



universität
wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Factors contributing to food allergy elicited by gastric acid suppression in a murine model“

verfasst von

Vera Elisabeth Assmann

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:

A 490

Studienrichtung lt. Studienblatt:

Diplomstudium Molekulare Biologie

Betreut von:

o. Univ. Prof. Dr. Thomas Decker

Diploma Thesis

Factors Contributing to Food Allergy Elicited by Gastric Acid Suppression in a Murine Model

Vera Elisabeth Assmann

2013

Academic advisors:

o. Univ. Prof. Thomas Decker

Department of Microbiology, Immunobiology and Genetics
Max F. Perutz Laboratories (MFPL)
University of Vienna

Assoc. Prof. DDr. Eva Untersmayr-Elsenhuber

Department of Pathophysiology and Allergy Research
Center of Pathophysiology, Infectiology and Immunology
Medical University of Vienna

The work was carried out at the
Department of Pathophysiology and Allergy Research,
Medical University of Vienna under the supervision of
Assoc. Prof. DDr. Eva Untersmayr-Elsenhuber

Data Sheet

Title:	Factors contributing to food allergy elicited by gastric acid suppression in a murine model.
Author:	Vera Elisabeth Assmann
Email:	vera.assmann@gmail.com
Academic advisors:	o. Univ. Prof. Thomas Decker Department of Microbiology, Immunobiology and Genetics Max F. Perutz Laboratories (MFPL) University of Vienna Assoc. Prof. DDr. Eva Untersmayr-Elsenhuber Department of Pathophysiology and Allergy Research Center of Pathophysiology, Infectiology and Immunology Medical University of Vienna
Work carried out at the:	Department of Pathophysiology and Allergy Research, Medical University of Vienna
Data of this diploma thesis are summarized in the manuscript:	Diesner SC, Schultz C, Pfitzner B, Assmann V, Krishnamurthy D, Weichselbaumer M, et al. Characteristics associated with anaphylaxis in an experimental food allergy model under gastric acid suppression. Manuscript submitted for publication.

Acknowledgments

The making of this diploma thesis has been an ongoing project that would not have come together as it did without the help, support and encouragement of a number of people. Though it will not be enough to express my gratitude in words, I would still like to thank them all.

First and foremost, I would like to thank Assoc. Prof. DDr. Eva Untersmayr-Elsenhuber. Her expertise, valuable guidance, critical review of this thesis and engagement as my mentor made it possible for me to complete this work. I could not have asked for a better supervisor! Also, I would like to thank Prof. Dr. Thomas Decker for his excellent supervision as my academic advisor at the University of Vienna.

Special thanks go to everyone who was or is part of the gastrointestinal immunology working group: Dr. Susanne Diesner, PhD, for all her advice and support, Cornelia Schultz for her continuous help and valuable technical assistance and Kristina Kreiner for her camaraderie and contribution to the read-out of the animal experiments.

Gratitude is also extended to all past or present members of the faculty and staff at the Institute for Pathophysiology and Allergy Research, especially to those who were involved in this project: Durga Krishnamurthy, PhD, Dr. Marlene Weichselbaumer, Philipp Starkl, PhD, Prof. Dr. Isabella Pali-Schöll and Prof. Dr. Erika Jenson-Jarolim.

My sincere thanks also go to Mag. Barbara Pfitzner, Dr. Michael Rothballer and Dr. Michael Schmid for their excellent analysis of the microbiome community composition and additionally to David Endesfelder, PhD, Dr. Gerhard Welzl and Prof. Dr. Thomas Rattei, who are also to thank for their statistical input of these.

I would also like to acknowledge Dr. Thomas Eiwegger, Prof. Dr. Zsolt Szépfalusi, Prof. Dr. Heinz Fehrenbach and Prof. Dr. Anton Hartman for their valuable cooperation.

I deeply cherish the unconditional love and support I received from my family throughout my studies and also during these years abroad. A thank-you must also go to my boyfriend Martin for his patience and encouragement.

Last but not least, I am indebted to my grandmother for her unwavering faith in me.

Table of Contents

ABSTRACT	1
ABBREVIATIONS	3
1. INTRODUCTION	5
1.1. Food allergy	6
1.1.1. Clinical Manifestations of Food Allergy	7
1.1.2. Epidemiology	8
1.1.3. Diagnosis and Treatment	8
1.2. Pathophysiology of Type I Food Allergy	9
1.3. The Immune System in the Gut	12
1.3.1. Innate Immunity in the Gut	14
1.3.2. Adaptive Immunity in the Gut	14
1.3.3. Oral Tolerance	16
1.3.4. Characteristics of Food Allergens Affecting Food Allergy	20
1.3.5. Host Factors Affecting Food Allergy	22
1.4. Murine Models and Food Allergy	23
1.4.1. A Mouse Food Allergy Model under Anti-Acid Medication	24
1.5. Aim of Study	25
2. MATERIALS	27
2.1. Chemicals	27
2.2. Medication	28
2.3. Kits	28
2.4. Antibodies	29
2.5. Cell Media	29
2.6. Buffers and Solutions	30
3. METHODS	31
3.1. Animals	31
3.2. Immunization Protocol	31
3.2.1. Immunization Readout	33
3.3. Sampling of Feces	34
3.4. Analysis of Feces	34

3.5. Oral Provocation	35
3.5.1. Anaphylaxis Readout	36
3.6. Sacrifice	37
3.6.1. Heart.....	38
3.6.2. Spleen	38
3.6.3. Stomach and Small Intestine.....	42
3.7. Statistics.....	46
 4. RESULTS.....	 49
4.1. Analysis of serum antibody titers in IgE responders versus non-responders.....	49
4.2. Readout of anaphylactic symptoms.....	50
4.3. Measurement of stomach pH levels of mice after treatment with anti-acid medication	51
4.4. Evaluation of the IgA levels in intestinal lavages	51
4.5. Cytokine profile and T cell counts of stimulated spleen cell supernatant	52
4.6. Differential blood cell count analysis during anaphylaxis	53
4.7. Histological evaluation of the gastrointestinal samples	54
4.8. Intestinal bacterial composition	55
 5. DISCUSSION	 57
 6. REFERENCES.....	 63
 7. LIST OF TABLES	 73
 8. LIST OF FIGURES.....	 75
 9. APPENDIX	 77
9.1. Zusammenfassung.....	77
9.2. Curriculum Vitae	79

Abstract

Food allergy is an important public health problem as approximately 3 to 6% of the population in Western countries is affected by immunologically mediated adverse reactions to food and incidence rates seem to be rising. Avoidance of the offending allergen to prevent food allergic reactions, the most severe of which is anaphylaxis, is the only causative treatment option available today. Therefore, investigations looking at the sensitization and effector phase of food allergic reactions are imperative to finding new options to diagnose and treat food allergy.

Every day a countless number of dietary antigens come in contact with the gastrointestinal immune system but only a limited fraction of these trigger allergic reactions. This is due to the acquired immune tolerance, the result of interactions between the innate and adaptive immune system in the gastrointestinal tract. Hence, the breakdown of oral tolerance has been implicated as an important agent in Th2 skewing, eliciting food allergy. Investigations have also focused on how food allergen characteristics and features of affected patients contribute to the development of allergic reactions to understand factors contributing to the breakdown of oral tolerance.

Previously, the hindrance of gastric protein digestion by acid-suppressive drugs has been shown to represent a risk factor for food allergy. The neutralization of gastric acidity interferes with the activation of gastric and pancreatic enzymes and promotes sensitization to digestion-labile proteins by allowing them to gain access to the mucosal immune system. Hence, we used a previously established murine model of food allergy whereby mice were orally immunized with the egg allergen OVA under concomitant acid suppression to induce food allergic reactions in inbred BALB/c mice. However, as in humans, not all mice developed oral hypersensitivity reactions. Therefore, the objective of this study was to investigate factors influencing the heterogeneity of the response in mice subjected to identical immunization procedures.

In conclusion, an increased gastric pH, which was observed in all mice, induced anaphylactic reactions only in the IgE responders. OVA-specific IgE, rather than IgG1, mediated anaphylactic responses in mice in this model of food allergy. This was underlined by mast cell degranulation, elevated Th2 cytokines and alterations in differential blood cell counts of the allergic mice. No substantial differences in the histological evaluations of the intestine and stomach or T cell counts were observed. However, significant variation in the intestinal bacterial composition between the groups was revealed on single bacterial strain level only and could contribute to the heterogenic immunological responses. Without any doubt, further studies need to be conducted to evaluate the time-dependency of microbiome changes during sensitization to determine its contribution to food allergy induction in some, but not all mice.

Abbreviations

APC	Antigen-presenting cell
BSA	Bovine serum albumin
CD25	Interleukin-2 receptor α -chain
ConA	Concanavalin A
DMP	Dry milk powder
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FcϵRI	High affinity IgE Fc receptor
FcγRIIb	Low affinity inhibitory IgG Fc receptor
FcγRIII	Low affinity stimulatory IgG Fc receptor
Foxp3	Forkhead box p3
IFN-γ	Interferon- γ
Ig(A, E, G1, G2a, M)	Immunoglobulin (A, E, G1, G2a, M)
i.g.	Intragastric
IL- (2, 4, 5, 10, 13, 17, 22)	Interleukin- (2, 4, 5, 10, 13, 17, 22)
IL-2R	Interleukin-2 receptor
IL-4Rα	Interleukin-4 receptor, alpha
i.v.	Intravenous
LPS	Lipopolysaccharides
MALT	Mucosa-associated lymphoid tissue
M-cells	Microfold cells
MHC	Major histocompatibility complex
MIS	Mouse immune sera
mMCP-1	Mouse mast cell protease-1
npMANOVA	Multivariate analysis of variance
OTU	Operational taxonomic unit
OC	Oral challenge
OVA	Ovalbumin, from chicken
PAF	Platelet-activating factor
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline with Tween-20
PCA	Principle component analysis
PP	Peyer's patch
PIS	Pre-immune sera
PPI	Proton pump inhibitor
SPT	Skin prick test
TGF-β	Transforming growth factor- β
TLR	Toll-like receptor
TMB	3,3', 5,5' Tetramethylbenzidine
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cell
Tris	Tris(hydroxymethyl)aminomethane
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20

1. Introduction

Allergic reactions seem to be a phenomenon of our time with a brief, yet concrete historic development. Even though the first documented anaphylactic reaction dates back to 2640 before Christ when Pharaoh Menes died due to wasp venom allergy (1), allergic reactions were not defined as such until early in the 19th century. In fact, because the mechanisms of the human immune system had not yet been elucidated, such hypersensitivity reactions were viewed as, among other things, neuroses. The idea that these afflictions may stem from a common source and shared pathology was rooted in the fast-paced biomedical advancements of the 20th century. Based on this knowledge, allergy quickly gained the pervasive presence and its place in modern culture that it still holds today.

In the 1800's, the consensus in the scientific community was that the immune response against a foreign agent was always protective. In 1796, Edward Jenner inoculated James Phipps, an 8-year-old boy, with cowpox taken from the hand of a milkmaid named Sarah Nelmes. After a subsequent smallpox challenge, he noticed that the boy was immune (2–4). Although he was not the first to vaccinate, his experiments were the first proper scientific account of an effective and safe vaccination against smallpox, which was declared eradicated by the World Health Organization in 1980 (5,6).

In 1902, Charles Richet, in collaboration with Paul Portier, was the first to discover and describe a negative immune reaction (7). They injected dogs with a toxin from a sea anemone. While attempting to determine the lethal dose of the toxin, a number of dogs survived. They expected that initial exposure would leave behind diminished susceptibility or *prophylaxis* produced by immunization. However, they were surprised to find that a second injection of a much lower dose killed the dogs. Since this was the opposite of a protective immune reaction and showed an increase in susceptibility, Richet termed it *anaphylaxis* (8). Charles Richet was even awarded the Nobel Prize in Medicine/Physiology in 1913, in recognition of his work on anaphylaxis (9).

Von Pirquet first coined the term “allergy” in 1906 to describe a changed or altered biological reactivity. This was a direct result of his study with the cowpox vaccination and his interpretation of the observed skin reaction thereafter. Von Pirquet believed that the early “reaction” of immunity after vaccination (the exanthema of smallpox) and the immune state of an individual after vaccination that Jenner had first described were associated but contradicted each other. Therefore, he suggested the term “allergy”, for a more general term for the change in ability to react. He also proposed that the state of “immunity” should only be expressed as such when a foreign entity does not result in a clinical reaction (10).

Then, in 1921, Prausnitz and Kuestner performed a groundbreaking experiment to describe the origins of allergy. Prausnitz, who was subcutaneously injected with Kuestner’s serum (who was allergic to fish), developed an immediate skin response at the site of injection (11). Therefore, they were able to conclude that a transferable humoral factor specific to an allergen, later termed “reagin” (12), was responsible for the allergic reaction to that allergen in a healthy individual. It was not for another 40 years, that this reagin was identified as a new class of immunoglobulins (Ig) (13,14), termed immunoglobulin E (IgE) (15).

1.1. Food allergy

Today, food allergy is described as immunologically mediated hypersensitivity to a dietary compound (16). Food adverse reactions are in a first step categorized as toxic or non-toxic responses (Fig. 1). Reactions due to toxins develop in anyone as long as the dose is high enough. This includes reactions to pharmacologically active food components (e.g., migraines caused by tyramine in aged cheeses) or food borne pathogens (e.g., scombroid fish poisoning¹). Non-toxic reactions, also called food hypersensitivities, depend on the individual’s susceptibility. Here it is important to distinguish between immunologically mediated (food allergy) and non-immunologically mediated (food intolerance) adverse reactions (Fig. 1). The latter are often confused with food allergy (17). Food allergy can then be further classified as

¹ Under-cooked scombroid fish contaminated by Salmonella organisms produce toxins and histamine-like reactions in some sensitive individuals.

IgE- or non-IgE-mediated mechanisms (Fig. 1). IgE-mediated allergic reactions are the most common form of food allergy and are characterized by an immediate onset of symptoms after ingestion triggered by specific IgE antibodies. However, it is important to note that the detection of allergen-specific IgE (sensitization) does not necessarily indicate a clinical reaction (17). Non-IgE-mediated food allergy is less common. In contrast to IgE-mediated food allergy, non-IgE mediated allergic reactions mainly affect the mucosa of the gastrointestinal tract. The delayed reactions are most likely mediated by cellular immune responses and/or non-IgE antibodies (18).

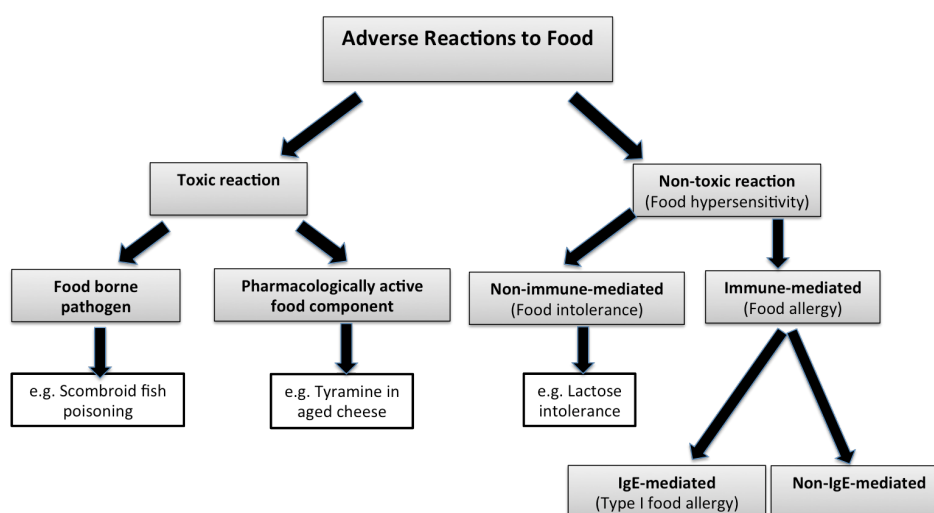


Figure 1: Classification of food adverse reactions. Adverse reactions to food can be classified as toxic or non-toxic reactions. Toxic reactions include reactions to food borne pathogens or pharmacologically active food components. Non-toxic reactions can then be further classified as food intolerances or food allergies. Food allergic reactions can be either IgE-mediated or non-IgE-mediated.

