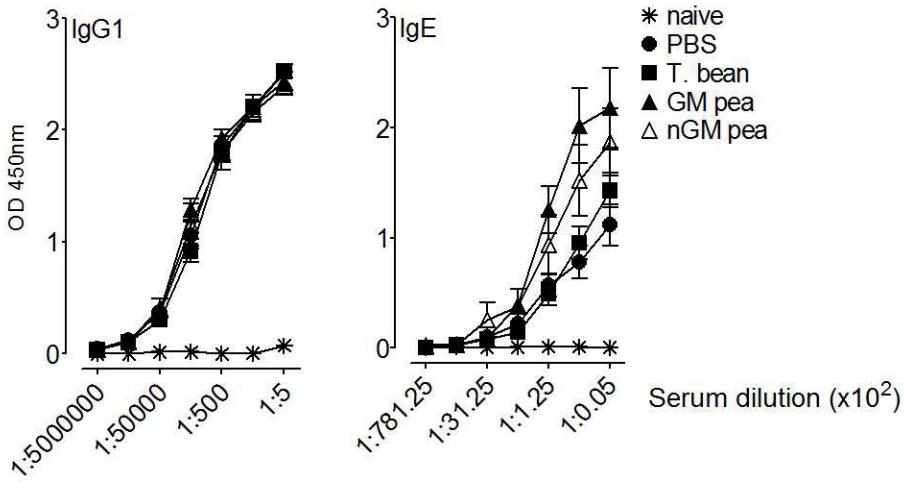


Figure 34. *OVA-specific IgG1 and IgE antibody titer in serum.* *The experimental design and feeding protocol are summarized in Figure 29. Serum samples were obtained by cardiac puncture after last aerosol challenge with OVA. ELISA was performed to measure OVA-specific IgG1 and IgE. Results are presented as O.D. ± SEM, n = 5.*

Table 7. *Mean reciprocal endpoint titer in serum*

	Exacerbation	
Group	**α-OVA IgG1**	**α-OVA IgE**
naive	250.5 ± 249.5	1.000 ± 0.0000
PBS	$23x10^6 \pm 11.02x10^6$*	380.2 ± 150.0
T. bean	$22.1x10^6 \pm 11.42x10^6$*	181.0 ± 113.7*
nGM pea	$22.1x10^6 \pm 11.42x10^6$*	969.0 ± 731.2
GM pea	$27.5x10^6 \pm 12.99x10^6$*	400.2 ± 139.1

*Serum samples were obtained by cardiac puncture after last aerosol challenge with OVA. Data are presented as mean reciprocal titer ± SEM. n = 5, Mann Whitney test *p<0.05 vs naive.*

Feeding native AAI bean in mice with OVA-induced allergic asthma had higher AAI-specific IgG1 antibody responses compared with mice fed with GM pea. Our previous results showed that mice with acute onset and relapse allergic asthma produce OVA-specific antibodies that are higher in exacerbation compared with acute onset disease. Furthermore, we measured AAI-specific IgG1 antibody titers and observed that mice with allergic asthma irrespective of disease phase, acute or relapse, both induced a high IgG1 response to AAI bean protein but higher at relapse (Figure 35). We detected an increase of AAI-specific IgG1 antibodies in GM pea-fed mice. In our previous experiment, naïve mice were fed with GM peas and challenged with AAI (Figure 22) only induced a slightly increase of transgenic AAI-specific IgG1. However, mice with OVA-induced allergic asthma had a remarkably high transgenic AAI-specific IgG1 response. It is still unclear what reason for this difference is, but an explanation might be related to OVA. Because the immune system is in an active modus against OVA; it is able to react in a faster or more intense way against another antigen in body.

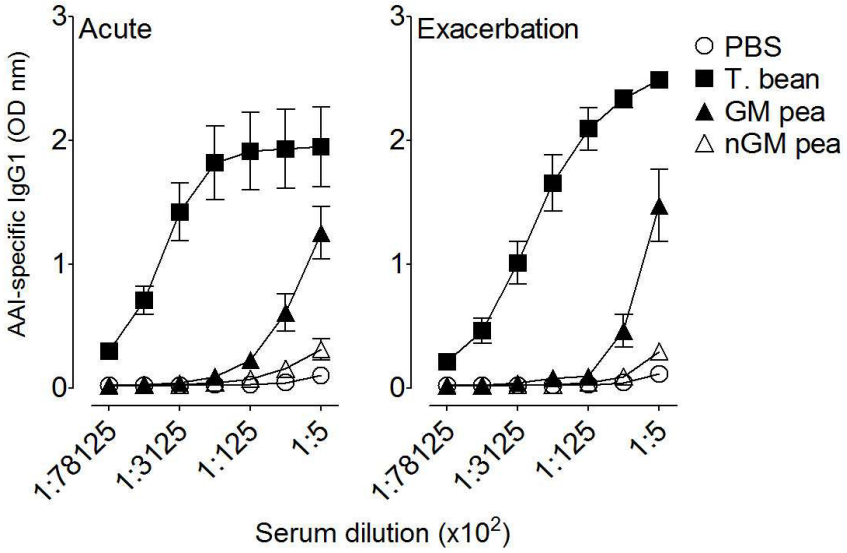


Figure 35. *AAI-specific IgG1 antibody secretion in serum of mice with allergic asthma.* *Experimental sensitization and feeding protocol are described in Figure 23 and Figure 29. Serum samples were used from mice with allergic asthma in acute onset and exacerbation. Blood samples were collected by cardiac puncture after last aerosol challenge with OVA and centrifuged to obtain sera. ELISA was performed to measure AAI-specific IgG1. Briefly, plates were coated with either transgenic AAI (10 µg/ mL) and added sera (1:500 dilution) from mice fed with GM pea or nGM pea or plates were coated with native AAI (10 µg/ mL) and added sera (1:500 dilution) from mice fed with Tendergreen bean. Results are presented as O.D. ± SEM, n = 5.*

Another point which caught our attention was that the nGM pea which does not express AAI induced a mild antibody response against AAI as well. This result indicates that there is a cross-reactive protein in the seed meal that induces an antibody response against AAI. We hypothesized that it might be probably pea lectin which has a 38-54% structure similarity to AAI.

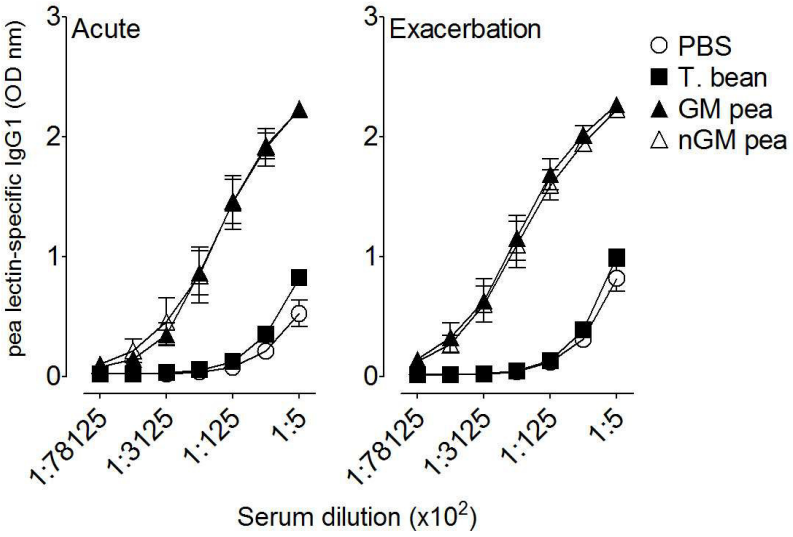


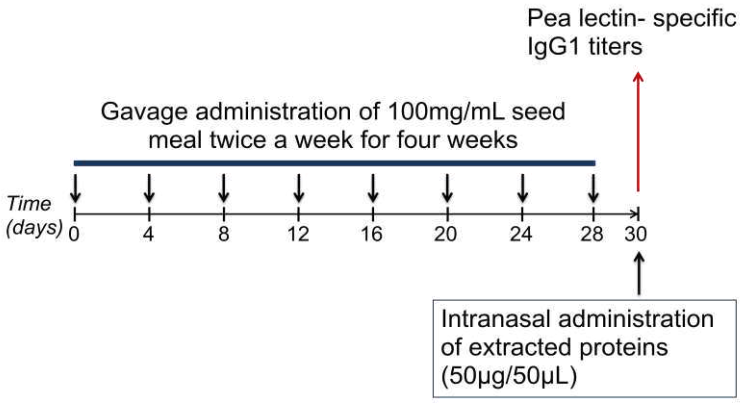
Figure 36. ***Pea lectin-specific IgG1 antibody response in serum of mice with OVA-induced allergic asthma.*** *Experimental sensitization and feeding protocol were induced as described in Figure 23 and Figure 29. Serum samples were used from mice with allergic asthma in acute onset and exacerbation phase. Blood samples were collected by cardiac puncture after last aerosol challenge with OVA and centrifuged to obtain serum. ELISA was performed to measure AAI-specific IgG1. Briefly, plates were coated with either transgenic AAI (10 µg/ mL) and added sera (1:500 dilution) from mice fed with GM pea or nGM pea or plates were coated with native AAI (10µg/ mL) and added sera (1:500 dilution) from mice fed with Tendergreen bean. Results are presented as O.D.± SEM, n = 5.*

Native and transgenic AAIs are cross-reactive to pea lectin. In our previous studies, we observed an enhanced AAI-specific IgG1 titer in mice fed with nGM pea which does not express AAI. To analyze this phenomenon, we tested the possibility of a cross-reactive response to pea lectin, because it has a similar amino acid structure as AAI[242]. To our surprise, we observed increased pea lectin-specific IgG1 in mice fed with GM pea and nGM pea and a lower response in mice fed with Tendergreen bean (Figure 36). An explanation for a positive pea lectin antibody response in Tendergreen bean-fed mice might be due to the similarity between pea lectin and lectin PHA found in all varieties of beans. This observation confirms the structure similarity to AAI and explains the detection of AAI-specific antibodies in

mice fed with nGM pea. Alternatively, pea lectin induces a B cell response which might explain the enhanced AAI-specific antibody response in the GM pea-fed group. The AAI antibody does not only recognize AAI, but also binds to pea lectin which thus increases IgG1 antibody titers.