1.1.1. Clinical Manifestations of Food Allergy

Symptoms of food allergy may occur locally (affecting the skin, gastrointestinal tract, and/or respiratory system) or systemically (additionally involving the cardiovascular system resulting in anaphylaxis). Skin reactions are the most common. Angioedema, urticaria and flushing represent the most common cutaneous symptoms. Gastrointestinal tract symptoms of food allergy involve abdominal pain, vomiting, nausea and diarrhea while respiratory tract symptoms include laryngeal edema, bronchospasm and rhinorrhea (17). The oral allergy syndrome is the mildest form of the IgE-mediated hypersensitivity reaction

(symptoms include itching and tingling of the mouth, tongue and throat). The most severe, potentially fatal allergic reaction is anaphylaxis.

1.1.1.1. Anaphylaxis

Today, the leading cause of anaphylaxis is food allergy (19). An anaphylactic reaction to food can occur within minutes to hours after ingestion of the offending food (20). Anaphylactic reactions are frequently the result of allergic reactions to fish, shellfish, peanuts and tree nuts (21,22). It often affects multiple organ systems and is accompanied by the classic manifestations of the IgE-mediated food allergic reaction, as described (in section 1.1.1.). Initially, symptoms appear in the throat or oral cavity (although they should not be confused with the oral allergy syndrome), followed by gastrointestinal symptoms and finally cardiovascular symptoms along with airway obstruction, which are potentially life-threatening (20). Skin symptoms occur in 80-90% of cases (22,23). Interestingly, the severity, timing and sequence of the symptoms may vary among individuals and even within the same patient.

1.1.2. Epidemiology

Nearly 3 to 4% of adults and 6% of young children in westernized countries are affected by food-induced allergic disease (17,24). Furthermore, incidence rates seem to be increasing (25,26), making food adverse reactions an important public health concern. Food allergy predominates in children in the first one or two years of life, yet it tends to disappear later on. About 80% of pediatric patients outgrow their allergies by 16 years of age (27,28); particularly allergies to milk, egg, soy and wheat may be resolved with age (29). Peanut, tree nut, fish, and shellfish allergies tend to last a lifetime, although 20% of peanut allergies may be outgrown (30).

1.1.3. Diagnosis and Treatment

To diagnose food allergy a detailed history, a physical examination and diagnostic tests are required. Conventional diagnostic tests for food allergy include skin prick tests (SPT) and serum-specific IgE tests (24,31). Larger SPT wheals are a

quick indicator of sensitization in IgE-mediated allergic reactions. Higher allergen-specific IgE titers in the blood determined by serum-specific IgE tests may also indicate sensitization or clinical reactivity. However, both SPT and serum-specific IgE tests do not predict the severity of an allergic reaction or allow prognostic conclusions (24). An oral food challenge, where the food is ingested in increasing doses under supervision (due to the risk of anaphylaxis), remains the gold standard in diagnosing food allergy (31). A food/symptom diary or elimination diet, whereby consumption of the allergen-containing food is avoided, can also be helpful during the diagnostic evaluation procedure (32).

To date, identifying the responsible allergen, eliminating the offending food from the diet and educating patients what to do in case of accidental ingestion remain the mainstays of diagnostic testing and treatment of food allergic patients. Epinephrine administration can be used in the event of an anaphylactic reaction due to accidental exposure (17,33). The fact that there are no treatment options other than avoidance of the offending food of course is not ideal, which is why other therapeutic strategies are being intensively investigated (24). One promising option is oral immunotherapy (an allergen-specific therapy), which involves gradually increasing exposure to the allergen with the intention of desensitizing the patient and/or developing tolerance (34–37). Additionally, anti-IgE treatment (an allergen-nonspecific therapy), which uses antibodies that bind to IgE, neutralizing the IgE and inhibiting it from binding to mast cells and basophils, is also being investigated for peanut allergy (34,38). Hence, the development of new treatment options goes hand in hand with advancements in understanding the pathophysiology of food allergy, which will hopefully provide exciting progress in this field in the future.

1.2. Pathophysiology of Type I Food Allergy

In IgE-mediated food allergic disorders, the first phase is called “sensitization”, which occurs when the allergenic food protein or an immunologically intact component of the allergen crosses the epithelial barrier. If a Th2 reaction is stimulated and B cells² initiate the production of IgE antibodies specific to this

² A Th2 response to allergens stimulates isotype switching to the IgE class in B cells.

allergen, the patient might react upon allergen re-exposure. IgE antibodies then bind to the high affinity IgE Fc receptor (FcεRI) on mast cells and tissue basophils. When patients are exposed to the food compound a second time, the allergen binds to the IgE antibodies, causing them to cross-link and triggering the release of preformed and newly formed mediators such as histamine, prostaglandins and leukotrienes, in what is called the “effector” phase³ (Fig. 2). This, in turn, leads to clinical reactivity and allergic symptoms including increased vascular permeability and smooth muscle contractions during immediate reactions (minutes after exposure), as well as a delayed inflammatory reaction (several hours after allergen exposure).

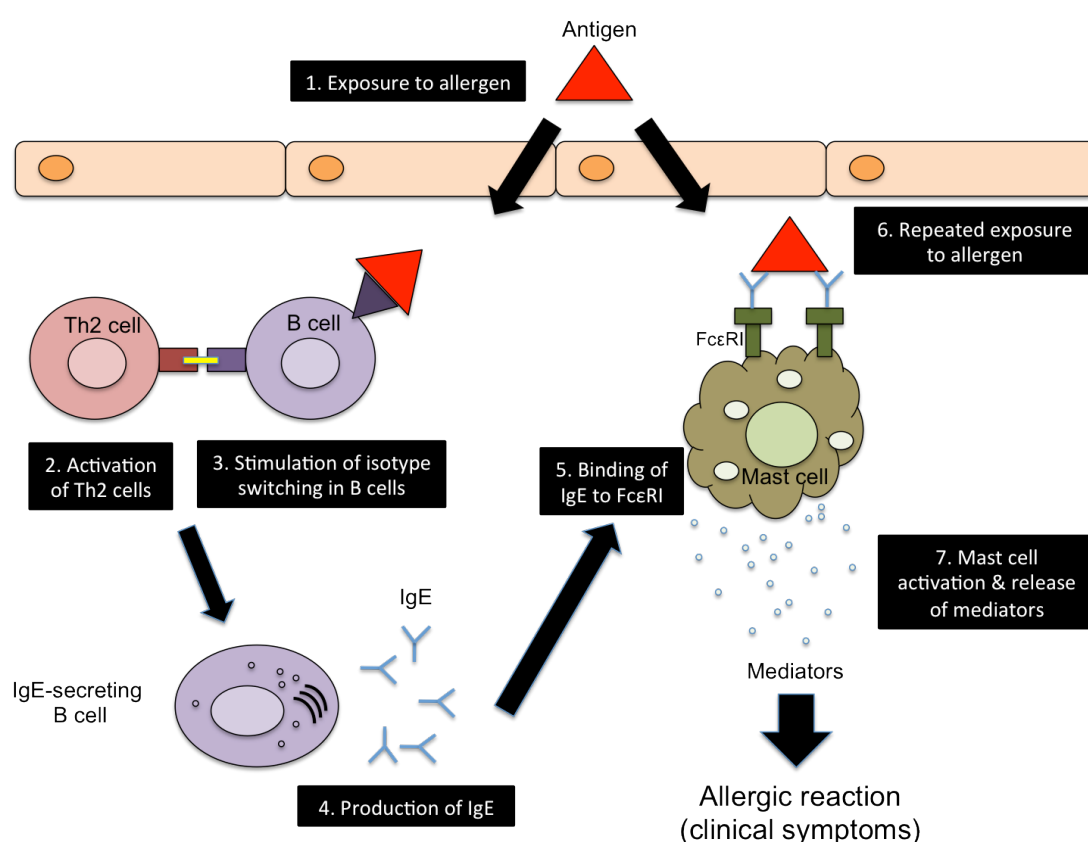


Figure 2: Pathophysiological events of type I food allergic reactions. First, the food allergen crosses the epithelial barrier. It then stimulates a Th2 response, which signals B cells to produce IgE by promoting isotype switching. Subsequently, IgE can bind to FcεRI on mast cells and allergy effector cells. If the patient is exposed to the offending food a second time, it comes to cross-linking of the IgE antibodies and the release of mediators such as histamine, prostaglandins and leukotrienes leading to the clinical symptoms of food allergy.

As mentioned above, the mildest form of IgE-mediated food adverse reactions is the oral allergy syndrome. It is mainly triggered in pollen-allergic individuals after

³ The activated mast cells also produce IL-3, IL-5 and IL-13 (Th2-type cytokines) that attract eosinophils and other inflammatory cells (139).

consumption of fresh fruits and vegetables due to the cross-reactivity of IgE antibodies (39). After the stimulation of IgE production to pollen, which sensitizes individuals via the respiratory route, the antibodies bind to (or “cross-react”) with certain homologous proteins found in uncooked fruit or vegetables (31,40). The most severe allergic reaction, on the other hand, is anaphylaxis. The immune mechanisms that cause food-induced systemic anaphylaxis are not yet well understood. Several experimental studies (41–44) showed that allergen injection can induce two different mechanisms for systemic anaphylaxis in mice: either the classic IgE, FcεRI and mast cell-dependent pathway or an alternative immunoglobulin G (IgG), low affinity stimulatory IgG Fc receptor (FcγRIII), basophil-dependent or macrophage-dependent mechanism as seen in Fig. 3. Also, histamine is the primary mediator for the classic pathway. Platelet-activating factor (PAF) also plays a pathophysiological role but to a lesser extent. On the other hand, PAF is primarily responsible for the anaphylactic reaction in the alternative pathway. Histamine and PAF increase vascular permeability (41,42), which promotes the clinical symptoms of food allergy.

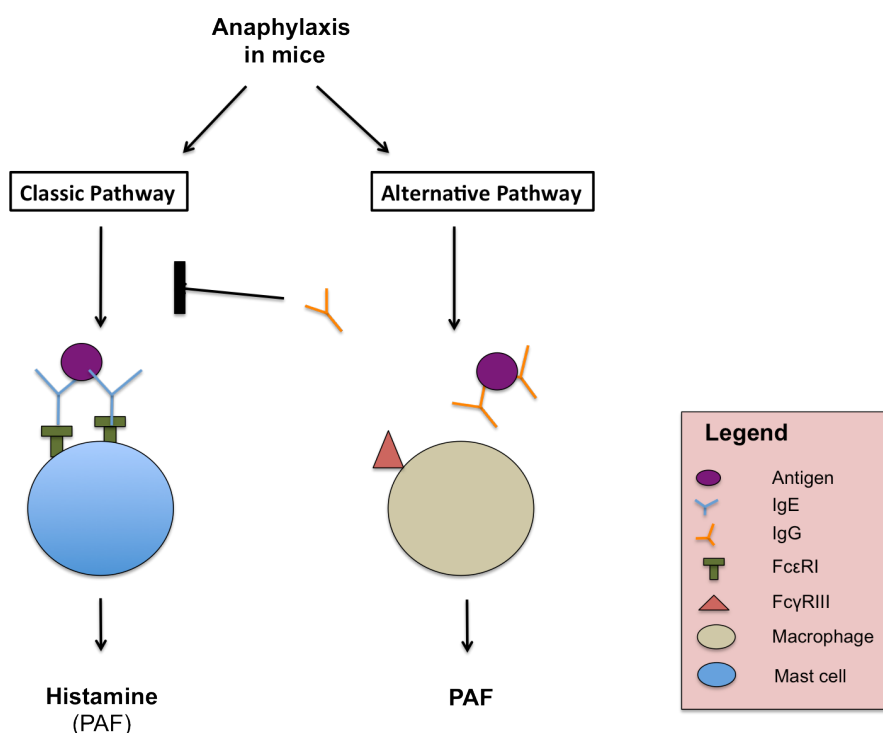


Figure 3: Mechanisms of anaphylaxis in murine models. Systemic anaphylaxis in mice may follow either the classic or alternative pathway. In classic IgE-dependent anaphylaxis, exposure to antigen causes cross-linking of IgE bound to FcεRI on mast cells, inducing the release of the primary mediator histamine and sometimes PAF. In the IgG-dependent mechanism, the antigen-IgG complex stimulates cross-linking of FcγRIII on macrophages and triggers the release of PAF. Both histamine and PAF enhance smooth muscle contractions and increase vascular permeability, leading to the clinical symptoms of anaphylaxis. If more IgG than IgE is present then antigen-specific IgG can block the classic pathway. This figure is adapted from Finkelman (41).

Less antigen and antibody are required to activate the IgE/FcεRI/mast cell-dependent pathway than the IgG/FcγRIII/macrophage-dependent pathway (41). Because most anaphylactic reactions in humans are induced by very low antigen doses, they are most likely dependent on the classic pathway (41). It also seems that when there is more IgG than IgE but both are present, which is commonly the case in allergic patients, antigen-specific IgG can prevent IgE-mediated anaphylaxis by neutralizing antigens and hinder them from cross-linking mast cell FcεRI-associated IgE (45). IgG antibodies also decrease effector cell activation by cross-linking the FcεRI and the low affinity inhibitory IgG Fc receptor (FcγRIIb) (45). Finally, it seems that interleukin (IL)-4 and IL-13, which act through the IL-4 receptor α (IL-4Rα), as well as nitric oxide, endogenously regulate the classic and alternative pathways of anaphylaxis because they increase the responsiveness to mediators and herewith enhance the susceptibility to anaphylaxis (41). IL-4, IL-13⁴ and nitric oxide promote vascular permeability and vasodilation (46).

1.3. The Immune System in the Gut

The gastrointestinal system consists of a single epithelial cell layer on top of a basement membrane (Fig. 4). The tight junctions between these columnar epithelial cells act as a physical line of defence and a thick mucus layer on top of the epithelium contains factors that trap pathogenic organisms (such as bacteria and viruses) and other particles. The lamina propria, a layer of loose connective and supportive tissue containing lymphatic vessels, blood vessels and mucosa-associated lymphoid tissue (MALT)⁵, lies underneath (Fig. 4). The more distant lymph nodes are also important for the induction and amplification of the adaptive immune response. Specialized cells of the epithelium (Fig. 4) sample antigens and deliver them to the MALT or mesenteric lymph nodes (further described in section 1.3.3.).

⁴ IL-4 and IL-13 both activate the transcription factor STAT6 (140)

⁵ The MALT is a collection of immune cells including lymphocytes, dendritic cells, macrophages and mast cells; it is the site of initiation for specialized adaptive immune responses.

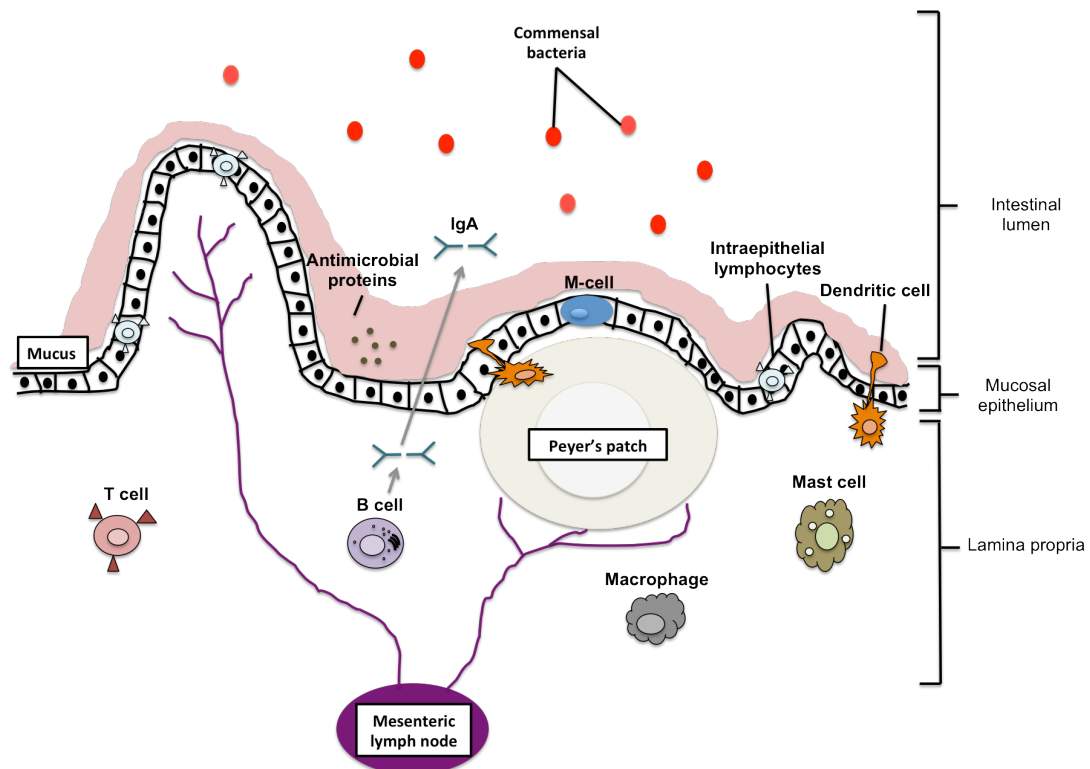


Figure 4: Immunity in the gastrointestinal system. A huge number of commensal bacteria can be found in the intestinal lumen. A monolayer of epithelial cells spans the intestinal tract. A mucus layer on top of the epithelium contains antimicrobial substances and factors that trap pathogenic organisms. Together, they comprise the mucosal epithelium, which serves as a primary barrier against microbial invasion. The underlying supportive tissue is called the lamina propria. Specialized epithelial cells such as dendritic cells, M-cells and intestinal epithelial cells sample antigens and deliver them to the MALT within the lamina propria or lymph nodes, where components of the innate (macrophages, epithelial cells) and adaptive (T cells, IgA-producing B cells and cytokines) immune system must either induce tolerance to non-harmful foreign substances or recognize and respond to invaders.

The gastrointestinal tract has two extraordinary features. Firstly, it is the largest immune organ of the human body; the surface area of the intestinal epithelium (forming mainly small intestinal villi and microvilli) spans approximately 300 m² (47). Secondly, approximately 100 trillion bacteria reside in the lumen of the gut, growing on the surface of even healthy individuals as commensals (48–50). So the mucosal immune system has the difficult task of recognizing friend from foe. It must respond to the few pathogenic organisms that can become part of the gut flora by ingestion of contaminated food or water while being confronted with and having to tolerate a staggering number of commensals (51). For this reason, components of the innate (macrophages, natural killer cells, epithelial cells, toll-like receptors [TLRs]) and adaptive (lymphocytes, Peyer's patches [PPs], secretory immunoglobulin A [IgA] and cytokines) immune system of the gut have developed strategies to tolerate foreign substances that are not harmful to the body. These components in turn collaborate with other immune cells (antigen-presenting cells [APCs] and regulatory T cells

[Tregs]) and together, they play a crucial role in the development of oral tolerance (52,53).

1.3.1. Innate Immunity in the Gut

The first line of defence of innate immunity in the gastrointestinal tract is the mucosal epithelial lining; the tight junctions between these epithelial cells act as a barrier (54). The epithelial cell layer is coated not only with mucus but also with a macromolecular matrix called the glycocalyx, consisting of membrane-bound mucins and various glycolipids. It serves as a barrier as well, to prevent microbes from contacting the epithelium. TLRs are expressed by intestinal epithelial cells to promote inflammatory and antiviral responses but they are in turn regulated to hinder immune responses to commensals. Studies in C3H/HeJ mice, who do not have a functional TLR4 that recognizes bacterial lipopolysaccharides (LPS), showed that the susceptibility to food allergy in mice is inhibited by TLR signals provided by the commensal gut flora (55). Dendritic cells and macrophages in the gut are capable of promoting immune responses to pathogenic microbes that cross the barrier into the lamina propria but they may also minimize the inflammatory response in the lumen (56).

1.3.2. Adaptive Immunity in the Gut

Humoral immunity is a major form of adaptive immunity in the gastrointestinal tract; primarily dimeric IgA antibodies that are transported across the epithelium, as well as IgG and immunoglobulin M (IgM) antibodies that are also present in the gut lumen, mediate humoral immunity (57). Secretory IgA antibodies bind antigens in the lumen and prevent adsorption (termed “immune exclusion”) (58). However, their importance has been a matter of debate. It was thought that mucosal IgA binds and neutralizes the dietary antigen prior to systemic absorption to prevent anaphylaxis (59). For example, knockout mice lacking the polymeric immunoglobulin receptor that facilitates the secretion of IgA and IgM were more sensitive to IgG-mediated anaphylaxis (60). However, the mice developed tolerance after oral feedings of the allergen, mediated at least in part by Tregs, which suggests that cellular factors can compensate for impaired immune exclusion (60). In addition, another study showed

that antigen-specific IgA and IgG in the serum, not antibodies in the gut lumen, protect against systemic IgE-mediated anaphylactic reactions to food implying that ingested allergens must be absorbed systemically to induce such severe food allergic reactions (61).

A number of different CD4⁺ T cells are involved in the development of adverse reactions to food or oral tolerance on the other hand (refer to Fig. 5). CD4⁺ T cells are mainly found in the epithelial layer, throughout the lamina propria and within PPs and their effector functions depend on the cytokines they produce (59). Th1 cells are associated with the production of pro-inflammatory cytokines tumor necrosis factor α (TNF- α) and interferon γ (IFN- γ) and protect against intracellular bacteria and viruses (62). Th17 cells seem to be important in maintaining the barrier function of the epithelial layer because they produce the cytokines IL-17 and IL-22; their receptors are expressed on intestinal epithelial cells and induce pro-inflammatory epithelial defense mechanisms, such as the expression of mucins and defensins (63). Th2 cells produce cytokines IL-4, IL-5 and IL-13, which increase mucus secretion and induce smooth muscle contraction (64).

Activated dendritic cells interact with T cells and when this occurs in a suitable milieu (large amounts of IL-4, IL-5, IL-13), the T cell becomes a Th2 cell, which signals B cells to produce IgE antibodies. A study in mice demonstrated these variations in the Th1/Th2 response after food allergen sensitization as the spleen cells of C3H/HeJ mice showed significantly increased IL-4 and IL-10 secretion, whereas BALB/c mice revealed significantly increased IFN- γ secretion (65). It seems that the allergic reaction is maintained by a Th2-dependent IgE response; however, the way it is initiated in naïve individuals remains the fundamental question. The traditional hypothesis that over-activation of Th2 cells to ingested food proteins is the primary elicitor of food allergy, may not hold up to be true (53). It has now been recognized that intestinal homeostasis (the result of complex interactions between the innate and adaptive immune system), or rather the lack thereof due to failure of regulatory mechanisms, plays a significant role in the Th2 skewing (66).

1.3.3. Oral Tolerance

Despite the huge number of dietary antigens exposed to the gastrointestinal tract on a daily basis, only few lead to allergic reactions in certain individuals. The emerging concept is that this is due to the development of oral tolerance (52) and that food hypersensitivity reactions are a result of the failed induction or breakdown of adaptive immune tolerance (24,33).

If allergens survive digestion and evade immune defences in the lumen, they will contact the intestinal surface and be sampled and taken up by either microfold cells (M-cells), epithelial cells or dendritic cells (Fig. 4) (59), after which they will be taken up by APCs in the MALT in conjunction with signals from the commensal flora (64). Interplay between a number of food allergen characteristics (refer to Section 1.3.4) and host factors (refer to Section 1.3.5.) will determine the immune response to the ingested food protein.

1. Dendritic cells

Dendritic cells are important professional APCs present in PPs, propria lamina and mesenteric lymph nodes of the gut (56). They present peptides of food allergens via the major histocompatibility complex (MHC) class II complex to T cell receptors on CD4⁺ T cells. It seems that dendritic cells are deciding factors for whether active immunity or tolerance will develop in naïve individuals (64). Effector dendritic cells (defined by the marker fractalkine receptor CX3CR1) extend dendrites between the epithelial cells to the lumen to sample antigens and then migrate to the mesenteric lymph nodes to activate and induce differentiation of pro-inflammatory effector T cells (that in turn produce IL-17 and IFN- γ) (67). Another subset of dendritic cells, regulatory dendritic cells (defined by the marker α_E integrin CD103), which originate in the lamina propria and migrate to the mesenteric lymph node, induce differentiation of naïve T cells to Tregs (68) most likely via a transforming growth factor- β (TGF- β) and retinoic acid-dependent mechanism (69). The role of dendritic cells in allergic disease in response to dietary antigens has not been completely understood yet. However, a study by Chambers et al. showed that an adoptive transfer of dendritic cells from mice with type I milk hypersensitivities induced antigen-specific IgE

responses in naïve mice in the absence of an antigen challenge (70). This indicates that dendritic cells do play a role in allergen-specific IgE responses, at least in the murine system.

2. Epithelial cells

Intestinal epithelial cells take-up soluble antigens that escaped proteolysis in the lumen (71). They may also act as non-professional APCs (by expressing MHC class II molecules on their basolateral side) selectively activating CD8⁺ suppressor T cells, involved in suppressing the local immune system (72). After crossing the epithelium, antigens may encounter T cells or macrophages in the lamina propria or enter circulation (52).

3. M-cells overlying PPs

PPs are aggregates of lymphoid follicles, found predominantly in the ileum. They are composed of a germinal center consisting of B cells committed to IgA secretion surrounded by fewer T cells (52). Specialized epithelial cells called M-cells overlying PPs sample particulate antigens and deliver them to dendritic cells in the subendothelial dome region, that in turn ingest the antigens and finally deliver them to the B cell follicles of the PPs (73). IgA switching then occurs in the B cells, which is mediated by TGF- β -secreting T cells (74).

1.3.3.1. Effectors of Oral Tolerance

CD4⁺ T cells are divided into T helper cells (Th1, Th2, Th17 cells; refer to Section 1.3.2.) and Tregs as seen in Fig. 5. Tregs modulate the immune response and suppress the immune response of other T helper cells (75). After receiving environmental information from the APCs, it comes to the activation and differentiation of Treg subsets (natural and inducible Tregs) that, depending on the cytokines produced, may carry out diverse roles in tolerance development or food allergy. Tregs have therefore been described as effectors of oral tolerance. However, other mechanisms such as anergy and deletion can also affect the establishment of

oral tolerance (53). Additionally, other types of T cells such as CD8⁺ suppressor T cells, $\gamma\delta$ T cells and natural killer T cells have also been implicated in oral tolerance (53).

Natural Tregs (T_R ; CD4⁺CD25⁺Foxp3) arise in the thymus and express the IL-2 receptor α -chain (CD25⁺), as well as the transcription factor forkhead box P3 (Foxp3), which enables these cells to inhibit both Th1 and Th2 immune responses (75). The local production of TGF- β is one factor that promotes Foxp3 expression (69). It seems that both TGF- β and IL-10 help maintain homeostasis in the gut immune system (76,77).

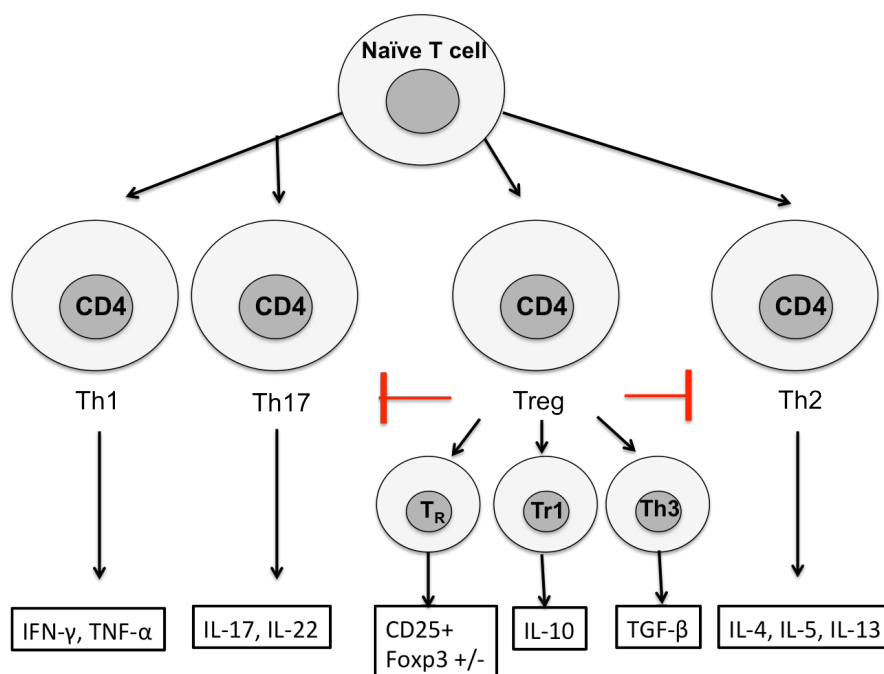


Figure 5: Subsets of CD4⁺ T cells. Naïve T cells can differentiate to CD4⁺ T cell subsets. They can become Th cells (Th1, Th17 and Th2 cells) or Tregs. Depending on their cytokine profile, they may have different effector functions. Th1 cells are associated with the production of pro-inflammatory cytokines such as TNF- α and IFN- γ . Th17 cells, on the other hand, produce IL-17 and IL-22. Th2 cells produce cytokines IL-4, IL-5 and IL-13. Tregs can either be natural or inducible Tregs. Natural Tregs (T_R) express CD25 as well as Foxp3. Inducible Tregs can be further classified as TGF- β -secreting Th3 cells and IL-10-secreting Tr1 cells. Tregs can block Th17 and Th2 responses. This figure is adapted from Brandtzaeg (47).

Inducible Tregs are CD4⁺ cells that acquire their regulatory properties and can differentiate from naïve cells after being exposed to the antigen in the periphery (53). Most acquire Foxp3 expression. There are at least two different forms of inducible Tregs (53): Th3 cells (secrete TGF- β) and Tr1 cells (secrete IL-10). While natural Tregs are more significant for peripheral tolerance to self antigens, inducible Tregs

are crucial for the induction of tolerance to exogenous allergens (53). Consequently, defective Treg activity has been linked to the development of food allergy (78–80). While this has been shown numerous times in mice (78), there have been less human studies conducted. One study looked at children with non-IgE-mediated cow's milk allergy. After a milk-free period, the children were orally challenged and the T cell responses were determined. Compared with children who remained clinically intolerant, children who became tolerant to cow's milk showed an expansion of CD4+CD25+ Tregs (79). In an *in vitro* study, lymphocytes of milk-allergic individuals were cultured in the presence of milk. After being restimulated, the cells showed low levels of TGF- β and IL-10, while displaying a characteristic Th2 response, suggesting a malfunction in inducible Treg activity (80).

1.3.3.2. The Microbiome and Oral Tolerance

Furthermore, the gastrointestinal immune system of the gut is shaped by the intestinal bacterial composition (49). Dietary factors also have an impact on the intestinal immune system and the microbiome (47,52). Hence, the neonatal immune system matures by responding to the colonizing bacteria and ingested dietary compounds (53). To develop a protective immune response the gut must be exposed to colonizing bacteria, which normally begins at birth when the newborn enters the birth canal and comes in contact with vaginal and colonic bacteria of the mother. Therefore, any aberration of the intestinal flora at such an early stage can lead to disturbances of the immune system (81).

The intestinal bacteria produce a Th1 cytokine profile. Furthermore, retarded bacterial colonization may hinder APCs from providing signals to T cells favouring a Th1 response. This leads to a Th2 predominance and as a result allergy. This was shown in children delivered by caesarean section (especially those with a genetic predisposition), who were more prone to develop food allergy (82). Colonization is important for the generation of defense mechanisms including the secretory IgA response and the development of an appropriate Th cell response (81). This has been determined in studies of germ-free animals and their intestinal immune responses (83). In a germ-free state, mucosal IgA is neither secreted nor does oral tolerance

develop (84,85). Thus, inadequate colonization can lead to a Th2 skewing and hypersensitivity reactions.

The ‘hygiene hypothesis’ is one attempt to explain the recent rise in atopic disorders in Western countries. According to the hygiene hypothesis, the reduction in the microbial burden and the resulting imbalances in the gut flora are due to increased hygiene (86). Pattern recognition receptors (present on lymphoid and epithelial cells in the gut), such as TLR-4, are also influenced by the bacterial colonization of the gut, which affects the innate immune response to bacterial molecular patterns and in turn the acquired immune response (55). As the communication between the bacteria and the gut is highlighted, clinicians are attempting to adjust the responses using probiotics to selectively stimulate certain beneficial bacteria (87).

These local immunological factors may affect the induction of oral tolerance, however the breakdown of oral tolerance can be triggered by host factors (e.g. genetic disposition, use of anti-acid medication) as well as food allergen characteristics (e.g. solubility, abundance).