Next, we assessed whether consumption of either raw or cooked Tendergreen bean, Pinto bean, GM pea or nGM pea induces a pea lectin-specific antibody response. To address this question, mice were fed with different varieties of seed meals and i.n. challenged with one dose of pure AAI (Figure 37A). Serum was obtained 48 h after i.n. challenge and pea lectin-specific IgG1 was measured. We observed a strong enhancement of IgG1 antibodies in mice fed with raw GM pea and nGM pea and decreased after heat-treatment (Figure 37B). These data indicated that feeding of nGM pea which does not express AAI can stimulate a response against pea lectin. Tendergreen bean- and Pinto bean-fed mice had a small increase of pea lectin-specific IgG1 antibody. These data indicate that there is amino acid structure similarity of native AAI expressed in bean and pea lectin which were unaffected with heat-treatment.

A.



B.

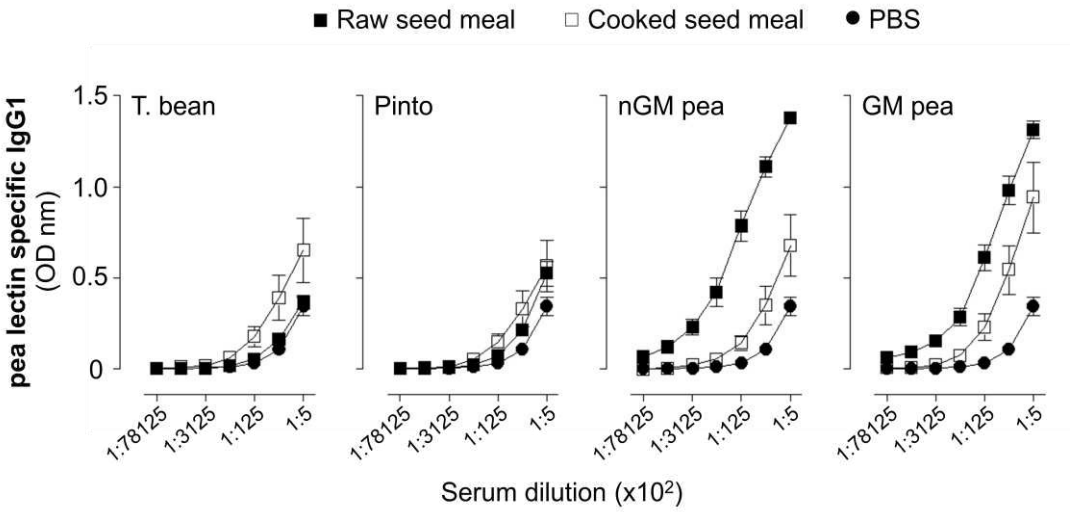


Figure 37. ***Feeding of bean and GM pea induced pea lectin-specific IgG1 antibody response in serum.*** *(A) Feeding protocol. Briefly, groups of mice (n = 6 - 7) were force-fed by gavage 100 mg/ mL seed meal twice a week for 4 weeks. Two days after last feeding mice were i.n. challenged with extracted proteins (50 µg/ 50 µL). Antibody titers were measured after challenged. As negative control mice were force fed with PBS and challenged with PBS. Blood samples were collected by cardiac puncture and centrifuged to obtain serum. (B) ELISA was performed to measure pea lectin-specific IgG1. Briefly, plates were coated with 10 µg/ mL pea lectin and sera (1:500 dilution) were added from mice fed with Tendergreen bean, Pinto bean, GM pea or nGM pea. Results are demonstrated in O.D. ± SEM values, n = 6 - 7.*

We performed another experiment to confirm the structure similarity between AAI and pea lectin. We used the same experimental approach as in our previous experiment (Figure 38A) to analyze the allergenicity of pure AAI. Briefly, mice were immunized i.n. with 6 doses of pure protein isolated from Tendergreen bean, Pinto bean or GM pea.

Forty-eight hours later blood was collected by cardiac puncture to obtain serum to measure AAI-specific and pea lectin-specific IgG1 antibodies. We observed an increase of IgG1 antibody to AAI-bean, AAI-pinto and AAI-pea and an increase of pea lectin-specific IgG1 antibody titers in mice immunized with AAI (Figure 38B), but lower response compared with AAI-specific IgG1.

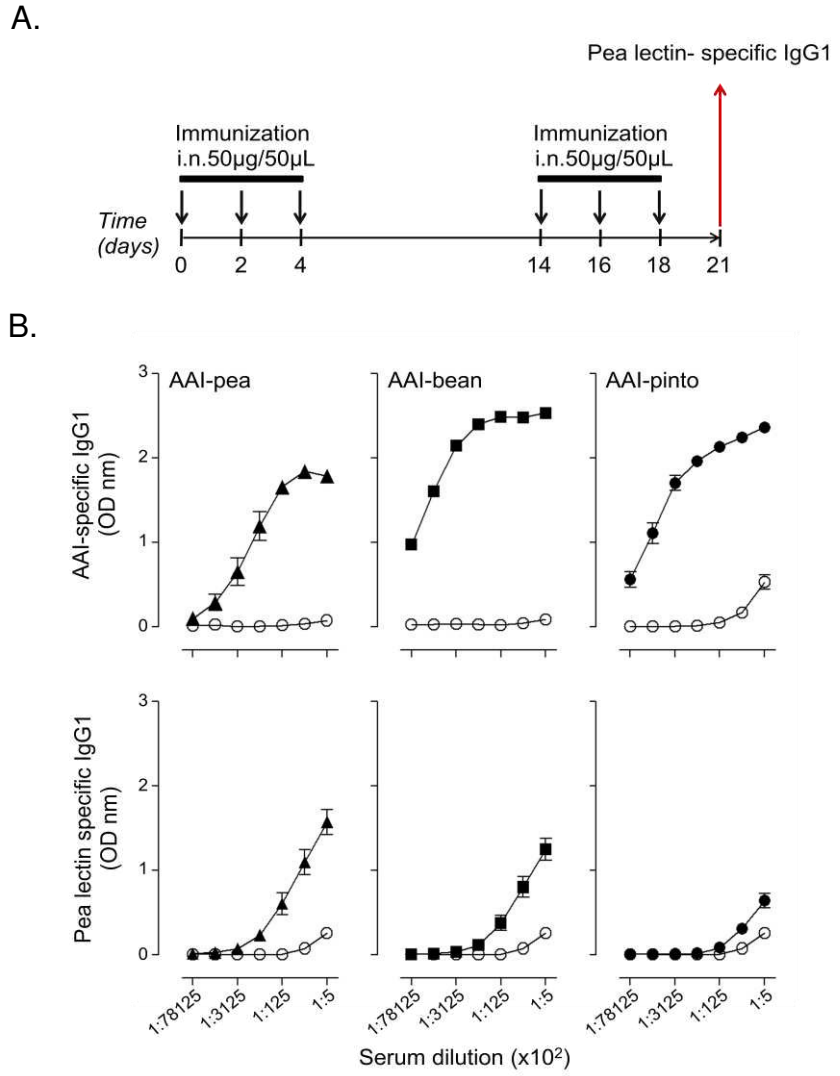


Figure 38. *I.n. administration of AAI induces pea lectin-specific IgG1 antibody response in serum.* *(A) Immunization protocol. Animals were instilled 6 times i.n. with purified protein (50 µg in 50 µL PBS) or only 50 µL PBS. Forty-eight hours after the last i.n. challenge, antibody titers were measured. Blood samples were collected by cardiac puncture and centrifuged to obtain serum. (B) ELISA was performed to measure AAI-specific and pea lectin-specific IgG1. Results are presented as O.D. ± SEM, n = 6 - 7.*

Our results demonstrate that not only is there the structure similarity between native AAI expressed in beans and pea lectin, but also confirms the similarity between transgenic AAI and pea lectin. Despite modifications in post-translational glycolysation of AAI in GM pea, pea lectin a common source in peas can be recognized from both native and transgenic AAI epitope.

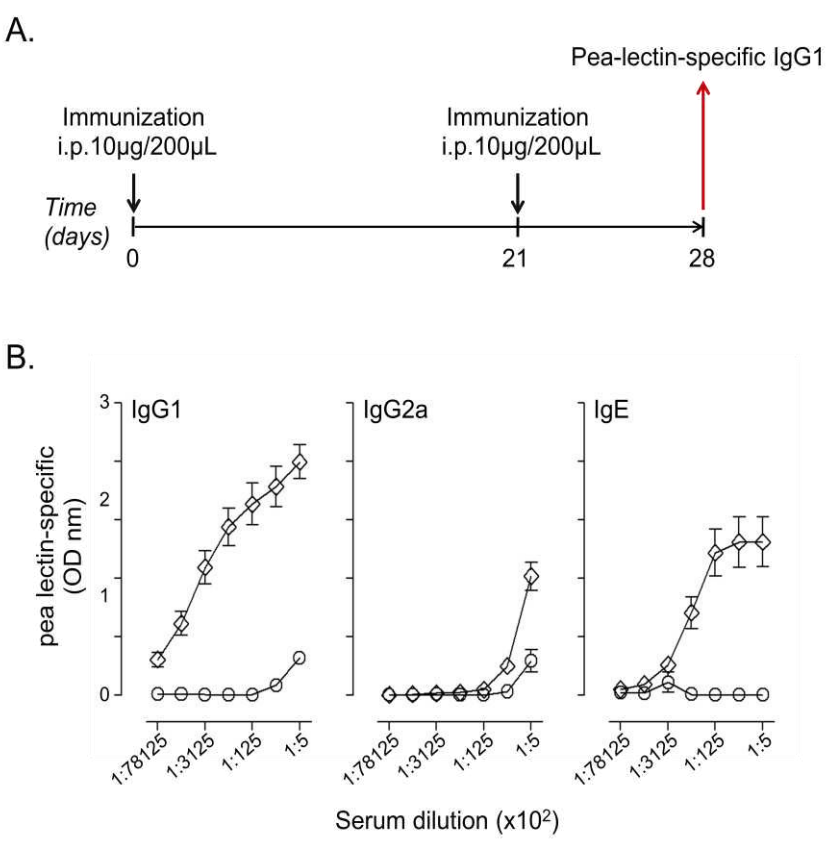


Figure 39. *I.p. immunization of pea lectin induces pea lectin-specific IgG1 antibody response in serum.* *(A) Immunization protocol. BALB/c mice were immunized by i.p. injection of purified AAI-bean, AAI-pinto, AAI-pea (10 µg in 200 µL PBS) or only with 200 µL PBS on days 0 and 21. Seven days after last immunization blood was collected by cardiac puncture and centrifuged to obtain serum. (B) ELISA was performed to measure pea lectin-specific IgG1. Briefly, plates were coated with 10 µg/ mL pea lectin and serum obtained from immunized mice was added (1:500 dilution). Results are presented as O.D. ± SEM, n = 6 - 7.*

Our results show that feeding of nGM peas without AAI induce an allergic AAI response which is probably due to a cross-reactive response to pea lectin. To confirm the immunogenicity of pea lectin, mice were immunized i.p. with 50 µg/ 200 µL pea lectin on days 0 and 21 (Figure 39A). One week later, pea lectin specific antibody titer level was measured in sera. We observed that pea lectin induced specific IgG1 and IgE antibody response (Figure 39B). In addition to IgG1 and IgE, we detected pea lectin-specific IgG2a antibodies. Based on these data, we concluded that pea lectin induces mixed Th2 and Th1 responses. Our results support *Lavelle et. al.*[46] findings that i.n. and oral administration of plant lectin stimulates the production of specific IgG in mouse serum.