1.3.4. Characteristics of Food Allergens Affecting Food Allergy

Although it seems that almost any food can cause food allergy, the so-called “major” allergens accounting for over 90% of all allergic reactions are milk, egg, peanut, tree nuts, shellfish, fish, wheat and soy; allergy to preservatives and additives are rare (88). Certain features of food allergens may render them more allergenic than others. Characteristics that lead to their allergenicity have been the focus of extensive research efforts to answer the fundamental question: what characteristics make a food protein become an allergen?

It is the protein component of these foods that leads to allergy- specifically small, water-soluble glycoproteins (normally 10 to 70 kDa in size). General characteristics of food proteins that have been described are (89):

- Most of the major food allergens are abundantly present in food

- They must be polyvalent molecules (with at least two or more IgE binding sites) and
- Should be relatively stable to heat, acid and proteases⁶.

Only recently, the impact of carbohydrates or rather the carbohydrate epitope of some glycosylated proteins on allergenicity became the focus of research (90–93). In fact, studies have shown that the high temperature (>180°C) when roasting peanuts seems to increase stability and allergenicity by way of the Maillard reaction, which leads to glycated end products (90,91). Another study was able to show that IgE antibodies directed at a carbohydrate moiety (galactose- α -1,3-galactose) led to clinical symptoms of food allergy in response to red meat (92) and another study showed that the glycosylated form, rather than the deglycosylated form, of the major peanut allergen Ara h 1 acts as a Th2 adjuvant and induces a Th2 response (93).

Additionally, it seems that different structural motifs (sequential or conformational epitopes) are important for IgE induction and the initiation of the disease by sensitization (94–96). Sequential epitopes are amino acid chains along the protein backbone recognized by IgE, while conformational epitopes are amino acid chains brought close together that depend on the tertiary structure (24). One study showed that 70 to 80% of infants and children allergic to milk and eggs can tolerate baked forms of the protein while responding to the raw form (94,95). This tolerance might be due to the fact that the children produce IgE antibodies mainly to the conformational epitopes, which are denatured more than linear epitopes by heat and it seems that these children are more likely to grow out of their food allergy (31).

It seems that food hypersensitivity reactions can also be induced via the respiratory route (cross-reactivity; refer to Section 1.2.) or by allergen exposure to the skin. This was demonstrated in a mouse model by Hsieh et al. (97) where mice were sensitized by epicutaneous administration of OVA, which resulted in high levels of allergen-specific IgE and subsequent oral provocation induced anaphylaxis. It was

⁶ Food proteins in most cases should be resistant to denaturation and can therefore remain intact even after processing, cooking, or digestion (32). However, this is not an absolute requirement because food allergens have been found that are not as resistant, as well as stable non-allergenic food proteins (89).

also observed in children with a peanut allergy, where sensitization occurred after using peanut oil on inflamed skin (98). This study additionally found that peanut allergy was independently associated with the intake of soy protein, which could have arisen from cross-reactivity (98).

1.3.5. Host Factors Affecting Food Allergy

There are various host factors that can additionally contribute to the development of food allergy. As epidemiological studies have shown, the pediatric population shows a higher rate of food allergy compared to the adult population (17,24), suggesting that the maturity of the gastrointestinal tract may play a role. However, another study showed that introducing allergenic foods at an early age might protect against food allergy; the prevalence of peanut allergy for Jewish children in the UK was 10-fold higher than for Jewish infants in Israel who were introduced to peanuts at a much earlier age (99). Two other studies suggested that time of allergen exposure plays a role, but to varying degrees depending on the food (100,101). The one study found that introducing cooked egg at four to six months of age might protect against egg allergy (100); the other study showed that introducing cow's milk within the first two weeks had a protective function, whereas infants introduced to cow's milk between four and six months of age exhibited an increased risk for cow's milk allergy (101). Furthermore, diet and culture also seem to play a role. It is thought that the increase in prevalence of peanut allergy in westernized countries is due to the fact that peanuts are consumed roasted, whereas in China peanuts are mainly fried or boiled (102).

The fact that genes also play a role in food allergy was indicated by a twin study, where monozygotic twins had a much higher concordance rate of peanut allergy than dizygotic twins (103). The genetic influence on food allergy was also demonstrated in a mouse model. The study showed that the induction of food allergy is strain-dependent; C3H/HeJ mice revealed significantly increased allergen-specific IgE and anaphylactic symptoms after sensitization and oral provocation with cow's milk and peanuts compared to BALB/c mice (although BALB/c mice also showed increased peanut-specific IgE) where no hypersensitivity reactions were detected (55).

Another important factor to consider is the disruption of the stomach gate-keeping function, which may be impaired due to changes of the gastric pH levels. Food allergens that are normally degraded are able to resist digestion due to pharmacologically elevated stomach pH. The hindered pepsin activation was found to represent a risk factor for food allergy induction (104–108). Furthermore, gastric digestion was found to reduce the allergenicity of the important allergens such as the egg protein ovalbumin (OVA) (109,110).

1.4. Murine Models and Food Allergy

Animal models, specifically rats and mice, have extensively been used to study both the sensitization and effector mechanisms of food adverse reactions. Animals are sensitized to the antigen with adjuvant systemically resulting in antigen-specific IgE production and allergic responses upon allergen provocation (111). However, only animal models that respond to an oral challenge without the use of immunologically active adjuvants can truly reflect the situation in food allergic humans. This is difficult to establish, as the normal response to oral exposure of antigens in the gut is active immune tolerance. It is the breakdown of oral tolerance though, that seems to play a predominant role in food hypersensitivity reactions (52). Hence, animal models where the normal pathways leading to oral tolerance are altered, by using mucosal adjuvants or by interfering with the epithelial barrier, are being investigated (111). One way of manipulating the epithelial barrier is by impeding the physiological digestion of foods, for example by treating mice with anti-acid medication (such as sucralfate given orally and omeprazole given systemically) (104,105,107,112–115). The neutralization of gastric acidity interferes with the activation of pepsin and promotes sensitization to digestion-labile proteins by allowing them to gain access to the mucosal immune system (107). Sucralfate contains aluminium and has adjuvant properties independent of its effects on gastric acid (116), however the fact that anti-acid medications in general enhance sensitization, supports the idea that conventional digestion processes are important for protecting against hypersensitivity reactions against food compounds (111).

1.4.1. A Mouse Food Allergy Model under Anti-Acid Medication

In a first human study, 152 adult patients who were previously non-allergic and had been treated with anti-acid medication (either proton pump inhibitors [PPIs] or H₂-receptor blockers) for three months were screened. Pre-existing IgE increased in ten percent of the patients and *de novo* IgE against dietary compounds⁷ increased in 15% of patients (117). In another study, three out of five patients who had developed hazelnut-specific IgE during treatment with anti-acid medication showed positive results to oral provocation even 12 months after therapy (112). Hereafter, a murine model of food allergy elicited by gastric acid suppression was developed. In this model, mice were treated three times on three consecutive days with the PPI omeprazole plus sucralfate (for an additional Th2-bias) and at the same time orally immunized with the egg allergen OVA, inducing an IgE-mediated response including positive oral provocation tests (110).

⁷ For example, carrots, celery, potato, orange, milk, apple, wheat and rye flour.

1.5. Aim of Study

The intake of anti-ulcer medication has been previously described as a risk factor for the development of food allergy (104,105,112,114,117). However, as in humans, not all mice developed oral hypersensitivity reactions upon being immunized with OVA accompanied by PPI treatment. The aim of this study was to investigate the heterogeneity of the response in inbred BALB/c mice subjected to identical immunization procedures. The mice were all treated and housed under the same conditions and hence the immunological responses should be comparable. Thus, we wanted to determine the factors contributing to food allergy in this murine model and more specifically, characterize differences in the immune response, histology and intestinal bacterial composition.

2. Materials

2.1. Chemicals

Table 1: Information regarding chemicals used.

Substance	Catalogue Number	Manufacturer
ACK lysing buffer	10-548E	Lonza, Cologne, Germany
Bovine serum albumin (BSA)	A3912	Sigma, Vienna, Austria
Concanavalin A (ConA)	C0412	Sigma, Vienna, Austria
Dry milk powder (DMP)		Maresi, Vienna, Austria
Fetal calf serum (FCS)	10270-106	Gibco Invitrogen, Lofer, Austria
Formaldehyde min. 37%	104003	Merck, Darmstadt, Germany
L-Glutamine	25030-024	Gibco Invitrogen, Lofer, Austria
Magnesium chloride hexahydrate	105833	Merck, Darmstadt, Germany
Ovalbumin (OVA)	A5503	Sigma, Vienna, Austria
Paraformaldehyde	104005	Merck, Darmstadt, Germany
Penicillin/Streptomycin	15140-122	Gibco Invitrogen, Lofer, Austria
Potassium di-hydrogen phosphate	104873	Merck, Darmstadt, Germany
Potassium chloride	1.05001.1000	Merck, Darmstadt, Germany
Protease inhibitor cocktail tablets	04693124001	Roche Diagnostics, Mannheim, Germany
RPMI-1640	31870-025	Gibco Invitrogen, Lofer, Austria
Sodium bicarbonate	106329	Merck, Darmstadt, Germany
Sodium carbonate	106392	Merck, Darmstadt, Germany
Sodium chloride	106404	Merck, Darmstadt, Germany
Sulphuric acid 95-97%	100731	Merck, Darmstadt, Germany
Tris(hydroxymethyl)-aminomethane (Tris)	108382	Merck, Darmstadt, Germany
Tween-20	P1379	Sigma, Vienna, Austria

2.2. Medication

Table 2: List of medications used for anti-acid treatment of mice.

Name	Active Ingredient	Manufacturer
Losec®	Omeprazole	AstraZeneca GmbH, Wedel, Germany
Ulcogant®	Sucralfate	Merck, Darmstadt, Germany

2.3. Kits

Table 3: Content and catalogue information of kits used.

Kit	Catalogue Number	Manufacturer
TMB Substrate Reagent Set	555214	BD OptEIA™, BD Biosciences, Vienna, Austria
Mouse regulatory T cell staining kit	88-8111	eBioscience, Vienna, Austria
Mouse TH1/TH2/TH17/TH22 13plex Kit Flowcytomix	BMS822FF	eBioscience, Vienna, Austria
NucleoSpin Kit for Soil	740780	Macherey-Nagel, Dueren, Germany
QiaQuick PCR Purification Kit	28106	Qiagen, Hilden, Germany
Quant-iT™ PicoGreen dsDNA Quantification Kit	P11496	Invitrogen, Paisley, UK
Mouse MCPT-1 (mMCP-1) Ready-SET-Go!	88-7503-88	eBioscience, Vienna, Austria
Contents		
10x ELISA Coating Buffer	00-0000-53	
Capture Antibody: Purified Anti-Mouse MCP-1 (250x)	14-5503-68	
Standard: Mouse MCPT-1 Recombinant Protein (1 µg/mL)	39-5505-60	
Detection Antibody: Anti-mouse MCP-1 Biotin (250x)	13-5504-68	
Enzyme: Avidin-HRP (250x)	00-4100-94	
5x Assay Diluent	00-4202-55	
Substrate: 1x TMB Solution	00-4201-56	

2.4. Antibodies

Table 4: Antibody catalogue information.

Antibody	Catalogue Number	Manufacturer
<i>Standards</i>		
IgG1, IgG2a, IgA (0.1 mg/mL)	5300-01	Southern Biotech, Birmingham, Alabama, USA
IgE (0.5 mg/mL)	554118	BD Biosciences, Vienna, Austria
<i>Primary/Capture Antibody</i>		
Purified rat anti-mouse IgA (0.5 mg/mL)	Clone (C10-1) #556960	BD Biosciences, Vienna, Austria
Purified rat anti-mouse IgG1 (0.5 mg/mL)	Clone (A85-1) #553440	BD Biosciences, Vienna, Austria
Purified rat anti-mouse IgG2a (0.5 mg/mL)	Clone (A85-1) #553440	BD Biosciences, Vienna, Austria
Purified rat anti-mouse IgE (0.5 mg/mL)	Clone (R35-72) #553413	BD Biosciences, Vienna, Austria
Biotin rat anti-mouse IgA (0.5 mg/mL)	Clone (C10-1) #556978	BD Biosciences, Vienna, Austria
<i>Secondary Antibody</i>		
ECL™ Anti-rat IgG, Horseradish Peroxidase linked whole antibody (from goat)	NA935V	GE Healthcare, Vienna, Austria
Streptavidin-HRP	21126	Pierce, Thermoscientific, Rockford, IL

2.5. Cell Media

Table 5: Cell media composition.

Cell Media	Composition
Pre-lysis media	• 500 mL RPMI-1640 • 1% Penicillin/Streptomycin • 1% L-Glutamine
Full media	• 500 mL RPMI-1640

	• 10% FCS • 1% Penicillin/Streptomycin • 1% L-Glutamine
--	---

2.6. Buffers and Solutions

Table 6: Solutions for buffer preparation.

Buffers	Solutions
	4% Paraformaldehyde
	0.9% w/v Sodium chloride
	1.8 M Sulfuric acid
10x Phosphate buffered saline (PBS)	• 2 g potassium chloride • 2 g potassium di-hydrogen phosphate • 1 g magnesium chloride hexahydrate • 80 g sodium chloride ad 10 L distilled water (pH 7.4)
10x Tris buffered saline (TBS)	• 60.75 g Tris • 87.80 g sodium chloride ad 1 L distilled water (pH 7.4)
10x Tris Saline	• 30.25 g Tris • 87.6 g sodium chloride ad 2 L distilled water (pH 7.2)
Enzyme-linked immunosorbent assay (ELISA) Coating Buffer	• 1.96 g sodium carbonate • 2.64 g sodium bicarbonate ad 500 mL distilled water pH 9.6
1x PBS 0.05% Tween-20 (PBST)	• 200 mL 10x PBS • 1 mL Tween-20 (Sigma) ad 2 L distilled water
1x TBS 0.05% Tween-20 (TBST)	• 200 mL 10x TBS • 1 mL Tween-20 (Sigma) ad 2 L distilled water
Washing Buffer	• 200 mL 10x Tris Saline (pH 7.2) • 1 mL Tween-20 (Sigma) ad 2 L distilled water
Fluorescence activated cell sorting (FACS) Buffer	• 1x PBS • 1% FCS
FACS Fix	• FACS Buffer • 3.75% Formaldehyde

3. Methods

3.1. Animals

Six to eight week-old female BALB/c mice were obtained from Charles River Laboratories, Sulzfeld, Germany. All experiments were performed according to the European Union rules of animal care and with permission from the ethical board of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (permission number GZ BMWF-66.009/0051-11/10b/2008).

3.2. Immunization Protocol

A previously established mouse food allergy model (110) based on oral allergen gavages under concomitant anti-acid treatment was used to induce an IgE-mediated food allergy. In short, 64 mice were kept on an OVA-free diet (Ssniff, Germany) and repeatedly treated with a PPI for three consecutive days, followed by feedings of the model food allergen OVA in combination with sucralfate on days 2 and 3 (Fig. 6).

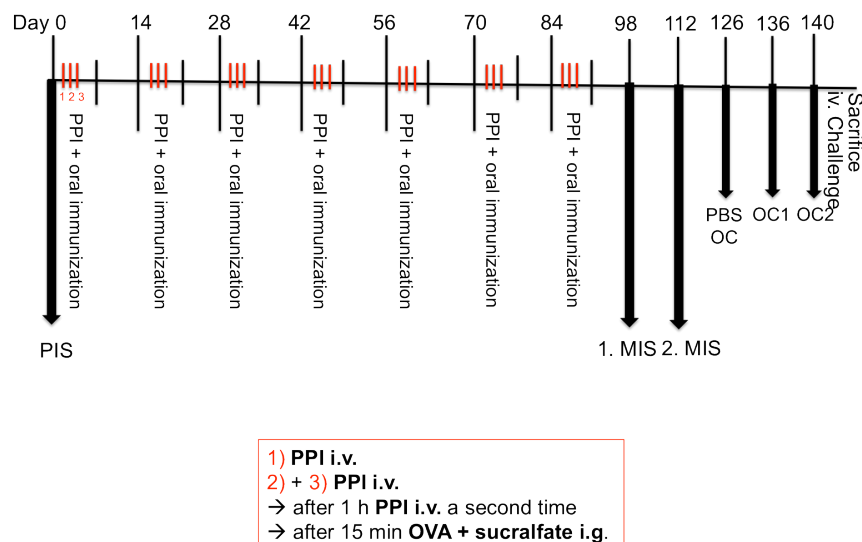


Figure 6: Immunization protocol in a murine model under gastric acid suppression. PIS were taken from tail veins of the mice before immunizations. The mice were then immunized seven times (PPI + oral immunization). They were treated with the PPI omeprazole (i.v. on three consecutive days and i.v. a second time one hour later on days 2 and 3) and then fed OVA with sucralfate on days 2 and 3 during each three-day immunization cycle. MIS was subsequently collected from tail veins for analysis of IgE, IgG1, IgA and IgG2a titers. Mice were orally challenged with PBS (PBS OC) and ten days later with OVA (OC 1). On the day of the sacrifice, mice were again orally challenged (OC 2) and after one hour i.v. challenged with OVA for evaluation of anaphylactic responses.

- One immunization cycle consisted of mice being intravenously (i.v.) administered 100 μ L of the PPI omeprazole (Losec[®], AstraZeneca GmbH, Wedel, Germany; 116 μ g diluted in sterile 0.9 % sodium chloride) on days 1, 2 and 3.
- On days 2 and 3, the first i.v. injection was followed by a second i.v. PPI injection one hour later.
- Fifteen minutes thereafter, the mice were orally gavaged with 0.2 mg OVA (Sigma Aldrich, Vienna, Austria) in sterile PBS in combination with sucralfate (2 mg; Ulcogant[®], Merck, Vienna, Austria).
- Blood samples were taken from the tail vein before immunizations on day 0 (pre-immune sera [PIS]) and two and four weeks after the last immunization cycle (mouse immune sera [MIS]).

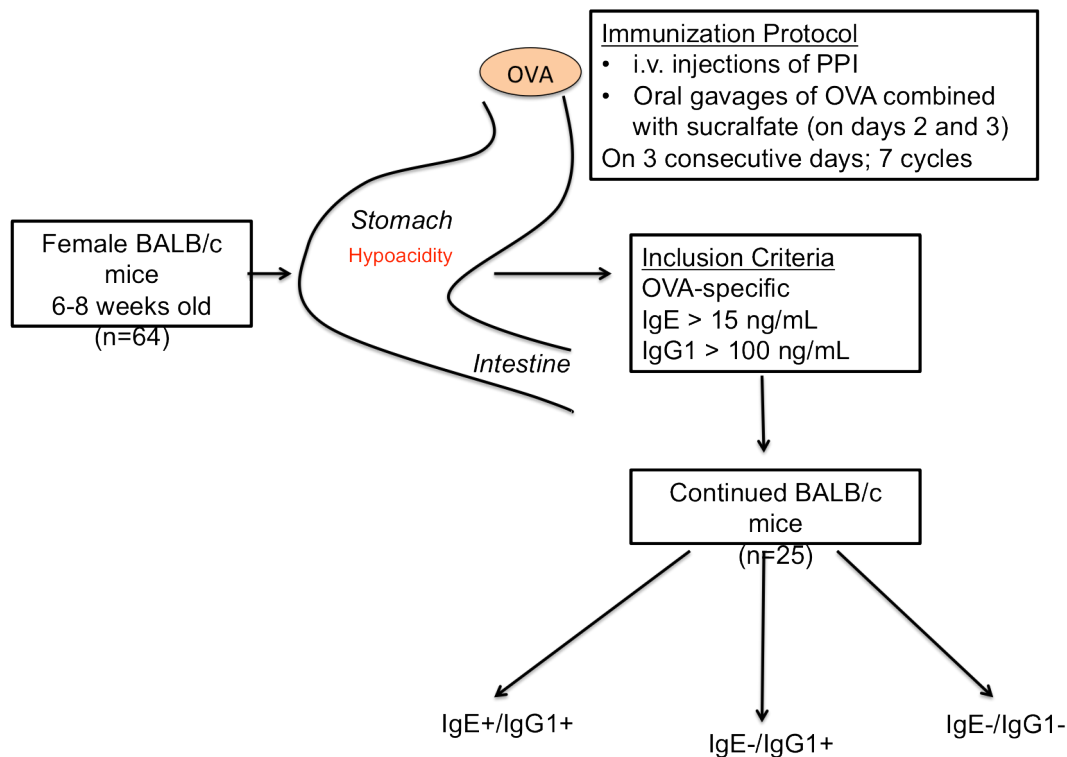


Figure 7: Inclusion criteria and allocation to the three groups of interest. 64 BALB/c mice were subjected to our immunization protocol under concomitant gastric acid suppression. After preliminary ELISAs, 25 mice were allocated to the three groups of interest according to their serological IgE and IgG1 titers and continued.

Mice were allocated to the three groups of interest (IgE-/IgG1-; IgE-/IgG1+; IgE+/IgG1+) after preliminary enzyme-linked immunosorbent assays (ELISA) based on the criteria shown in Fig. 7. Only animals revealing an explicit OVA-specific

immune response (meaning IgE greater than 15 ng/mL, IgG1 greater than 100 ng/mL or at detection limit for negative response) were continued and further investigated.

3.2.1. Immunization Readout

3.2.1.1. ELISA

Murine sera taken before immunizations as well as two and four weeks afterwards were screened for IgE, IgG1, IgA and IgG2a levels.

- The respective purified mouse antibody (refer to Table 4) was serially diluted in ELISA coating buffer to serve as a standard. The antigen of interest OVA (10 µg/mL) was also diluted in ELISA coating buffer. Both were coated (100 µL per well) on a 96-well flat-bottom microtiter plate (Maxisorb®, NUNC, Roskilde, Denmark) to immobilize them to the wells.
- The plate was incubated overnight at 4°C.
- The next day, the plate was washed four times with 200 µL per well TBST.
- Then, 200 µL per well of 1% w/v dry milk powder (DMP; Maresi, Vienna, Austria) in TBST were added to block non-specific binding sites on the surface of the wells and the plate was incubated for two hours at room temperature.
- The plate was then washed again four times with 200 µL per well TBST.
- Murine sera were diluted 1:100 (for IgG1, IgA, IgG2a ELISAs) or 1:20 (for IgE ELISAs) in TBST with 0.1% w/v DMP accordingly.
- 100 µL of the serum dilutions were added to the appropriate wells in duplicates. The plate was then incubated overnight at 4°C.
- On day 3, wells were washed four times with 200 µL per well TBST.
- Then, 100 µL per well of the respective primary antibody (anti-mouse IgE, IgG1, IgA or IgG2a; BD Biosciences, Vienna, Austria) diluted 1:500 in TBST with 0.1% w/v DMP were added and the plate was incubated for two hours at room temperature.
- After repeated washing, 100 µL per well of the secondary antibody peroxidase labelled goat anti-rat IgG (GE Healthcare, Vienna, Austria) diluted 1:1000 in

TBST with 0.1% w/v DMP were added and the plate was incubated for one hour at room temperature.

- For visualization of antibody binding, 100 µL per well 3,3',5,5'-tetramethylbenzidine (TMB; BD OptEIA™, BD Biosciences, Vienna, Austria) were added after repeated washing cycles. After a sufficient color development, the reaction was stopped with 100 µL 1.8 M sulphuric acid per well.

The absorbance of each well was measured using the Infinite® 200 PRO ELISA Reader (Tecan Group Ltd., Männedorf, Switzerland) at 450-630 nm.

3.3. Sampling of Feces

- Stool samples (30 to 50 mg) were freshly collected after the seventh immunization cycle from each individual animal to rule out cross-contamination. Animals were placed in a restrainer and the collected feces were then transferred to sterile Eppendorf tubes and immediately snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis.
- Samples of litter and food were also collected and analysed for bacterial contamination.

3.4. Analysis of Feces

Feces analysis was performed as a cooperation at the laboratory of the Department of Microbe-Plant Interactions, Helmholtz Centre, Munich by Mag. Barbara Pfitzner, Dr. Michael Rothballer and Dr. Michael Schmid. Bacterial composition of stool samples was investigated as described in Diesner et al. (118) as follows:

“Approximately 35 mg of cecal luminal contents of group IgE-/IgG1+, group IgE+/IgG1+ and group IgE-/IgG1- mice were used for microbiome analyses. Total bacterial genomic DNA was extracted using NucleoSpin Kit for Soil (Macherey-Nagel, Dueren, Germany) following manufacturer’s instructions. Amplification of the V6–V9 region of 16S rRNA gene was performed with primer 926F (5’-

AAACTYAAAKGAATTGACGG -3') (119) and 630R (5'-CAKAAAGGAGGTGATCC -3') (120) with the attached Roche 454 sequencing adaptors. For multiplexing purposes the forward primer included a 10-nt barcode sequence. Three independent PCRs were performed for each sample with Fast Start High Fidelity PCR System (Roche, Mannheim, Germany) containing 20 ng of template DNA with an optimal annealing temperature of 50°C and 22 cycles. PCR reactions were pooled together and purified using QiaQuick PCR Purification Kit (Qiagen, Hilden, Germany). After quantification using a Quant-iT™ PicoGreen dsDNA quantification kit (Invitrogen, Paisley, UK) samples were equally pooled. The sequencing of this amplicon library was performed on a Roche 454 GS FLX Pyrosequencer (Roche, Mannheim, Germany) using Titanium chemistry. Amplicons were sequenced unidirectionally as recommended in the manufacturer's instruction for amplicon Lib-L libraries. Sequences were processed and data were analysed with the software mothur v.1.29.0. (121). For quality filtering reads were denoised and barcodes, primers and sequences including more than 8 homopolymers were removed and sequences trimmed to a minimal sequence length of 200bp. For taxonomic analysis sequences were aligned against Silva SEED alignment database, chimeras were removed using uchime implementation (122) in mothur and taxonomic assignment was done using RDP trainset with a cutoff of 80% (123). To compare equal numbers of sequences of each fecal sample subsamples with 10429 sequences were generated. Sequences with similarity > 97% were combined to one Operational Taxonomic Unit (OTU). Prior to the statistical analysis all OTUs with less than 0.01% of the total abundance were excluded from the analysis."

3.5. Oral Provocation

After the last immunization cycle, mice were subjected to an oral PBS challenge and then to an oral OVA challenge.

- Mice were fasted overnight before each challenge.
- On day 126, mice were orally challenged with sterile PBS (100 µL/mouse) as a negative control (PBS OC).

- Blood samples were taken from the tail vein one hour after the oral PBS challenge for mouse mast cell protease-1 (mMCP-1) measurements.
- Five days thereafter, mice were orally challenged with 50 mg OVA dissolved in sterile PBS (OC 1).
- The rectal temperature of animals was measured to verify an anaphylactic response, using a thermometer (Thermalert TH5, Clifton, NJ), 20 and 40 minutes after both the PBS and OVA oral provocation.
- Blood samples were again taken from the tail vein, one hour after OC 1 for mMCP-1 measurements (see Fig. 8).

3.5.1. Anaphylaxis Readout

Rectal temperature decreases and mMCP-1 level readouts were used to evaluate the anaphylactic reaction. Murine sera from after the PBS challenge and OC 1 were screened for mast cell degranulation marker mMCP-1 using the READY-SET-GO! Mouse MCPT-1 kit (eBioscience, Vienna, Austria) by following the manufacturer's protocol. In short:

- A Corning Costar 9018 ELISA plate (included in the kit) was coated with 100 μ L per well of the capture antibody (anti-mouse MCP-1) in 1:250 in 1x coating buffer.
- The plate was sealed with Parafilm and incubated overnight at 4°C.
- After multiple wash steps using 250 μ L PBST, 100 μ L per well of the standard dilution series starting at 15,000 pg/mL and of the samples (diluted 1:30 in 1x assay diluent) were added to the appropriate wells.
- The plate was then sealed and incubated overnight at 4°C.
- After another five wash steps with PBST, bound antibodies were detected using 100 μ L per well of detection antibody (anti-mouse MCP-1 biotin) diluted 1:250 in 1x assay diluent and the plate was incubated at room temperature for one hour.
- The plate was then once again washed five times with 250 μ L PBST.
- Next, 100 μ L per well of the enzyme (Avidin-HRP) diluted 1:250 in 1x assay diluent were added and the plate was incubated at room temperature for 30 minutes.

- The plate was then washed seven times with 250 μ L PBST, leaving one to two minutes between each wash step.
- Finally, 100 μ L per well of TMB solution (also included in kit) were added and the plate was incubated for 15 minutes. Then 50 μ L of 1.8 M sulphuric acid were added to each well.

The plate was then read at 450-570 nm using the Infinite® 200 PRO ELISA reader (Tecan Group Ltd., Männedorf, Switzerland).

3.6. Sacrifice

Mice were sacrificed on day 140 (refer to Fig. 6).

- On the day of the sacrifice and five days after OC 1, mice were again orally challenged with 50 mg OVA in sterile PBS (OC 2) to induce a local anaphylactic response and the rectal temperature was taken (refer to Fig. 8).
- After one hour, mice were challenged i.v. with 50 μ g OVA in 50 μ L sterile 0.9% sodium chloride to induce a severe systemic anaphylactic response.
- Rectal temperature measurements were again performed 5, 10 and 15 minutes after challenge.
- Mice were killed 15 minutes thereafter with CO₂.

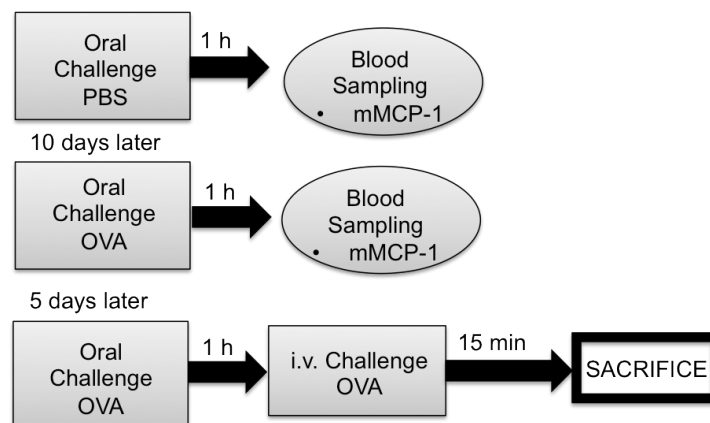


Figure 8: Oral provocation and sacrifice. Mice were first orally challenged with PBS as a negative control. On day 136, mice were orally challenged with OVA. The rectal temperature was taken and blood was collected by the tail vein after each oral provocation for evaluation of the anaphylactic responses. On the day of the sacrifice, mice were orally and i.v. challenged. After the sacrifice, blood was collected by cardiac puncture for differential blood cell count analysis.

3.6.1. Heart

3.6.1.1. *Blood collection by cardiac puncture*

- The dead animals were laid on their backs.
- A 1 mL syringe and a 27Gx3/4 needle (BD Biosciences, Vienna, Austria) were used.
- The syringe was pushed vertically through the sternum.
- Once blood appeared in the syringe, the plunger was pulled back to obtain the maximum amount of blood available.
- The blood was collected in EDTA tubes (Sarstedt, Wiener Neudorf, Austria).
- Tubes were inverted several times and immediately put on ice for subsequent differential blood cell count analysis.

The differential blood cell count analysis was performed at the Department of Biomedical Research at the Medical University of Vienna on an Advia® 2120i Hematology System (Siemens, Eschborn, Germany).

3.6.2. Spleen

3.6.2.1. *Spleen cell preparation*

- Spleens of mice were gently removed using sterile surgical equipment and were immediately transferred to a sterile petri dish containing approximately 5 mL pre-lysis medium
- Spleens were then gently minced between two frosted glass slides (the glass slides were first rubbed in 0.9% sodium chloride), filtered over a 40 µm nylon cell strainer (BD Biosciences, Vienna, Austria) into a sterile Falcon tube and diluted with pre-lysis medium to a total volume of 15 mL.
- Cells were incubated on ice until the next step.
- Thereafter, Falcon tubes were centrifuged for six minutes at 1,600 rpm and supernatants were discarded.
- The pellets were then resuspended in 5 mL ACK lysing buffer (Lonza, Cologne, Germany) and incubated for exactly five minutes at room temperature for lysis of erythrocytes.

- The lysis process was stopped with 5 mL full medium.
- The cell suspension was subsequently washed by repeated centrifugation and resuspended in 5 mL full medium.
- Finally, the pellets were resuspended in 5 mL full medium, filtered over a sterile nylon mesh and left on ice until further processing.