3.5 Effect of native bean and GM AAI consumption in Severe Combined Immunodeficiency (SCID) mice engrafted with PBMCs from legume allergic patients

Native and GM AAI induced eosinophils in airway of Hu-SCID mice chimera.
Several studies have attempted to investigate the pathogenesis of human allergy. However, *in vivo* human studies are limited because of practical and ethical considerations, and *in vitro* studies on human lymphocytes do not always absolutely reflect the *in vivo* situation. Since testing GM peas in human is not possible, CB.17 SCID mice were used to establish a model of human allergic lung inflammation. SCID mice were reconstituted with PBMCs from legume allergic patients or healthy donors. Two days later Hu-SCID mice were force fed with Tendergreen bean, GM pea and nGM pea twice a week for 4 weeks and then i.n. challenged with one dose of pure AAI as described in our feeding protocol (Figure 40A, B). Airway and lung inflammation were analyzed 72 h after i.n. challenge. To verify the successful engraftment of PBMCs, blood smears of SCID mice were performed 24 h and 48 h after PBMC transfer to detect lymphocytes (data not shown).

Sera from the selected legume allergic patient had skin (chronic urticaria, perioral dermatitis) and respiratory (polysensitized to airborne allergens with bronchial hyperactivity) symptoms to legumes. Prick to Prick test to native bean and pea resulted severe reactions (Score were 3). Negative sera did not show clinical symptoms to legumes (data not shown). The diagnosis of legume allergy specific to beans was confirmed using IgE immunoblot analyzed in sera of allergic patients (Figure 41). These data were kindly provided by our partner in Hungary (Central Food Research Institute Department of Food Safety). The representative blot illustrated from sera of one patient with an allergic response to several food antigens including legumes such as soybeans, lentils, green peas, beans, peanuts. But patient´s sera did not show cross-reactivity to food allergens as gliadin, ovalbumin or casein. To determine specific AAI-bean IgE in legume patients another immunoblot was performed and AAI-bean IgE was detected (Figure 42A, B).

A.

	Gavage administration of 100 mg/ mL in PBS	i.n. challenged (50 µg/ 50 µL)
Groups:	Naive	
	PBS	PBS
	Tendergreen bean	AAI-bean
	Pinto bean	AAI-pinto
	GM pea	AAI-pea
	nGM pea	AAI-pea

B.

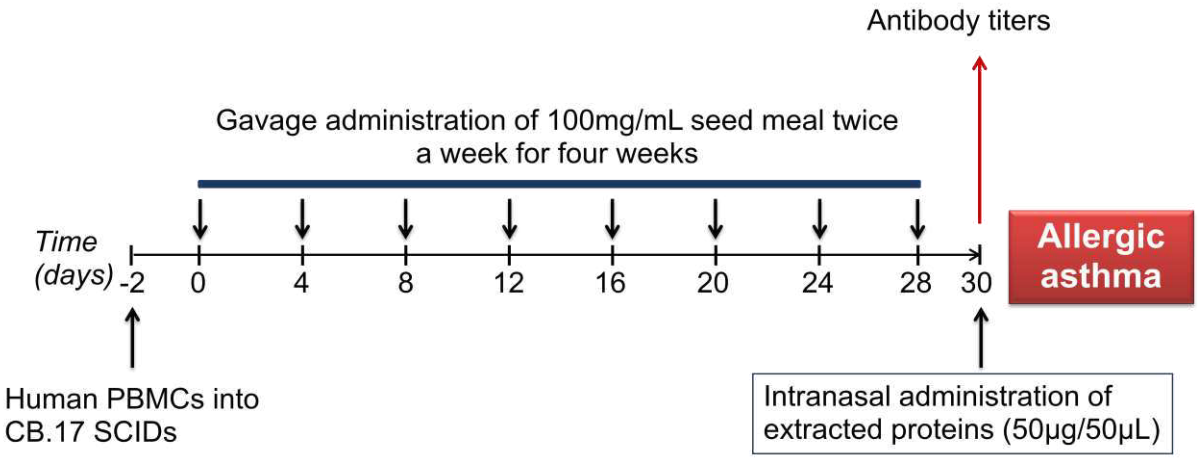
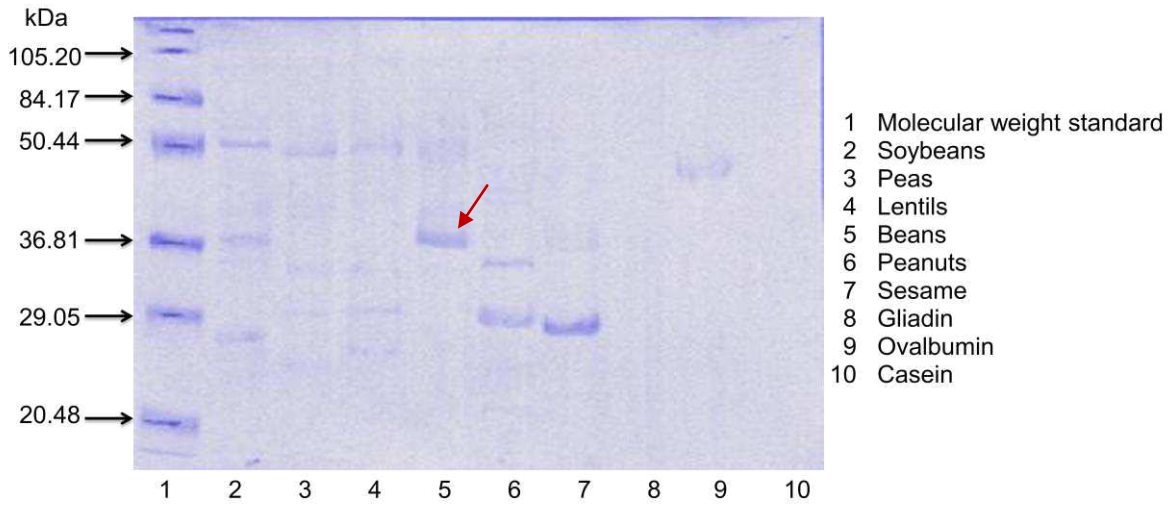
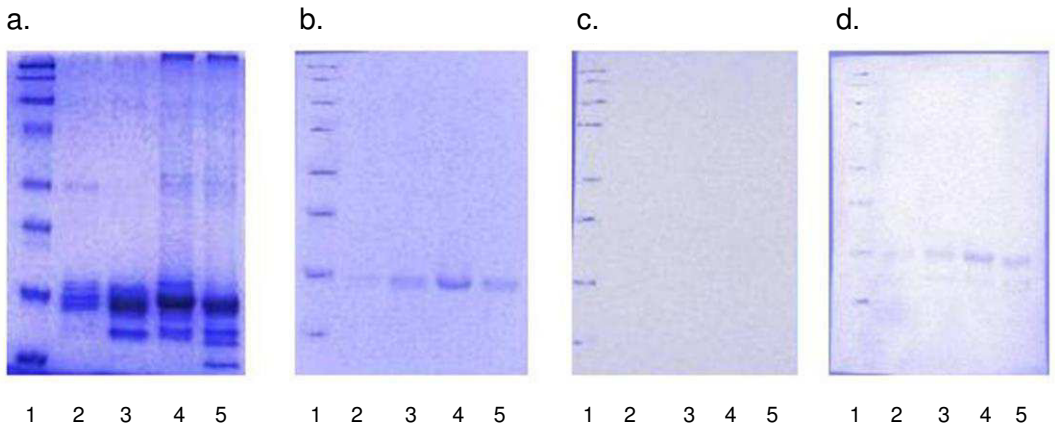


Figure 40. ***PBMCs of legume allergic patient and healthy donor were engrafted into CB.17 SCID mice.*** *Groups of mice (n = 5) were engrafted with 2.5 x 10^6 PBMCs either from patient or from healthy donor two days before feeding by gavage administration of 100 mg/ mL seed meal twice a week for 4 weeks. Two days after last feeding mice were i.n. challenged with extracted protein (50 µg/ 50 µL). Features of allergic asthma were analyzed 72 h after challenge. As negative control naïve SCID mice were untreated until assessment. (A) List of groups. (B) Experimental protocol.*



Dr. Éva Gelencsér, Central Food Research Institute – Budapest, Hungary

Figure 41. ***Immunoblot of anti-human IgE from serum of legume allergic patient.*** *Lane 1 represents the molecular standard weight. Lanes 2 – 10 indicate bands recognized by serum from patient positive and negative for specific IgE antibodies. Red arrow indicates the recognition of specific IgE against bean.*



1. MW standard, kDA: 199, 166, 80, 60, 37, 29, 20, 7 kDa
2. AAI-bean
3. AAI-pea
4. AAI-cowpea
5. AAI-chickpea

Dr. Éva Gelencsér, Central Food Research Institute – Budapest, Hungary

Figure 42. ***Immunoblot of anti-AAI IgE in serum of legume allergic patient.*** *Lane 1 represents the molecular standard weight. The proteins of the seed meal samples were separated by 15% SDS-PAGE (a). Separated proteins were transferred to a PVDF membrane from the gel. IgE reactive bands were identified by immunoblot using patient's sera (b). Separated proteins were transferred to a PVDF membrane from the gel and the*

carbohydrate epitopes were eliminated from the AAI meal by Na-metaperiodate at acid pH. Deglycosylation was proven by Schiff-staining on the membrane (c) and following the membrane was incubated with the human legume allergic patient's sera (d). The bound IgE antibodies were detected with horseradish peroxidase conjugated anti-Human IgE secondary antibody. The binding patterns were detected using substrate solution (4-chloronaphtol/H_2O_2).

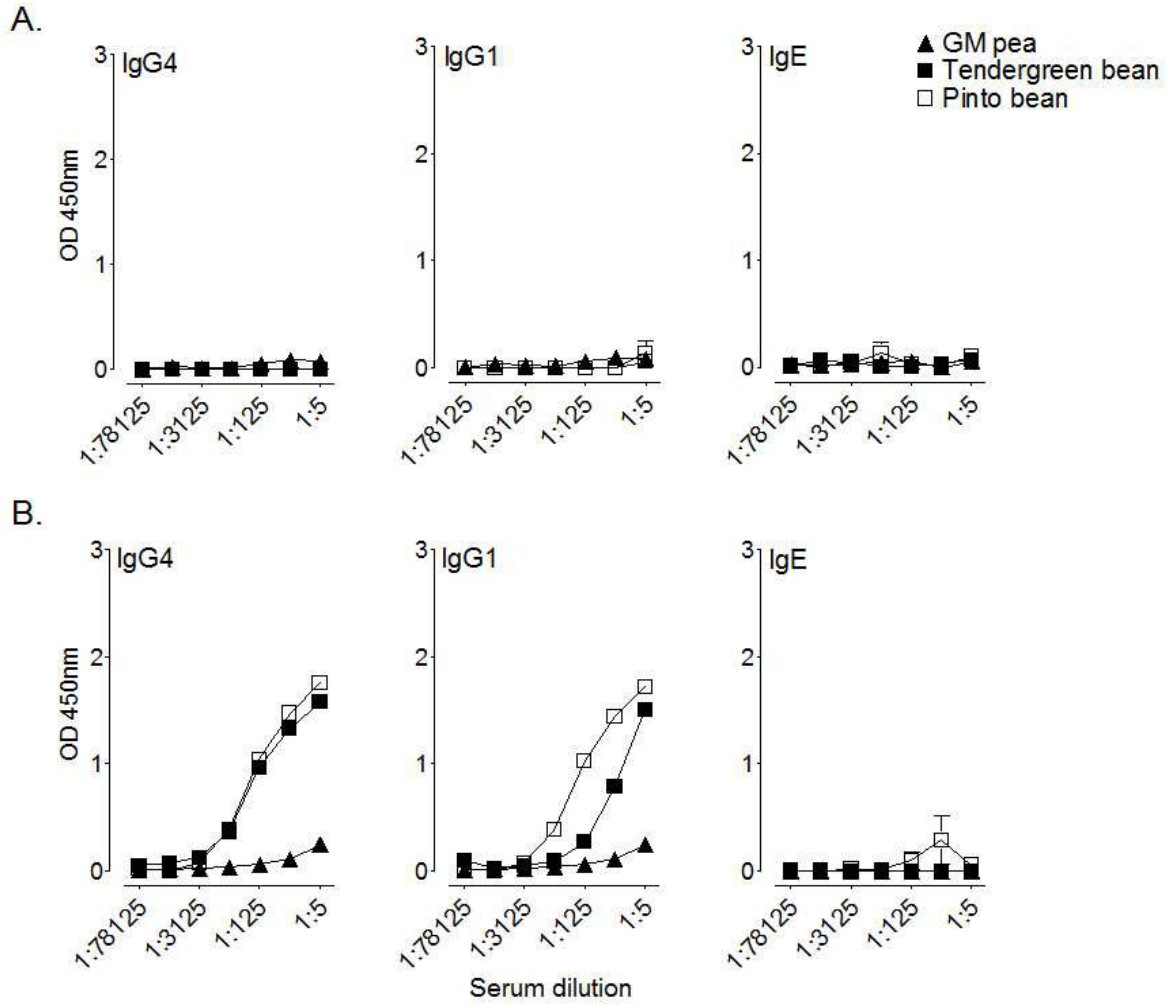


Figure 42. ***Human AAI-specific IgG4, IgE and IgG1 antibody titers in serum of legume allergic patient and healthy donor.*** *(A) Immunoglobulin level in serum of legume allergic patient (B) and healthy donor.*

We did not detect AAI-specific IgE or AAI-specific IgG4 in serum of legume patient by ELISA (Figure 43A). The reason for this observation remains unknown but suggests that the immunoblot is more sensitive than ELISA. But interestingly, AAI-specific IgG4 and IgG1 antibodies were detected in the serum of the healthy donor. Beans are a common source in human diet. Since legume allergic patient are allergic to beans

they would prevent beans on their daily meals. This patient was not challenged before measuring AAI-specific antibodies which could explain the low titer of antibodies. It was only detectable by Western blot a technique, which is a more sensitive to detect low level of AAI-specific IgE. In contrary, the healthy donor had consumed beans more regularly which induced antibodies production against AAI-bean.

Seventy-two hours later after i.n. challenged, Hu-SCID mice were assessed to measure airway and lung inflammation. In airway, we found lymphocytes, neutrophils, macrophages and eosinophils in both allergic (indicated by black bars) and healthy chimera (white bars), which is evidence for the successful engraftment of human PBMCs to build up a human system in this model. Interestingly, GM peas but also native beans had increased inflammatory cells in both allergic and healthy chimera. This observation was not observed in mice fed with nGM pea, Pinto or PBS. We found almost equal numbers of lymphocytes and macrophages, but higher numbers of neutrophils in Tendergreen bean-, Pinto bean- and GM pea-fed Hu-SCID mice (Figure 44).

Cellular infiltration and secretion of mucus were examined in Hu-SCID chimera. We detected low lung inflammation in SCID allergic chimera. GM pea-, Tendergreen bean- and Pinto bean-fed SCID mice have the same grade of cell infiltrates. In contrast, we did not detect inflammation in mice fed with nGM pea, and thus correlated with our observation in airways. In healthy Hu-SCID mice the inflammation was reduced compared to allergic SCID mice but statistically not significant (Figure 45). Mucus secretion was detected to a similar degree. In some groups, we observed higher mucus secretion in healthy SCID compare to allergic SCID mice, but they were not significantly different (Figure 45). These results indicate that SCID mice engrafted with human PBMCs subsequently fed GM peas developed allergic lung inflammation. However, we could not distinguish between healthy and allergic donors. We did not detect AAI-specific IgG4 and IgE in allergic and healthy Hu-SCID mice chimera (data not shown).

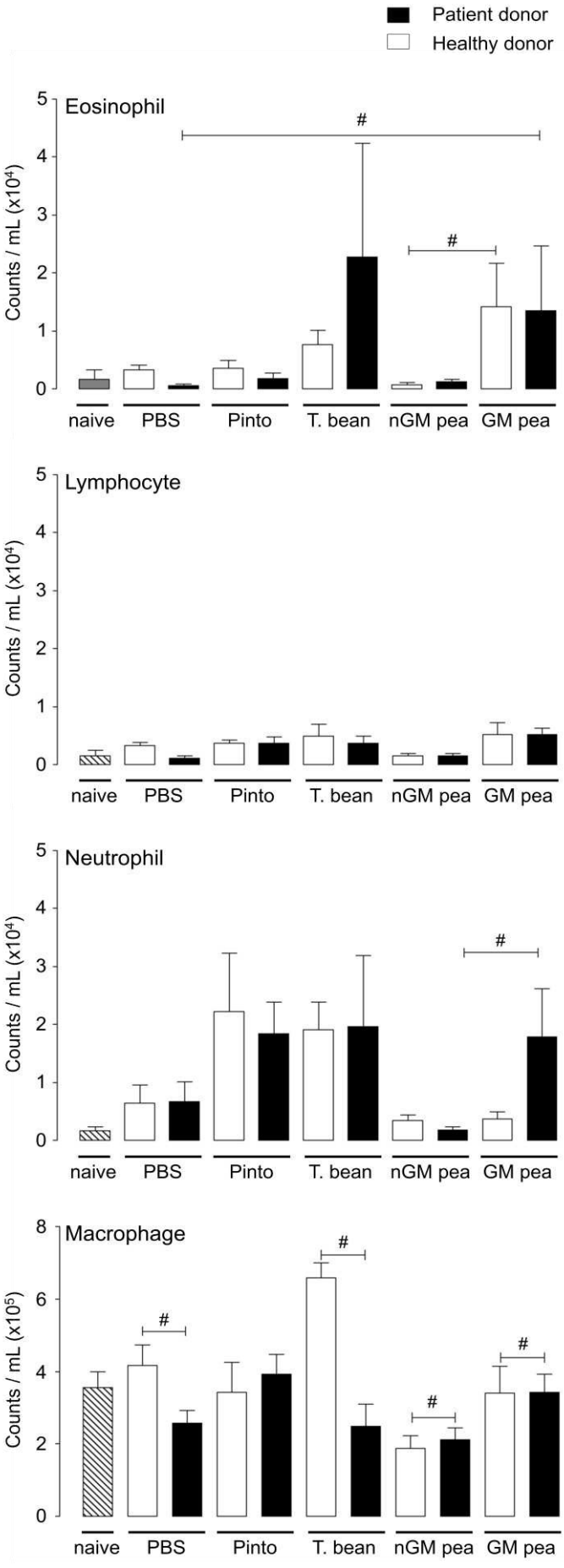


Figure 44. ***Characterization of inflammatory cells in BAL fluid retrieved from SCID mice reconstituted with PBMCs from allergic patient (black bars) and healthy donor (white bar), and fed with seed meals.*** *Seventy-two hours after challenged bronchoalveolar lavage fluid cells were collected. Cytospun cells were stained with Kwik-Diff and approximately 300 cells were counted from each mouse. Mean ± SEM, n = 5; Mann Whitney test $^{\#}p<0.05$*