3.6.2.2. Spleen cell stimulation

- After cell counting using a Coulter Counter (Beckman Coulter Z2, Vienna, Austria), cells were seeded in 96-well round-bottom cell culture plates (Corning Costar, New York, USA), 100 μ L cell suspension per well, for a total concentration of 5×10^5 cells per well (quadruplets).
- In a next step, 100 μ L of the stimulation substance OVA (Sigma, Vienna, Austria; 2 μ g/mL) diluted in full medium were added.
- 100 μ L per well Concanavalin A (ConA; Sigma, Vienna, Austria; 5 μ g/mL), diluted in full medium were added to cells as a positive control.
- 100 μ L full medium were added to cells as a negative control.
- Plates were then incubated for 72 hours at 37°C at a 5% CO₂ concentration.
- After 72 hours, 200 μ L supernatant per well were transferred to a new well on a 96-well round-bottom cell cluster plates (Corning Costar, New York, USA) using a multi-pipette.
- The supernatant was then stored at -20°C for later use.

3.6.2.3. Regulatory T Cell and Cytokine Evaluation using Flow Cytometry

Spleen cells were stained for CD4⁺ T cells and CD4⁺CD25⁺Foxp3⁺ Tregs with the mouse Treg staining kit (eBioscience, Vienna, Austria, #88-8111), according to manufacturer's protocol. In short:

- 100 μ L of the prepared cells (approximately 4×10^5) were added to each tube.
- The surface molecules were stained with 0.0625 μ g/test anti-mouse CD4 FITC and 0.03 μ g/test anti-mouse CD25 APC antibodies in fluorescence activated cell sorting (FACS) Buffer (1x PBS with 1% FCS) as seen in Table 7.

Therefore, a stock was prepared by combining 3.625 μ L anti-mouse CD4 FITC antibody with 4.35 μ L anti-mouse CD25 APC antibody in 1442 μ L FACS Buffer).

Table 7: Antibody concentrations for T cell count and cytokine production analysis using flow cytometry.

Antibody	Concentration (μg/test)	Isotype Control	Concentration (μg/test)
anti-mouse CD4 FITC	0.0625	Rat IgG2a FITC	0.0625
anti-mouse CD25 APC	0.03	Rat IgG1 APC	0.03
anti-mouse/rat Foxp3 PE	0.25	Rat IgG2a PE	0.25

- 50 μ L of the stock solution were added to the tubes, which were then incubated for 30 minutes at 4°C.
- The cells were then washed twice with 2 mL FACS Buffer. After pelleting the cells by centrifugation, the supernatant was discarded.
- Thereafter, 500 μ L of freshly prepared Fixation/Permeabilization working solution (3.625 mL Fixation/Permeabilization Concentrate with 10.8 mL Fixation/Permeabilization Diluent) were added to each sample. After pulse vortexing the samples, they were incubated at 4°C for 30 minutes.
- The cells were then washed twice with 2 mL Permeabilization Buffer (prepared from concentrate by 10x dilution with distilled water). The cells were centrifuged to pellet the cells and the supernatant was discarded.
- A stock solution of 0.25 μ g/test anti-mouse/rat Foxp3 PE antibody (refer to Table 7) or the isotype control to each sample in 1x Permeabilization Buffer (36.25 μ L anti-mouse/rat Foxp3 PE with 1413 μ L Permeabilization Buffer) was prepared. 50 μ L of the stock solution were added to each sample and the tubes were then incubated at 4°C for 30 minutes in the dark.
- The cells were then washed again twice with 1 mL Permeabilization Buffer. After centrifuging to pellet the cells, the supernatant was discarded.
- Finally, the cells were resuspended in 250 μ L FACS Fix (FACS Buffer + 3.75% formaldehyde).

Samples were analysed on a FACS Calibur machine (BD Biosciences, Vienna, Austria). Absolute numbers of CD4⁺ T cells and CD4⁺CD25⁺Foxp3⁺ T cells were calculated per spleen.

Spleen cell supernatants were screened for cytokine production using the mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit (eBiosciences, Vienna, Austria, #BMS822FF).

Preparation of Standard Mixture

- To start, the standard mixture was prepared. First, the standard vials were spun down for a few seconds to collect the lyophilized standard at the bottom.
- Then, the standards were reconstituted by adding distilled water according to the label on the vial.
- The vial was swirled thoroughly and after approximately 10 minutes, 10 µL of each reconstituted standard were added to a tube labeled Standard 1. Finally, 70 µL of 1x Assay Buffer (prepared from concentrate by 10x dilution with distilled water) were added for a final volume of 200 µL (1:20 dilution of each reconstituted standard).
- 100 µL 1x Assay Buffer were then added to six tubes labeled Standard 2 to 7. Then, 50 µL of Standard 1 were added to Standard 2, the contents were mixed, 50 µL of Standard 2 were added to Standard 3 and so on, for a total of seven standard dilutions.

Preparation of Bead Mixture

- Next, the bead mixture was prepared. Individual bead vials were vortexed and 1/20 of the final volume of each bead set was added to a centrifuge tube labeled “Bead Mixture” (110 µL per bead set). 1x Assay Buffer (1163 µL) was added to fill up to the final volume of 2593 µL.
- The tube was then centrifuged at 3000 g for five minutes.

Preparation of Biotin-Conjugate Mixture

- The Biotin-Conjugate Mixture was prepared by pipetting 1/20 of the final volume of each Biotin-Conjugate to a tube labeled “Biotin-Conjugate

Mixture” (259.4 μL per bead set). 1x Assay Buffer (1815.6 μL) was added to fill up to the final volume of 5187 μL .

Tubes

1. 25 μL of the Standard Mixture 1 to 7 were added to the designated tubes.
 2. 25 μL of the Standard Mixture 1 were added to an additional tube for cytometer setup.
 3. 25 μL 1x Assay Buffer were added to the blank tubes.
 4. 25 μL of the samples (approximately 4×10^5 of the prepared murine spleen cells) were added to the designated tubes.
- 25 μL Bead Mixture were added to all the tubes.
 - 50 μL of Biotin-Conjugate Mixture were then added to all the tubes.
 - The contents were then mixed and the tubes incubated for two hours at room temperature.
 - Next, 1 mL 1x Assay Buffer was added and the tubes were washed twice by centrifugation (five minutes at 200 g). The supernatant was discarded carefully, leaving 100 μL liquid in each tube.
 - 50 μL of Streptavidin-PE Solution (166 μL concentrated Streptavidin-PE in 5021.5 μL) were then added and the tubes incubated for one hour at room temperature.
 - The tubes were then washed twice (as above), again leaving 100 μL liquid in each tube after discarding the supernatant.
 - Finally, 400 μL 1x Assay Buffer were added.

Acquisition was done on a FACS Calibur machine (BD Biosciences, Vienna, Austria) and samples were analysed using the eBioscience FlowCytomix Pro Software.

3.6.3. Stomach and Small Intestine

3.6.3.1. Intragastric pH measurement

- Directly after sacrifice, the stomach was isolated and the cardia and pylorus were clamped using sterile surgical equipment.

- The stomach was immediately perfused with 150 μ L sterile 0.9% sodium chloride. The pH of the washing fluid diluted in 1.3 mL distilled water was then measured using a pH electrode (Greisinger Electronic GmbH, Regenstauf, Germany).
- 150 μ L sterile 0.9% sodium chloride diluted in distilled water were used as a control.

3.6.3.2. *Gastrointestinal lavages*

- Intestinal contents were lavaged with 2 mL cold PBS and collected using a syringe in Eppendorf tubes containing 2 mL of a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany; 1 protease inhibitor cocktail tablet in 10 mL sterile PBS).
- The samples were incubated on a shaker for 30 minutes at 4°C and then centrifuged at 16,000 g for 10 minutes.
- The clear supernatant was then stored at -20°C for mucosal IgA analysis.

3.6.3.3. *Evaluation of antibody production in gastrointestinal lavages*

3.6.3.3.1. OVA-specific IgA ELISA

- First, a serial dilution (in ELISA coating buffer) of the purified mouse IgA antibody (refer to Table 4) served as a standard. The antigen of interest OVA (10 μ g/mL) was also diluted in ELISA coating buffer. 100 μ L per well of both were coated on a 96-well flat-bottom microtiter plate (Maxisorb®, NUNC, Roskilde, Denmark) to immobilize them to the wells.
- The plate was incubated overnight at 4°C.
- On day 2, the plate was washed four times with 200 μ L per well washing buffer.
- Then, 200 μ L per well of 1% w/v bovine serum albumin (BSA; Sigma, Vienna, Austria) in washing buffer were added to block non-specific binding sites on the surface of the wells and the plate was incubated for two hours at room temperature.

- The plate was then washed again four times with 200 μ L per well washing buffer.
- 100 μ L of the undiluted gastrointestinal lavages were then added and the plate was incubated overnight at 4°C.
- On day 3, the plate was washed four times with 200 μ L per well washing buffer.
- Then, 100 μ L per well of the biotin rat anti-mouse IgA antibody (BD Biosciences, Vienna, Austria) diluted 1:250 in 0.1% w/v BSA in washing buffer were added and the plate was incubated for 30 minutes at room temperature.
- After repeated washing, 100 μ L per well of the secondary antibody streptavidin-HRP (Pierce, Rockford, IL) diluted 1:5000 in 0.1% w/v BSA in washing buffer were added and the plate was incubated again for 30 minutes at room temperature.
- For visualization of antibody binding, 100 μ L per well TMB (BD OptEIA™, BD Bioscience, Vienna, Austria) were added after repeated washing cycles.
- After a sufficient color development, the reaction was stopped with 100 μ L 1.8 M sulphuric acid per well.

The absorbance of each well was measured using the Infinite® 200 PRO ELISA Reader (Tecan Group Ltd., Männedorf, Switzerland) at 450-630 nm.

3.6.3.3.2. Total IgA ELISA

- A 96-well flat-bottom microtiter plate (Maxisorb®, NUNC, Roskilde, Denmark) was coated with (50 μ L/well) rat anti-mouse IgA (BD Biosciences, Vienna, Austria) using 1x Tris saline for dilution.
- The plate was incubated overnight at 4°C.
- The next day, the plate was washed four times with 200 μ L per well washing buffer.
- Then, 200 μ L per well of 1% w/v BSA (Sigma, Vienna, Austria) in washing buffer were added to block non-specific binding sites on the surface of the wells and the plate was incubated for two hours at room temperature.

- The plate was then washed again four times with 200 μ L per well washing buffer.
- Serial dilution (in 0.1% w/v BSA in washing buffer) of the purified mouse antibody (refer to Table 4) served as a standard. The gastrointestinal lavages of the mice were diluted 1:1000 in 0.1% w/v BSA in washing buffer.
- 100 μ L of the samples and standard dilutions were added to the appropriate wells in duplicates. The plate was then incubated for 30 minutes at room temperature.
- Next, the plate was washed four times with 200 μ L per well washing buffer.
- Then, 100 μ L per well of the biotin rat anti-mouse antibody (BD Biosciences, Vienna, Austria) diluted 1:250 in 0.1% w/v BSA in washing buffer were added and the plate incubated for 30 minutes at room temperature.
- After repeated washing, 100 μ L per well of the secondary antibody streptavidin-HRP (Pierce, Rockford, IL) diluted 1:5000 in 0.1% w/v BSA in washing buffer were added and the plate incubated again for 30 minutes at room temperature.
- For visualization of antibody binding, 100 μ L per well TMB (BD OptEIA™, BD Bioscience, Vienna, Austria) were added after repeated washing cycles.
- After a sufficient color development, the reaction was stopped with 100 μ L 1.8 M sulphuric acid per well.

The absorbance of each well was measured using the Infinite® 200 PRO ELISA Reader (Tecan Group Ltd., Männedorf, Switzerland) at 450-630 nm.

3.6.3.4. Histology

The “swiss roll” (124), a technique for the preparation of the murine intestine where the mucosa remains on the outside, was used for histological studies.

- First, the intestine was removed using sterile surgical equipment and arranged to be divided, with the duodenum on the left, then the jejunum (center), and the ileum on the right.
- The divided intestine was then be rearranged in such a way, that all caudal ends were nearest to the investigator.

- Starting from the caudal end, each segment was slit open and the feces removed with a wooden stick.
- The wooden stick is then placed under the slit and used to roll up the intestinal segments.

The ‘swiss roll’ of the intestine and the stomach were placed in 4% paraformaldehyde overnight and subsequently in sterile 1x PBS. The stomach was cut open longitudinally. By diagonally cutting the intestine using a random start and then cutting every two centimeters, transverse, uniform systemic random samples were attained, which were then embedded into paraffin. 3-4 μ m thick sections were stained with Periodic acid-Schiff reagent to detect epithelial goblet cells, chloracetate-esterase to reveal mucosal myeloid cells and haematoxylin/eosin to identify inflammatory infiltrates (106).

3.7. Statistics

Data was statistically evaluated by Tukey’s multiple comparison test and one-way ANOVA. Differential blood cell counts were compared with the Bonferroni post-test as well as two-fold ANOVA. $P < 0.05$ was regarded as statistically significant. All statistical analysis was done using GraphPad Prism 5 software.

The microbiome data was statistically evaluated as a cooperation at the laboratory of the Department of Microbe-Plant Interactions, Helmholtz Centre, Munich by Mag. Barbara Pfitzner, David Endesfelder, PhD, Dr. Michael Rothballer, Dr. Gerhard Welzl, Dr. Michael Schmid and Prof. Dr. Thomas Rattei as described in Diesner et al. (118) as follows:

“Statistical analysis of microbiome data was performed using R platform (R version 2.15.1; <http://www.r-project.org>) with the packages vegan (125) and ade4 (126) and custom R scripts. Hellinger transformed OTU abundances were used to compare community patterns between the three groups by Principal Component Analysis (PCA). In addition, a multivariate analysis of variance (npMANOVA), based on Bray-Curtis distance on relative OTU abundances was performed. Differences on OTU level between all three groups were analysed using ANOVA

and the resulting p-values were adjusted for multiple testing with the Benjamini-Hochberg method (127). Wilcoxon-Mann Whitney tests with Benjamini-Hochberg correction for multiple testing was performed on all taxonomic levels to compare all pairwise combinations of IgE+/IgG1+, IgE-/IgG1+ and IgE-/IgG1 as well as IgE responders (IgE+/IgG1-) to non-responders (IgE-/IgG1+ and IgE-/IgG1-) and IgG responders (IgE+/IgG1+ and IgE-/IgG1+) to non-responders (IgE-/IgG1-). Differences with an adjusted $p < 0.05$ were considered to be statistically different. The means of sequence abundances of the two sub strains were expressed in percentage.”

4. Results

4.1. Analysis of serum antibody titers in IgE responders versus non-responders

Sixty-four female BALB/c mice were subjected to the previously established oral immunization protocol, which is based on repeated feedings of OVA under gastric acid-suppression (110). Mouse sera taken after the last immunization were tested for OVA-specific IgE (Fig. 9A), IgG1 (Fig. 9B), IgG2a (Fig. 9C) and IgA (Fig. 9D). Ten mice revealed high OVA-specific IgE and IgG1 levels (IgE+/IgG1+; IgE responders), five were only IgG1 positive (IgE-/IgG1+) and ten animals did not show elevated antibody levels (IgE-/IgG1-; IgE non-responders).