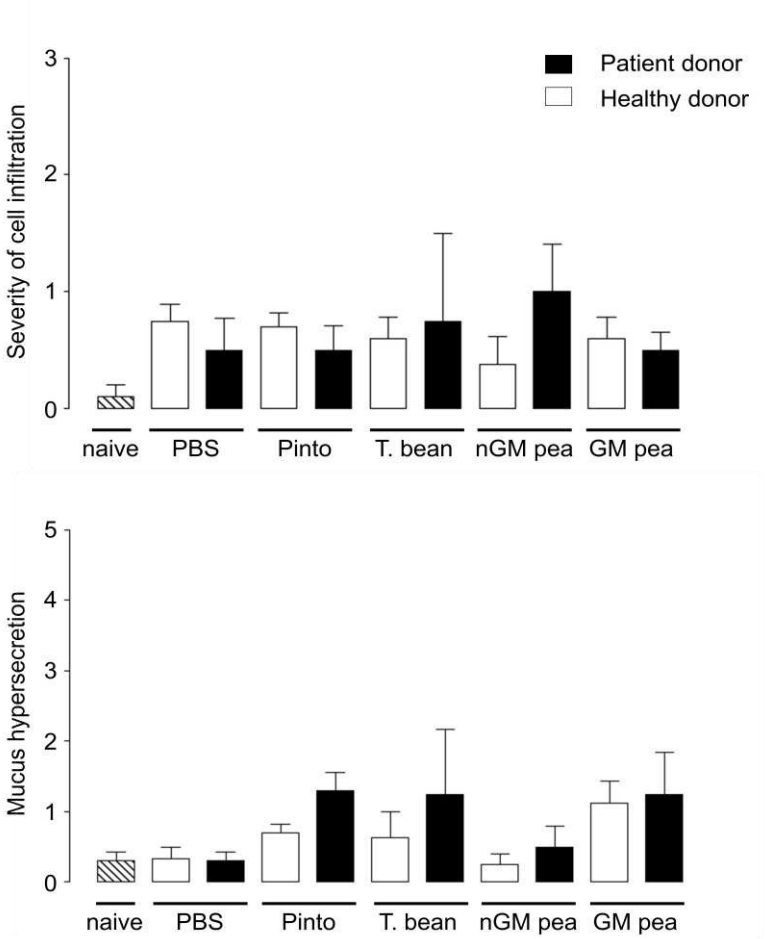


Figure 45. ***Accumulation of inflammatory cells und mucus hypersecretion in lung.*** *SCID mice were reconstituted with PBMCs derived from allergic patient (black bars) or healthy donor (white bars) and fed with seed meals. 3 µm frozen paraffin-embedded lung sections were stained either with H&E or periodic-acid Schiff reagent. Histological scores were performed by given grade numbers dependent on the severity of cell infiltrates, accumulation of eosinophils or mucus secretion by goblet cells. Histological scores were given for cell infiltrates from 0 - 3 and for mucus secretion from 0 - 5. Mean ± SD, n = 5*

Taken together, we observed that PBMCs isolated from healthy individuals and legume allergic patients transferred disease to Hu-SCID animals manifested by allergic asthma upon consumption of GM peas and i.n. challenge with AAI. Additionally, Hu-SCID mice fed with native beans and peas generated responses to AAI indicating that peas are not more allergenic compared with native beans. It was not possible to distinguish allergic and non-allergic individuals using the Hu-SCID model.

4. Discussion

Within the last decades the development of transgenic plants generated heated debates about the possibility of unidentified health effects. One of the main health issues in question is potential allergenicity of genetically modified plants and their products. It remains unknown whether the product of a novel gene expressed in a GM plant will have the potential to stimulate *de novo* sensitization or cross-react with a known allergen to elicit hypersensitivity reactions in previously-sensitized individuals[3, 40]. In kidney beans, AAI, an anti-nutrient, is denatured during the cooking process, and therefore is safe for human consumption. However, when AAI is inserted into a field pea, this same gene is differentially glycosylated and thereby, has the potential to induce allergic responses. Glycosylation is the process in which sugar chains are attached to proteins rendering them potential allergenic. Previous studies done by *Prescott et. al.* discovered that, in spite of sharing the same genetic background, glycosylation patterns were slightly different between AAI derived from kidney bean compared with AAI from GM pea[88, 243]. They tested GM pea in mice and observed that gavage feeding with GM pea twice a week for 4 weeks of young female BALB/c mice without adjuvant lead to robust IgE antibody production compared to Pinto bean-fed mice[88]. The aim of our studies was to repeat and adapt the model used in *Prescott et. al.* The present study was undertaken to evaluate the immunomodulatory effect of GM peas. The experimental data showed that the consumption of nGM and GM peas lead to allergic responses to AAI. We also observed that systematic and intranasal administration of transgenic AAI induced Th2 responses, however the same effect was also observed in mice immunized with AAI derived from Tendergreen bean or Pinto bean. Our results refute those in the *Prescott et. al.* study.

4.1 Intraperitoneal and intranasal administration induce AAI-specific IgG1 but not IgE synthesis

The BALB/c mouse strain is known to favor the development of Th2 type immune response and the production of IgE antibodies. Therefore, BALB/c mice were used to evaluate the immunogenic and allergenic potential of AAI. Additionally, these mice

were used in the previous studies by *Prescott et. al.* An important aim of our study was to compare immunogenicity between AAI proteins extracted from GM peas and beans. Therefore, we examined AAI-specific IgG and IgE antibody responses after i.p. administration of the allergen. Our results showed that immunization with AAI induced AAI-specific IgG1 antibodies. However, the response was higher in mice immunized with bean AAI compared to GM pea AAI which suggests that AAI derived from bean was as immunogenic as transgenic AAI. Anti-specific AAI IgG2a response was also detected but it was lower than IgG1. This observation was expected since we used protocols which are intended to skew responses towards allergic Th2 isotypes. Interestingly, both bean and GM pea AAI immunized mice did not developed IgE antibodies against AAI.

The reason that AAI-specific IgG1 was detectable but not IgE may be that AAI are immunogenic but not allergenic. *Knippel et. al.* showed that proteins, such as Ara h1 (purified from peanut), Pen a1 (purified from shrimp) or Ber e1 (2 S albumin from Brazil nut) were immunogenic in mice, inducing IgG antibody response at all tested doses after systemic (i.p.) administration[244]. However, only the potentially allergenic proteins provoked both IgG substantial levels of specific IgE. These data suggest that systematic administration of protein is a powerful approach to identify and characterize novel proteins. However, the question remains whether systemic exposure of proteins is a relevant route of exposure. Another study demonstrated that the induction of specific IgE antibody is invariably accompanied by the induction of specific IgG antibodies. If a negative IgE antibody response is observed in the presence of IgG response, then the lack of IgE antibody can be considered as good evidence for a non-allergenic potential[166].

A second explanation for the failure to generate an IgE antibody response might be due to the similarity of epitopes. IgG antibody reacts to the same or similar epitopes inhibiting the binding of IgE antibody. *Lehrer et. al.* indicated that IgG antibodies inhibit IgE antibody binding to allergen in both ELISA as well as immunoblot analysis[210]. They observed an augmentation of IgE antibody binding by depletion of IgG by protein G concomitant with an increase of the sensitivity in both ELISA and immunoblot analysis.

A third explanation for the absence of AAI-specific IgE may be that the application rate of AAI was not sufficient to stimulate IgE production. In our study, we administered 10 µg of AAI i.p. twice on days 0 and 21 without adjuvant, whereas another study animals were i.p. sensitized with 100 µg red kidney bean proteins once a week for a total period of 7 weeks. They observed a significant increase in IgE, IgG1, histamine, mast cell protease-1 and Th2 cytokine levels in comparison to control mice[241]. In addition, *Kumar et. al.* demonstrated that red kidney beans may induce allergic response in mice. This study group used a 10-fold-higher dose of protein and a longer experimental period compared to our experimental approach.

To compare the immunogenicity between AAI proteins extracted from GM peas and beans, we administered i.n. 50 µg of AAI suspended in 50 µL PBS, and observed that i.n. instillation induced an immune response. Bean AAI-specific IgG1 was detectable after 4 times instillations, whereas GM pea AAI-specific IgG1 response was induced after 6 instillations. These results indicate that intranasal exposure of protein can generate a specific antibody response. However, the antibody response was higher with i.n. instillation compare to i.p. administration. This is probably related to the higher total i.n. dose of 300 µg of AAI compared with a total AAI i.p. dose of 20 µg. Additional, AAI-specific IgE was detectable in mice treated intranasally with AAI extracted from Pinto bean.

Taken together, immunization by i.n. and i.p. routes demonstrates that, there is a difference in antibody responses to AAI from beans versus transgenic peas. Interestingly, the transgenic AAI were not more immunogenic or allergenic than bean AAI. This observation suggests that the variation in immune responses may be linked to differential post-translational modifications such as glycosylation as reported in *Prescott et. al.* study. However, immunogenicity and allergenicity of AAIs from beans and transgenic peas could not be correlated with the minor difference in glycosylation.

4.2 Consumption of GM AAI induces allergic response

In the experimental studies, oral administration is considered to mimic more accurately the relevant conditions of human allergen exposure. Therefore, in our second part of testing GM peas, we used force-feeding /intragastric route. Our results

showed that mice developed immune responses to the transgenic AAI when they ate the transgenic seed meals, a response similar to mice fed with Tendergreen bean, Pinto bean and nGM pea. Moreover, we observed that Tendergreen bean and Pinto bean fed groups had a higher eosinophil accumulation in the airways and lungs, and produced more AAI-specific IgG1 antibodies compared to mice fed with transgenic peas. These results are in contradiction to *Prescott et. al.* study, where they showed that feeding mice with GM pea but not Pinto bean or nGM pea seed meal induced an AAI-specific B-cell antibody response. In addition, mice also exhibited enhanced delayed-type hypersensitivity responses in skin[88]. In our study, we used the same experimental approach as *Prescott et. al.* with one exception: compared to their setup where only Pinto bean and nGM pea were taken as control groups, we included Tendergreen bean in our experiments since the original AAI was derived from Tendergreen bean. *Prescott et. al.* avoid to use Tendergreen bean, because it contains a high level of PHA, an anti-nutritional factor that induces dietary toxicity in rodents and birds, and has been demonstrated to stimulate T cells[245, 246]. It has been published that oral administration of PHA mainly stimulates the production of lectin-specific IgG1 antibodies in the blood and hence, a systemic Th2 cell response[76]. Therefore, they used a bean variety that contains very low PHA like Pinto bean as control. However, it is important to compare the immunogenicity and allergenicity of transgenic AAI in GM peas to the original AAI derived from Tendergreen bean. As a result, we observed that Tendergreen bean fed mice had approximately 2% higher eosinophils than Pinto bean, and there was no difference in antibody response between these both beans types. We speculate that due to PHA content in beans, we observed in general an increase of Th2 cell response. However, we did not detect significant differences between these two bean varieties.