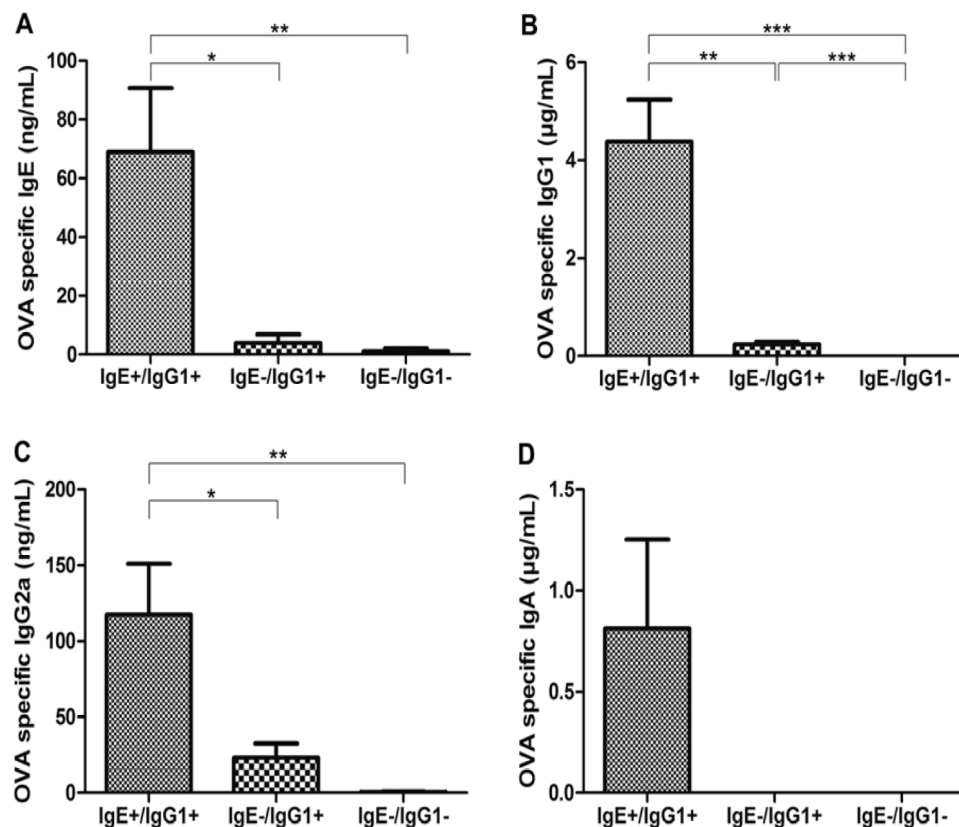


Figure 9: OVA-specific antibody titers of mice subjected to the oral immunization protocol under gastric acid suppression. Ten mice showed high OVA-specific IgE (A) and IgG1 (B) levels and were rendered IgE responders (IgE+/IgG1+). Five were only IgG1 positive (IgE-/IgG1+). Ten more were neither IgE nor IgG1 positive and classified as non-responders (IgE-/IgG1-). OVA-specific IgG2a (C) and IgA (D) were also increased in the IgE responder group. ***P<0.001, **P<0.01, *P<0.05.

Only the animals who revealed a clear-cut OVA-specific immune response based on the following criteria: IgE greater than 15 ng/mL, IgG1 greater than 100 ng/mL or antibody levels at detection limit for a negative response, were included in further investigations. IgE responders also showed significantly higher levels of OVA-specific IgG2a, as well as substantially higher OVA-specific IgA titers.

4.2. Readout of anaphylactic symptoms

Mice were i.g. challenged twice (OC 1 and OC 2) to trigger a local inflammation and additionally i.v. to induce a strong systemic response for evaluation of anaphylactic shock symptoms. After a single OC, the mast cell degranulation and anaphylaxis marker mMCP-1 (Fig. 10A) was significantly elevated only in the IgE responders (IgE+/IgG1+).

To further assess anaphylactic symptoms, which cause blood centralization and therefore a reduction of body temperature, the rectal temperature of immunized animals was measured before and 5, 10 and 15 minutes after i.v. allergen provocation (Fig. 10B). Anaphylactic responses were confirmed in the IgE responders by a drop in rectal body temperature (defined as a drop of at least 0.5°C).

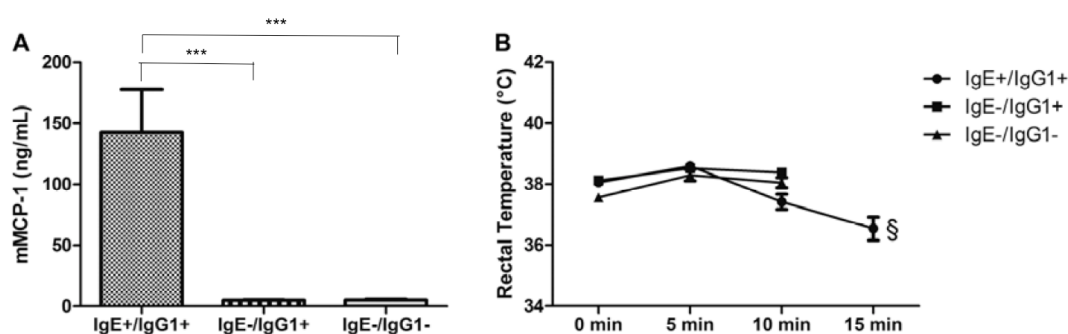


Figure 10: Evaluation of anaphylactic symptoms. Mouse mast cell protease-1 (mMCP-1) levels (A), measured after the first oral challenge (OC1), were increased significantly in the IgE responder group. This was accompanied by a drop in rectal body temperature (B) measured before and 5, 10 and 15 minutes after intravenously (i.v.) challenging mice with OVA. The IgE+/IgG1+ group is represented by dots, the IgE-/IgG1+ group by squares and the IgE-/IgG1- by triangles. ***P<0.001, §P<0.001.

4.3. Measurement of stomach pH levels of mice after treatment with anti-acid medication

The gastric pH levels of mice were analysed one hour after i.v. injection with a PPI (Fig. 11). In line with previous measurements (110), all animals showed an elevated gastric pH after being treated with anti-acid medication. However, there were slight but statistically not significant differences in pH levels between the three animal groups (IgE+/IgG1+, IgE-/IgG1+ or IgE-/IgG1-).

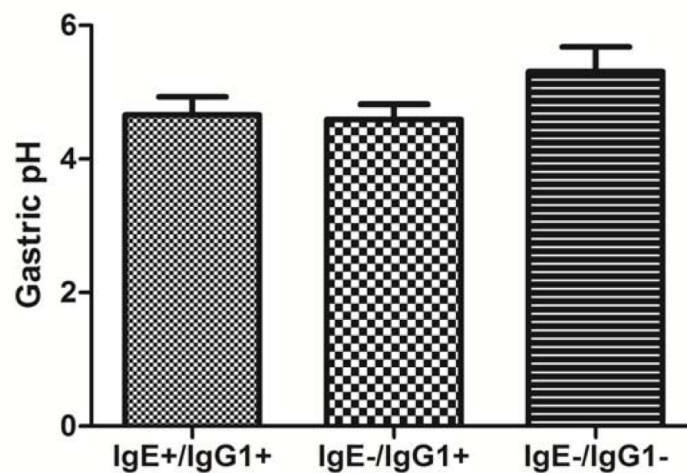


Figure 11: PH levels of the stomach in mice one hour after i.v. injection with a PPI. No significant differences were measured between the three groups.

4.4. Evaluation of the IgA levels in intestinal lavages

Total and allergen-specific IgA levels (Fig. 12A and B respectively) were evaluated in the intestinal lavages. A significantly higher level of total IgA was found in the IgE responders. Additionally, elevated levels of OVA-specific IgA were detected in both the IgE+/IgG1+ and IgE-/IgG1+ groups compared to the negative responders.

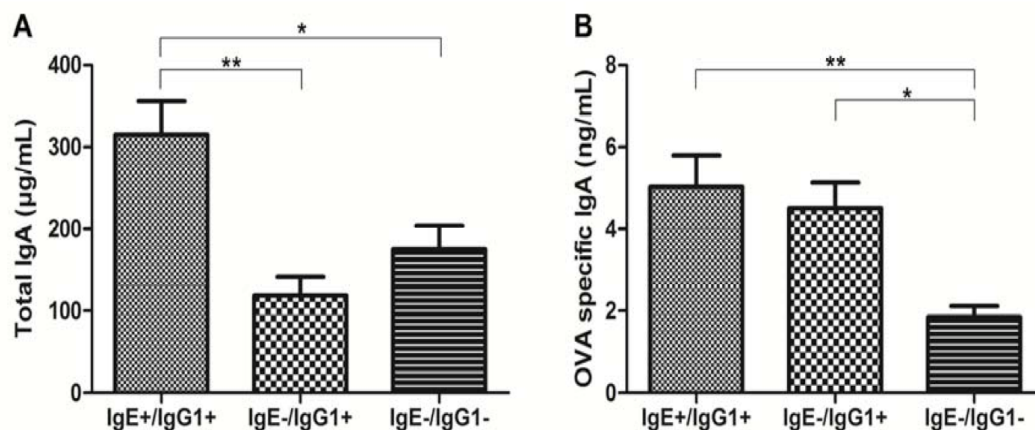


Figure 12: Total IgA (A) and OVA-specific IgA (B) titers in the intestinal lavages. IgE responders showed significantly increased levels of total IgA. Both the IgE responders and the IgG1-positive group showed significantly increased levels of OVA-specific IgA. **P<0.01, *<0.05.

4.5. Cytokine profile and T cell counts of stimulated spleen cell supernatant

The spleen cells of the immunized mice were stimulated with the model food allergen OVA or full medium as a control for 72 hours.

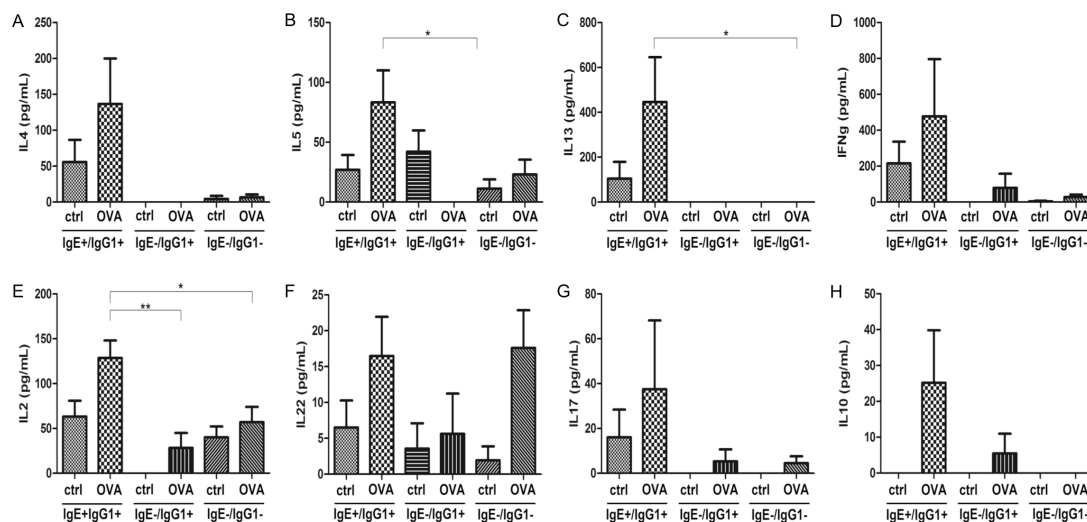


Figure 13: Cytokine production of the three groups of interest (IgE+/IgG+; IgE-/IgG+, IgE-/IgG-). IL-4 (A), IL-5 (B), IL-13 (C), IFN-γ (D), IL-2 (E), IL-22 (F), IL-17 (G) and IL-10 (H) levels were measured using the mouse Th1/Th2/Th17/Th22 13plex Flowcytomix kit by stimulating spleen cells of mice with OVA and full medium as a control (ctrl) for 72 hours. Th2 cytokine production (IL-4, IL-5, IL-13) was increased in the IgE responder group. IL-2 and IL-17 were also elevated in this group. IL-22 was additionally higher in the IgE non-responder group. **P<0.01, *<0.05.

Using the mouse Th1/Th2/Th17/Th22 13plex Flowcytomix kit, the supernatant of the stimulated spleen cells was screened for cytokine production (Fig. 13). It has previously been demonstrated that antigen feedings under gastric acid-suppression induces Th2 cytokine production (110). Also here allergic mice revealed substantially elevated levels of IL-4, and even significantly elevated levels of IL-5 and IL-13, all Th2 markers, compared to the IgE/IgG1 negative group. IFN- γ , a Th1 marker, as well as the T cell regulatory cytokine IL-10, were also elevated in the IgE+/IgG1+ group. Interestingly, the allergic animals revealed higher IL-2 and IL-17 levels compared to the other groups. However, IL-22 was additionally increased in the IgE non-responder group.

To rule out differences in T cell counts as the cause for the heterogenic immune response, spleen cells were also analysed for CD4+ and CD4+CD25+Foxp3+ cells in flow cytometry by staining. No substantial differences for CD4+ T cells (Fig. 14A) or CD4+CD25+Foxp3+ Tregs (Fig. 14B) could be observed between the three groups.

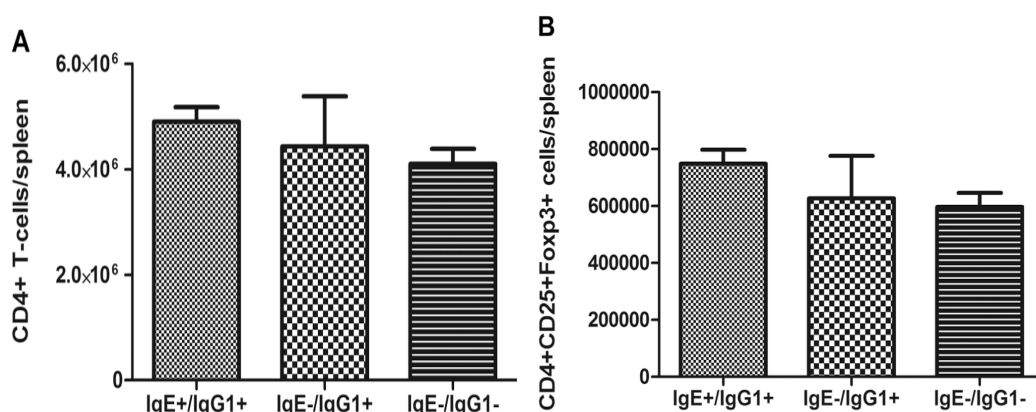


Figure 14: T cell count determined in spleens by flow cytometry. No significant differences between CD4+ T cell (A) and CD4+CD25+Foxp3+ Treg (B) counts were detected between the three groups of interest.

4.6. Differential blood cell count analysis during anaphylaxis

Murine blood was taken for differential blood cell count before provocation and 15 minutes after the i.v. challenge to evaluate the recruitment and significance of allergy effector cells during the anaphylactic response in our model (Fig. 15). The IgE

responders revealed significantly decreased numbers of lymphocytes (Fig. 15A) but significantly elevated levels of red blood cells (Fig. 15D) and monocytes (Fig. 15B) during anaphylaxis compared to the non-responders. Furthermore, the hematocrit (Fig. E) was significantly increased in the IgE responders. In comparison, the red blood cells and hematocrit were also significantly decreased in the IgE-/IgG1+ group compared to the IgE responders. Interestingly, neutrophils (Fig. 15C) and platelets (Fig. 15F) were lower in the IgE-/IgG1+ group, the latter even significantly, than in the other groups.

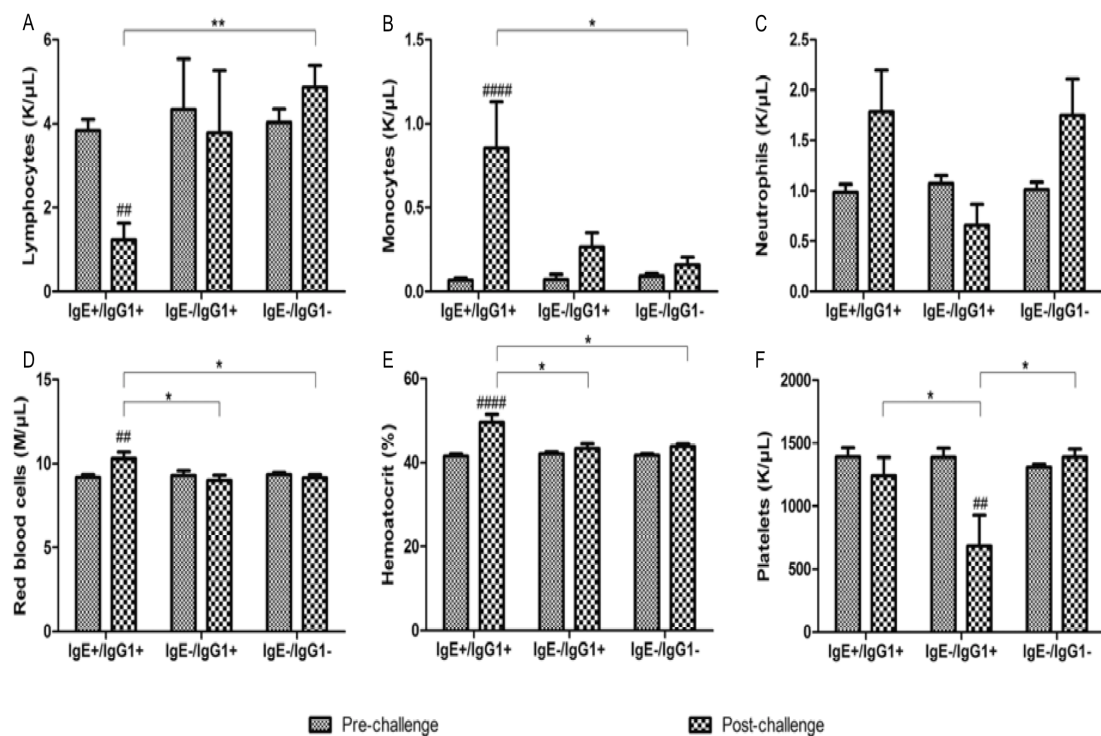


Figure 15: Differential blood cell count analysis during anaphylaxis. Levels of lymphocytes (A), monocytes (B), neutrophils (C), red blood cells (D), hematocrit (E) and platelets (F) were determined in blood samples taken before provocation (pre-challenge) and 15 minutes after i.v. challenging mice (post-challenge). Lymphocytes were decreased while red blood cells, hematocrit and monocytes were increased in the IgE responders. Neutrophils and platelets were decreased in the IgE-/IgG1+ group. The cells before and after the OVA challenge were determined using the two-way ANOVA and Bonferroni post-test; ##### $P < 0.0001$, ## < 0.01 . Differences within the groups were determined by one-way ANOVA and Tukey's multiple comparison test; ** $P < 0.01$, * < 0.05 .