Consumption of transgenic AAI induces allergic response but non-transgenic AAI can also induce an elevated Th2 immune response, suggesting that GM AAI is not more allergenic than bean AAI. These results are contrary to *Prescott et. al.* observation. One explanation for these dissimilar findings could lay in the differences of daily diet and the origin of the animals. Our experimental mice originated from Charles River Germany were kept in our mouse house, whereas the Australian study group used mice from Jackson Laboratory, which were subsequently bred at The John Curtin

School of Medical Research by sibling mating for at least 70 generations in an SPF Unit. For the daily diet, our mice received feeds from SSNIFF, whereas the experimental Australian mice were fed with Gordon´s Specialty Stock Feeds P/L in New South Wales. The main differences between these both feeds consists in the sources of dietary protein (animals vs. plants), fatty acid type, level of soluble fiber and level of vitamin supplementation (Table 8-10)[247]. Taken together, we observed that feeding BALB/c mice with transgenic and non-transgenic legumes leads to similar allergic responses. These results indicate that transgenic AAI is unlikely to be more allergenic than AAI derived from Pinto and Tendergreen bean.

Table 8. Comparison of ingredients between Australian and Austrian diets

Australian Diet	Austrian Diet
Wheat	Wheat and wheat flour
Sorghum	
Soybean meal	Soybean meal (full fat)
Pollard	
Bran (wheat)	Bran (wheat)
Meat and bone meal	
Blood meal	
Lucerne meal	
Vegetable oil	Soybean oil
Sunflower meal	Sunflower meal
Salt	
Vitamin and mineral premix	Vitamin and mineral
Lysine	
Choline chloride	
	Barley
	Cornan corn products
	Sugar beet pulp

4.3 Heat treatment of legume seed meals decreases allergenicity

In general, beans and peas are not consumed by humans in raw form. To mimic real life conditions, we included in our experimental setup feeding with cooked beans and peas. Our findings demonstrated that when given heated nGM pea and GM pea seed meals mice developed decreased inflammatory cell accumulation in airway and lung with reduced specific antibody responses (Figure 20-22).

Table 9. Comparison of crude materials between Australians and Austrian diets

	Australian Diet	Austrian Diet
Min. Crude Protein	23%	22.1%
Min. Crude Fat	6%	4.5%
Max. Crude Fibre	5%	3.9%
M.E. (Min.)	13 MJ/ kg	(estimated: 14.5 MJ/ kg)
Dry substance	(suggested: 90%)	90.4%
Crude Ash	(suggested: 7%)	6.7%
N-free extract materials	(estimated: 49%)	53.3%
Starch		35.8%
Sugar		5.2%

Table 10. Nutrient analysis Austalian and Austrian diets

Amino Acids	g/ kg	% (g/ kg)
Lysine	9.8	1.41 (14.1)
Methionine & Cystine	5.9	0.83 (8.3)
Threonine	8.4	0.76 (7.6)
Histidine	5.0	0.51 (5.1)
Leucine	15.2	1.5 (15)
Arginine	12.1	1.31 (13.1)
Valine	10.2	1.00 (10)
Isoleucine	8.0	0.89 (8.9)
Phenylaline & Tyrosine	16.4	1.64 (16.4)
Tryptophan	3.7	0.28 (2.8)
Methionine	-	0.46 (4.6)
Leucine	-	1.5 (15)
Phenyalanine	-	0.97 (9.7)
Glycin	-	0.91 (9.1)
Minerals	-	-
Calcium	10.1g/ kg	1% (10g/ kg)
Phosphorus	7.7g/kg	0.7% (7g/ kg)
Potassium	5.4g/kg	1% (10g/kg)
Magnesium	1.8g/kg	0.24% (2.4g/kg)
Iron	97.0mg/kg	174 mg/kg
Copper	10.6mg/kg	16 mg/kg
Manganese	87.4mg/kg	73 mg/kg
Zinc	48.1mg/kg	98 mg/kg
Iodine	1.15mg/kg	2.2 mg/kg

Selenium	0.1mg/kg	0.3 mg/kg
Sodium	0.3%	0.25 mg/kg
Cobalt	-	2.2 mg/kg
Fats	**% of lipid**	**% of lipid**
Saturated Fat	21.3%	16.4
Mono-unsaturated	42.9%	22.4
Poly-unsaturated	30.7%	61.4
Fatty acids		**% of diet**
C 14:0	-	0.01
C 16:0	-	0.56
C 16:1	-	0.02
C 18:0	-	0.14
C 18:1	-	0.96
C 18:2	-	2.42
C 18:3	-	0.31
C 20:0	-	0.02
C 20:1	-	0.02
C 20:5	-	-
C 22:6	-	-
Vitamins	**g/kg**	**per kg**
Vitamin A	170 µg/100g	25000IE (750µg/100g)
Vitamin B1	4mg/kg	87 mg
Vitamin B2	5mg/kg	32 mg
Vitamin B6	6mg/kg	32 mg
Vitamin B12	0.005mg/kg	150 µg (0.150 mg/kg)
Vitamin C	150mg/kg	-
Vitamin D	200i.u/kg	1.000 IE
Vitamin E	50mg/kg	138 mg
Vitamin K	5mg/kg	20 mg
Niacin	10mg/kg	165 mg
Pantothenate	12mg/kg	62 mg
Folic Acid	10mg/kg	10 mg
Biotin	0.06mg/kg	730 µg (0.73 mg/kg)
Cholin-Cl	-	3.300 mg
Inositol	-	100 mg

It is known that heat treatment of proteins can lead to alterations in their structure with increase or decrease allergenicity by formation of new allergens or non-allergens[248]. Typically, loss of tertiary structure is followed by reversible unfolding, while loss of secondary structure (70 - 80°C) leads to the formation of new intra/ intermolecular interactions as well as rearrangements of disulfide bonds (80 - 90°C) and formation of aggregates (90 - 100°C). Heating in the presence of sugars found in foods leads also to modification through the Maillard reaction (non-enzymatic browning)[248]. Free primary amino groups are attacked by carbonyl compounds during the Maillard reaction, leading to the formation of stable advanced glycation end products. Most investigations dealing with the influence of heat treatment on legume allergenicity have been focused on peanut and soybean, such as roasting of peanuts which reduced IgE-binding in contrast to frying or boiling [249, 250]. Whereas another study demonstrated that heating at 100°C up to 60 min of soybean protein extracts did not significantly decrease IgE. However, boiling for 120 min and microwave heating of soybean seemed to decrease allergenicity[251]. Our results indicate that heated pea seed meals decreased allergic immune response. However, we observed that mice fed with beans developed higher airway eosinophilia when fed with heat-treated compared to raw seed meals. This result was unexpected because AAI is moderately heat stable and is inactivated by proper cooking. This suggests that except AAI, there may be other components in the seeds that affect the immune response and influence during heat-treatment. It has been published that the AAI activity in kidney beans alters the level of inhibitory activity dependent on temperature[252]. Heating at 80°C for 30 min did not significantly alter the level of inhibitory activity in beans. Cooking at 90°C for 30 min greatly reduced, but did not completely abolish the inhibitory activity of the beans. However, aqueous heat treatment of fully beans at 100°C for 5-10 min abolished AAI activity. In our experiment, legume seed meals were heated at 100°C for 25 min which suggests full inhibition of AAI activity. In summary, heat treatment of pea seed meals decreased the allergic immune response compared to raw seed meals. By contrast, cooked beans fed to mice increased inflammatory cell infiltrates in lung probably due to other components in the seeds.

4.4 Transgenic AAI does not worsen allergic responses to egg allergen

Prescott et. al. demonstrated that transgenic AAI promotes immune responses to other oral antigens. To verify this observation, we tested the effect of GM pea consumption on the initiation and exacerbation of allergic diseases to chicken egg white protein OVA. In our study, we observed that GM pea diet did not worsen pre-existing allergic disease in mice. We did not observe any significant differences in numbers of inflammatory cell infiltrates in airway and lungs between OVA-treated GM-fed groups versus OVA-treated isogenic-fed groups or OVA alone treated mice. Moreover, there was no difference in OVA-specific IgG or IgE antibody response between the groups. In contrast, *Prescott et. al.* have shown that transgenic AAI enhanced OVA-specific immunogenicity[88]. They reported that consumption of transgenic AAI together with OVA promoted a stronger Th2 inflammation compared to mice treated either only with OVA or OVA mixed with bean AAI. We do not have the exact reason for these dissimilar findings. One explanation is maybe due to the different experimental approach: naïve or relapsing mice were fed 4 consecutive weeks/ twice a week before aerosol challenge with OVA to induce allergic disease. By contrast, *Prescott et. al.* administered intragastrically purified AAI (5 µg) with OVA (1 mg/ mL) followed by intratracheal challenged with OVA (25 µL) three times a week for 2 weeks. We suspect that different dosage of transgenic AAI could explain the results disparity in these two studies (we did not use pure AAI protein but legume seed meals with approximately 2-4% AAI content of total seed protein). To verify, we performed two additional experiments in which we either added additional pure AAI to the seed meals, or we increased the dose of seed meals. Nevertheless, we did not observe any OVA-specific immunogenicity enhancement. Taken together, our results showed that consumption of transgenic and non-transgenic AAI did not affect OVA-specific allergic disease.

4.5 Cross-reactivity between AAI and pea lectin

Surprisingly, we detected AAI-specific antibody response upon feeding nGM peas which does not express AAI. One explanation could be that maybe another protein expressed in the nGM pea might cross-react to the transgenic protein. From the literature, we know that pea lectin has a homology structure to AAI. Therefore, we

measured cross-reactivity between AAI and pea lectin. Our results showed that GM pea AAI was cross-reactive with pea lectin, but Tendergreen bean- and Pinto bean-fed mice also developed pea lectin-specific antibody response with less intensity compared to GM pea AAI. These results demonstrate that not only there is a structure similarity between transgenic AAI expressed in GM peas and pea lectin, but also confirms the similarity between bean AAI and pea lectin. This suggests that despite minor modifications in post-translational glycolysation of AAI in GM pea, pea lectin a common source in peas can be recognized at both native and transgenic AAI epitope. Because pea lectin may play a role in the immunogenicity, we administered pea lectin either by intraperitoneal or intranasal routes. Interestingly, we observed that pea lectin was able to induce an immune response with elevated specific antibody production against pea lectin which cross-reacts with pea, bean and pinto AAI. These results support *Lavelle et. al.*[46] findings that i.n. and oral administration of plant lectin stimulates the production of specific IgG in mouse serum. Taken together, our results demonstrate that feeding with transgenic and non-transgenic peas induces anti-pea lectin responses, which are cross-reactive with AAI.

4.6 Hu-SCID mice developed allergic responses upon consumption of transgenic AAI

Data derived from our animal experiments may not be directly extrapolated to humans. Therefore, to circumvent this difficulty, we reconstituted SCID mice with human PBMCs to build a human-mouse chimera with a functional human antigen reactive immune system. This allowed us to perform an *in vivo* study of human immune responses. SCID mice are of particular interest in our study because of the absence of mature and functional T and B lymphocytes. The SCID mutation impairs the recombination of antigen receptor genes and causes an arrest in the early development of B and T lineage-committed cells. Thus, SCID mice can tolerate a graft with human cells and particularly those of purified PBMCs administered by i.p. injection[253, 254]. The need for humanized mice evolved from several key needs: first, human and rodent immune systems are different, and results obtained in animal models, even extremely promising, were not always translated into human therapies. Additionally, there are a number of human diseases that do not have appropriate

animal models, or the animal models that do exist have significant differences from the human counterpart[228]. Therefore, for our experiments, we reconstituted Hu-SCID mice with PBMCs isolated from legume allergic patients and healthy donors. Approximately 2.5 x 10^6 human PBMCs were engrafted into SCID mice. The relatively low number of human PBMCs was chosen due to the lack on blood samples from legume allergic patients but sufficient to induce disease. The number of transferred human cells is an important issue, since engraftment of high numbers of human PBMCs (10-80 x 10^6 PBMCs) developed graft-versus-host-disease (GvHD) in mice. But in the event of the transfer of lower cell numbers (5 x 10^6 PBMCs), there was no symptoms of GvHD reported within an experimental period of up to 40 days[231]. Another important factor is allergen exposure. Re-stimulation of animals with allergen is necessary to trigger IgE production; otherwise in the absence of allergen no IgE antibody titers can be detected. Therefore, we re-stimulated SCID mice by force feeding with beans and GM peas followed by i.n. challenged with AAI. Hu-SCID mice engrafted with PBMCs from legume allergic patients and force fed with Tendergreen bean revealed a higher eosinophilic accumulation in airway compared to SCID mice reconstituted with PBMCs from healthy donors. This observation was not repeated in Hu-SCID mice fed with GM pea. Both healthy and allergic Hu-SCID showed only a slight increase of eosinophilic numbers in the airways. Inflammatory cell infiltrates and mucus hypersecretion were low or undetectable in lung. Moreover, we were unable to detect AAI-specific IgG4 and IgE antibodies (data not shown) in mice serum. Although, AAI-specific IgE antibodies were detected by immunobloting, we failed to detect AAI-specific IgE in serum of legume allergic patient by using ELISA technique. The reason AAI-specific IgE was not detected may be that ELISAs are not sensitive enough at low titers of specific antibodies.

Furthermore, our results showed that SCID mice engrafted with PBMCs derived from patients had similar level of allergic disease as SCID mice engrafted with PBMCs from healthy donors. In this experimental model we could not distinguish any difference between healthy and allergic Hu-SCID mice. An explanation for the similar response to seed meals from non-allergic and allergic donors may be due to the low PBMCs engraftment. The number of PBMCs derived from patients is probably not sufficient to develop a significant allergic immune response to legumes in Hu-SCID

mice. Another explanation could be the lack in re-challenge of the legume allergic patients. Unfortunately, due to ethical issues, it is not possible to challenge patients with the allergen. Further studies, such as stimulating PBMCs with bean AAI and GM pea AAI before engraftment, are important to add more experimental insight into this controversial and interesting area.

Conclusion

Our studies show that transgenic AAI is not more allergenic than bean AAI; even there are minor differences in glycosylation. We observed that feeding mice with peas and beans induced an immune and allergic response to AAI and pea lectin which differs from the *Prescott et. al.* study, probably due to different laboratory conditions such as food supply or housing. Therefore using mice for testing allergenicity to food has to be careful evaluated. Furthermore, we tested allergic responses upon consumption of peas in Hu-SCID mice chimeras engrafted with PBMCs from legume allergic patients. Hu-SCID mice generated responses to AAI upon consumption of native beans and peas showing that peas are not more allergenic than native beans. Our results revealed that Hu-SCIDs can be used for studying responses to GMOs and novel foods, but for these GM peas, it was not possible to distinguish allergic and on-allergic individuals. Therefore, it is still not clear whether these peas and beans can induce symptomatic allergic responses in human. Further data on validation and quality testing will be required for safety and efficacy evaluation of GM peas.

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Curriculum Vitae

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Education and training

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Poster and oral presentations

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Publication

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

- **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, T. J. V. Higgins. Agric Food Chem., May 5 2011*

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PLOS ONE

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

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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.

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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

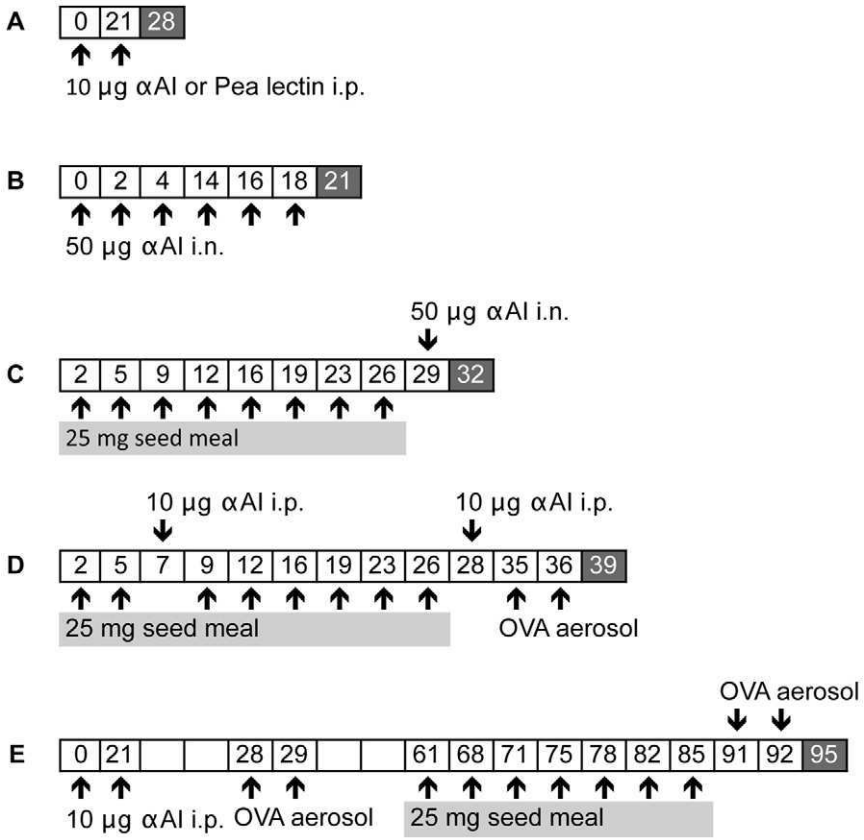

Figure 1. Experimental protocols. A. *Intraperitoneal immunization with purified proteins to assess protein immunogenicity.* On days 0 and 21 mice were immunized with 10 µg of purified αAIs from the transgenic peas, Tendergreen bean, Pinto bean, or pea lectin purified from non-transgenic peas. On day 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

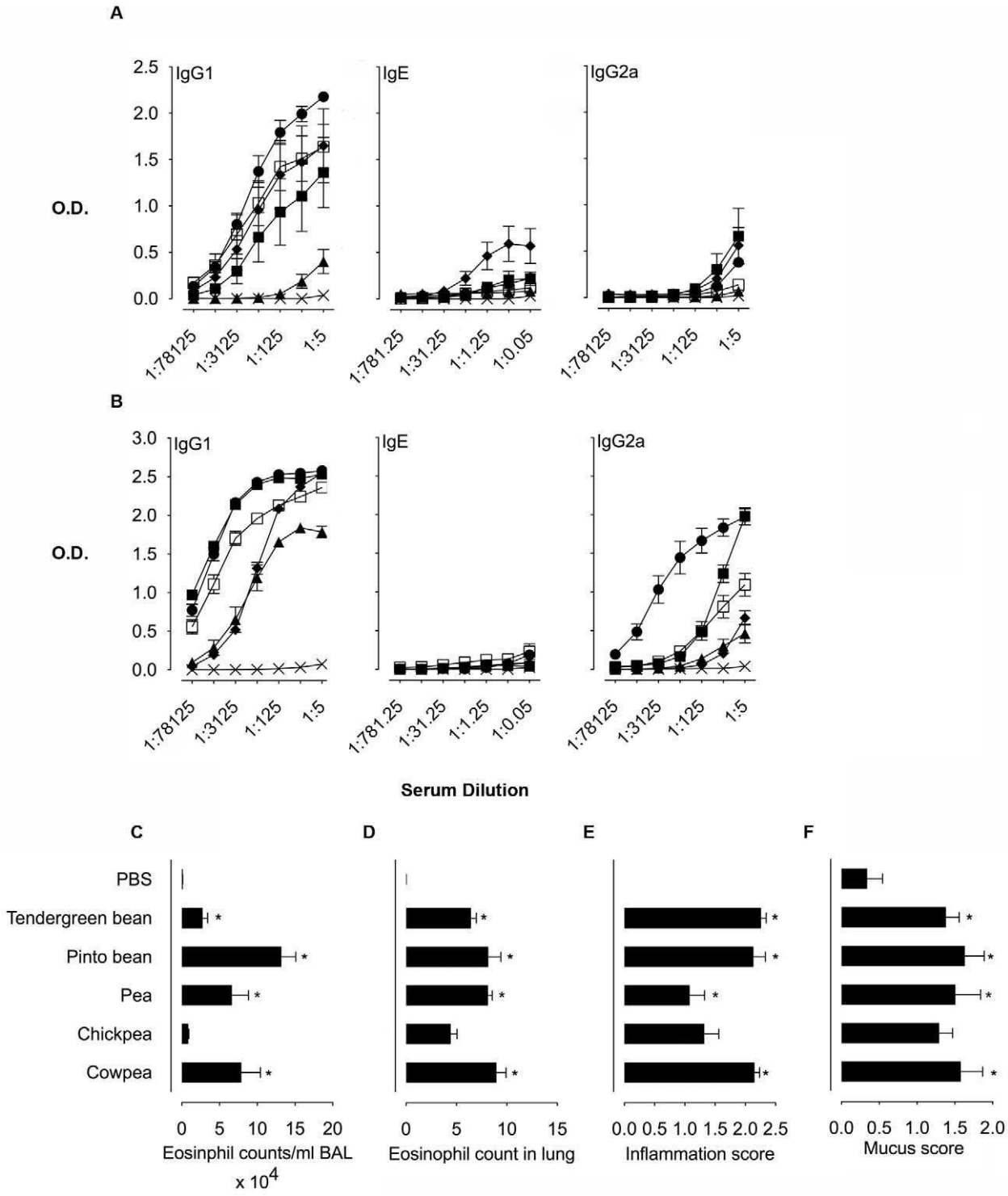

Figure 2. Immune responses to αAIs upon intranasal and intraperitoneal immunization. Serum antibody titres for Anti-αAI IgG1, IgE and IgG2a from A. i.p. αAI immunized mice and B. from i.n. αAI immunized mice. The treatment groups for A and B include PBS only ×, purified αAI proteins from Tendergreen 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 $\times 10^3$. 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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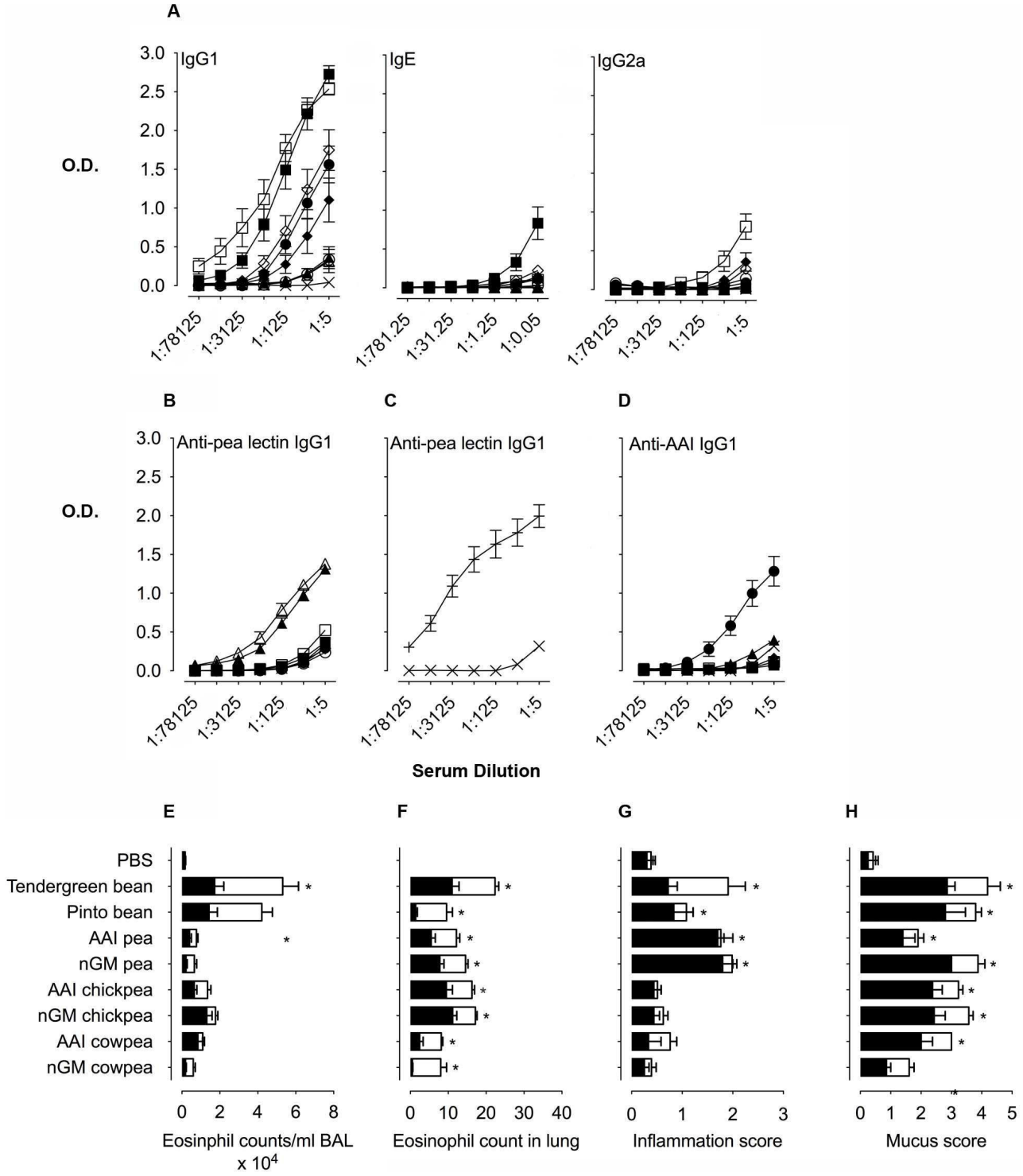

Figure 3. Immune responses following consumption of raw or cooked seed meal from αAI pea and bean. A. Serum antibody titres for anti-αAI IgG1, IgE and IgG2a from mice gavaged PBS or seed meals. B. Serum antibody titres for anti-pea lectin IgG1 from mice gavaged PBS or seed meals. C. Serum antibody titres 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 $\times 10^3$. 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.
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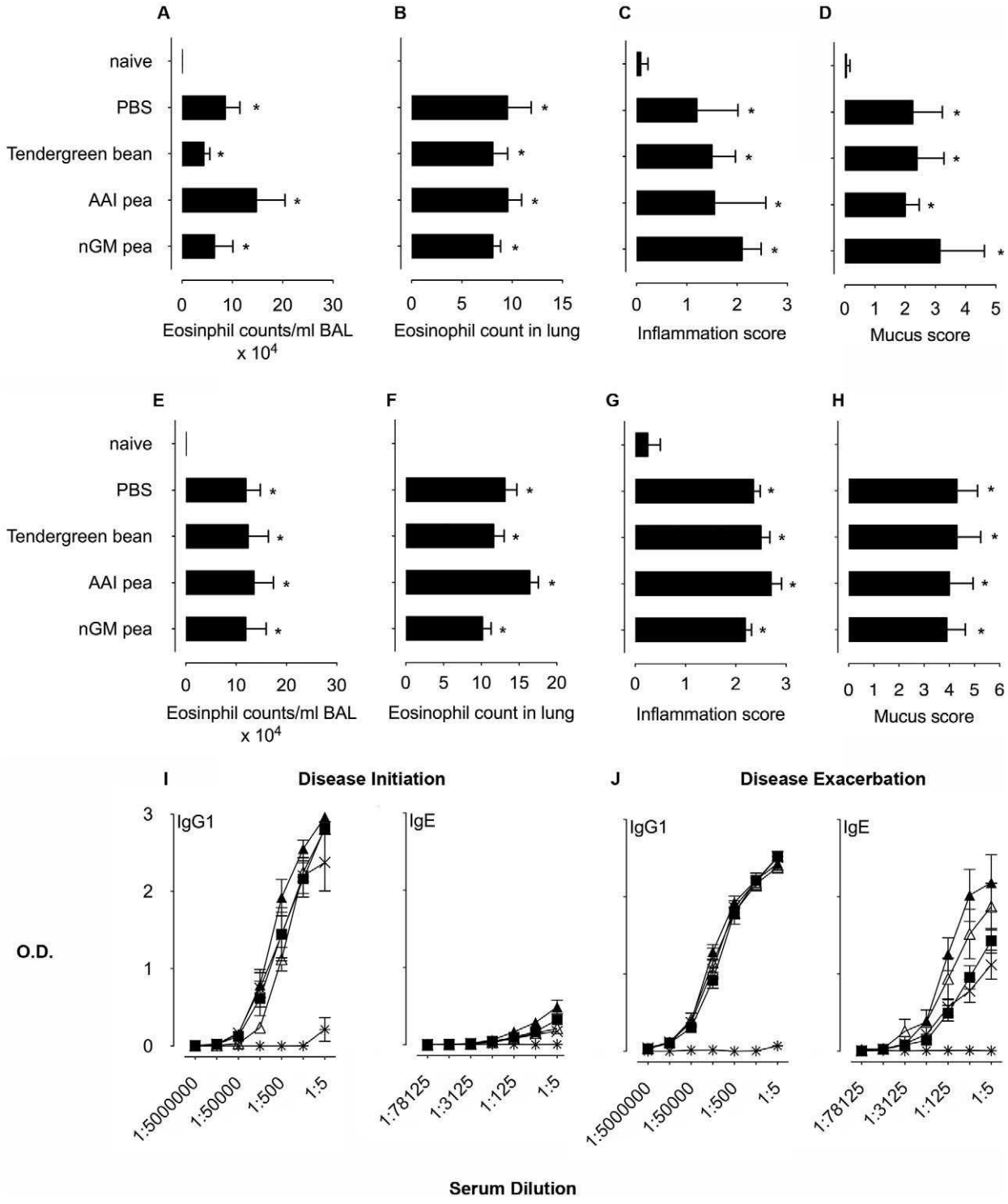

Figure 4. Adjuvant effect of αAI pea and bean consumption. Naïve BALB/c mice were compared with OVA-immunized and challenged mice gavaged with either PBS, or Tendergreen bean, αAI peas, non-transgenic pea seed meal. A. Eosinophil counts in BAL fluid from mice at disease initiation and e. exacerbation. 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 $OD_{450\ nm}$ ± SEM; n = 8, duplicate samples. For IgE, dilutions are expressed $\times 10^3$. 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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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)

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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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