4.7. Histological evaluation of the gastrointestinal samples

For histological evaluations, sections of the stomach and the intestine were stained for goblet cells, myeloid cells in the mucosa and inflammatory infiltrates. However,

distinct differences were not observed in the gastrointestinal morphology between the IgE responders, IgG1 positive animals or the non-responders.

4.8. Intestinal bacterial composition

Samples of feces from each mouse were examined to determine the intestinal bacterial composition. The V6-V9 region of the 16S rRNA gene was 454-pyrosequenced. 10429 sequences per sample were obtained succeeding a quality check and subsampling. As one OTU includes all sequences with a minimum of 97% similarity, 409 OTUs were identified. PCA showed only very finite OTU clustering in the feces of individual members of the same group (Fig. 16A). Additionally, no clear-cut differences between the bacterial communities of all three groups based on OTU data were detected after analysis by npMANOVA. Furthermore, greater concurrence within the groups as compared to between the groups was not seen after visual inspection of a Bray-Curtis distance matrix heat map (Fig. 16B).

Nonetheless, some significant distinctions in the microbial community composition between the three groups of interest were revealed. Following a taxonomic classification scheme based on data of sequence abundance however, significantly more sequences belonging to the *Synthrophaceae* (*Deltaproteobacteria*) family assigned to the genus *Smithella* (OTU 253) were detected in the IgE⁻/IgG⁻ group compared to the other two groups⁸ (Fig. 16C). In the IgE⁺/IgG1⁺ group, two OTUs were significantly more abundant. Both OTUs belong to the *Porphyromonadaceae* family and were assigned to the *Barnesiella* (OTU 185) and *Tennerella* (OTU 213) genus (Fig. 16D). These two OTUs found only in the IgE responders may be especially important, as these bacterial strains were not detected in either of the other two groups (IgE⁻/IgG⁺ or IgE⁻/IgG1⁻). In conclusion, bacterial components between the three groups of interest were overlapping but not identical. When bacterial communities were compared between the groups, small, but significant single bacterial OTU differences could be observed.

⁸ This was found succeeding correction for multiple testing.

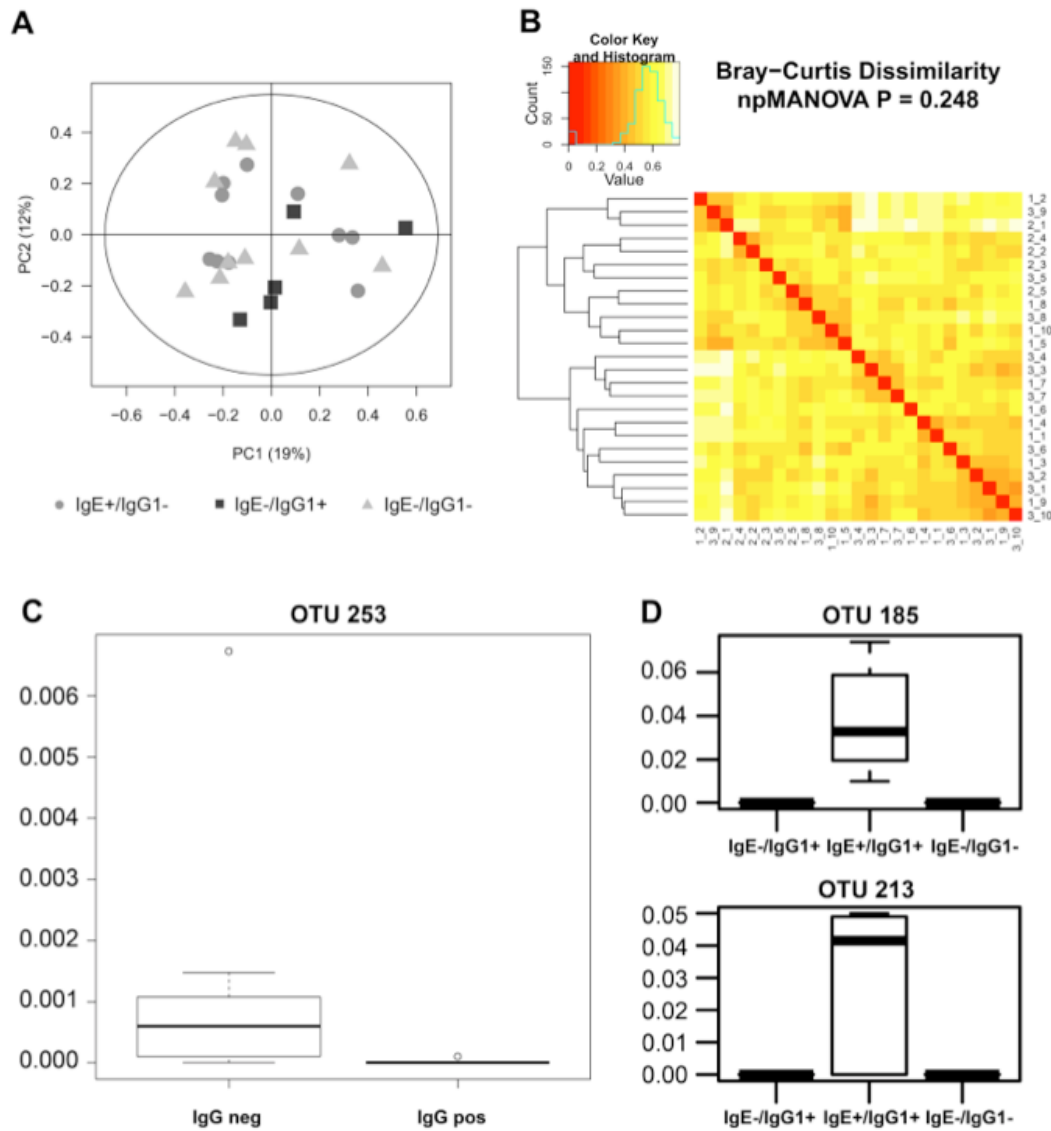


Figure 16: Bacterial community composition analysis in fecal samples of mice. Murine fecal samples of the three groups of interest were examined by PCA (A). 19% of variances in data are described by principle component (PC) 1 and 12% by PC 2. (B) shows the Bray-Curtis distance matrix heat map on OTU data, in which each mouse is depicted (1=IgE+/IgG1+; 2=IgE-/IgG1+; 3=IgE-/IgG1-). ((C) adj. P=0.04) shows the relative abundance of *Smithella* (OTU 253) for the IgE+/IgG1+ and IgE-/IgG1+ groups (IgG-positive) and the IgE-/IgG1- group (IgG-negative). The boxplot diagram in (D) illustrates the relative abundances of *Barnesiella* (OTU 185) and *Tennerella* (OTU 213) between the three groups. The median is shown as a black line while the boxes depict the inner quartile value range.

5. Discussion

Nearly 3 to 4% of adults and 6% of the pediatric population in industrialized countries are affected by immunologically mediated adverse reactions to food and prevalence rates seem to be increasing (25,26), making food allergy an important public health concern (17,24,33). Most food allergic patients suffer from IgE-mediated food allergic disorders. The initial sensitization phase, which can occur when an allergenic food protein crosses the epithelial barrier, stimulates Th2 reactions and leads to IgE formation. When the food compound is subsequently ingested, the allergen binds to the IgE antibodies on allergy effector cells and triggers the release of mediators. This may lead to allergic symptoms, the most severe of which is anaphylaxis. Today, avoidance of allergen exposure is the only therapeutic option in food allergy as there is no causative treatment available (17). Therefore, investigations looking at the sensitization and effector phase of food allergic reactions are imperative to finding new treatment options.

Traditionally, a skewed Th2 response to ingested food proteins was held responsible as the primary elicitor of food allergy, however the paradigm has shifted (53). The gastrointestinal tract is exposed to huge amounts of dietary antigens every day but only a limited number of proteins are responsible for most allergic reactions. The emerging concept is that development of food adverse reactions is impeded by oral tolerance (52). Oral tolerance, the result of a complex interactions between the innate and adaptive immune system, or rather the lack thereof due to the failure of regulatory mechanisms, is now viewed as an important perpetrator of the Th1/Th2 skewing (66). Much focus has also been on investigating food allergen characteristics (e.g. abundance, solubility) and host factors (e.g. genetic disposition, the maturity of the gastrointestinal system, intestinal bacterial composition, the time of allergen exposure, diet and culture) contributing to the development of allergic reactions. It is thought that these factors play a role in the breakdown of oral tolerance.

Another important factor to consider is the disruption of gastric digestion, which exerts a barrier function under physiological conditions, due to changes in the

stomach pH. For a long time only digestion-resistant food proteins were accepted to trigger food adverse reactions. Nowadays, food allergens that are able to resist degradation due to the hindrance of gastric digestion are also considered to be potential elicitors of food allergy (104,106–108,128). Based on this knowledge, a murine model of oral immunizations under concomitant gastric acid suppression was developed to establish food allergy (110), which mimics the situation in human patients (105). We have applied this immunization protocol to induce food allergic reactions in inbred BALB/c mice, treated repeatedly with a PPI omeprazole plus sucralfate and concomitantly orally immunized with the egg allergen OVA. However, as in humans, not all mice developed IgE-mediated adverse reactions to food. Previous studies have described variations in the humoral immune response between rats and mice, as well as different responses after treatment with allergens with varying degrees of allergenicity following different routes of exposure (65,129). However, for this study we used genetically identical BALB/c mice that were all subjected to the same immunization protocol and housed under the same conditions. Yet after preliminary ELISAs, only twenty-five animals revealed a clear-cut OVA-specific immune response (IgE greater than 15 ng/mL, IgG1 greater than 100 ng/mL or at detection limit for a negative response). Based on serological evaluations, animals were allocated to three groups: (1) IgE responders, as they revealed high OVA-specific IgE and IgG1 levels (IgE+/IgG1+); (2) the IgG1-positive group, with higher IgG1 levels (IgE-/IgG1+); (3) IgE non-responders, who did not show elevated antibody levels (IgE-/IgG1-). These three groups of interest were then further included in subsequent investigations to determine factors affecting food allergic reactions in this murine model of food allergy elicited by gastric acid suppression.

Literature has described two independent pathways of anaphylaxis in mice: the classic IgE, FcεRI and mast cell-dependent mechanism and the alternative IgG, FcγRIII, basophil-dependent or macrophage-dependent pathway (41–44). In this model however, anaphylactic responses are based on allergen-specific IgE antibodies. IgE responders showed significantly elevated levels of mast cell degranulation evidenced by the anaphylaxis and mast cell activation marker mMCP-1, reflecting IgE-mediated anaphylaxis (130,131). A drop in rectal body temperature confirmed anaphylactic responses. Additionally, IL-4 levels were substantially and IL-13 levels even significantly elevated in the IgE responder group. Both IL-4 and IL-13 have

been revealed to endogenously regulate the pathways of anaphylaxis by increasing the responsiveness to mediators produced by mast cells and macrophages and herewith enhance the susceptibility to anaphylaxis (41).

Total and allergen-specific IgA levels in the intestinal lavages were also evaluated. The significance of secretory IgA is still a matter of debate. It is thought that mucosal IgA binds the antigen prior to systemic absorption to prevent anaphylaxis (59). However, a significantly higher level of total IgA was found in the allergic mice and elevated levels of OVA-specific IgA were found in both the IgE responders and IgG-positive groups compared to the non-allergic mice. Given that IgE responders presented anaphylactic symptoms, these data indicate that secretory IgA is not capable of protecting against anaphylaxis in our model. In addition, Strait et al. showed that systemic, not enteric IgA or IgG antibodies, protect against systemic IgE-mediated anaphylactic reactions to food (61). In our model though, allergic mice additionally had significantly higher levels of OVA-specific IgG2a, as well as substantially higher OVA-specific IgA titers in the serum, which were not protective against anaphylaxis.

In addition, allergic mice showed variances in the differential blood cell count. They showed significantly decreased numbers of lymphocytes but significantly elevated levels of red blood cells and monocytes in peripheral blood during anaphylaxis compared to the IgE non-responder group. Furthermore, the hematocrit, an indicator of vascular leakage during anaphylaxis, was increased in the IgE responders. Interestingly, neutrophils and platelets were lower in the IgG1-positive group than the other groups. While it has been suggested that both neutrophils and platelets may contribute to the anaphylactic reaction, their defined role is not fully elucidated (132). PAF is also considered to be the primary mediator of the alternative IgG, FcγRIII, basophil-dependent or macrophage-dependent pathway of anaphylaxis (41–43). The fact that after challenges the IgG1-positive animals had significantly decreased levels of PAF could have implications for the lack of anaphylactic responses in this group of mice.

The murine gastric pH levels were analysed one hour after i.v. injection of a PPI. Gastric acid levels are decisive for the release and activation of proteases in the

gastrointestinal tract required for the digestive process of food proteins (108). When anti-acid medication neutralizes gastric acid, protein digestion is impaired and food allergens may persist in an intact form, which allows even digestion-labile food proteins to cross the epithelial barrier structurally intact and gain access to the mucosal immune system (104,108,114). This in turn, enhances the risk of sensitization and elicitation of allergic reactions. All animals from all three groups of interest (IgE+/IgG1+, IgE-/IgG1+ or IgE-/IgG1+) showed elevated gastric pH levels after being treated with anti-acid medication however, only slight but not statistically significant differences in pH levels were determined between the different groups. Thus, variations in response to acid-suppressive medication can be ruled-out as the cause for the heterogeneity of immunological responses observed.

Mouse studies further showed that antigen feedings under gastric acid-suppression induced a Th2-biased immune response (110). Additionally, anti-acid medications that contain aluminium compounds, such as sucralfate, act as Th2-adjuvants (116). In this study too, the allergic mice showed substantially elevated levels of IL-4, and even significantly elevated levels of IL-5 and IL-13, all Th2 markers, compared to the non-responders. IFN- γ , a Th1 marker, as well as the regulatory cytokine IL-10, were also increased in the IgE responder group. The fact that allergic mice showed higher Th1 and Th2 levels as well as increased levels of IL-10, indicates an overall enhanced immune response and may support the hypothesis that a skewed Th1/Th2 response is not the primary elicitor of food allergic reactions, as indicated previously (24,33,66). The allergic mice also showed higher IL-2 (which promotes the proliferation of activated T cells and B cells) (133) and IL-17 levels compared to the other groups. Interestingly, IL-22 was additionally increased in the non-allergic mice. IL-22 is a member of the IL-10-related cytokine family (134). It has both proinflammatory and tissue protective functions, regulated by IL-17 (135). Because of the dual role of this regulatory cytokine it is crucial to look at its function in combination with other immune modulatory factors under varying conditions. On the one hand, IL-22 can enhance inflammation, which could be its role in the IgE responder group. On the other hand, IL-22 signaling can help maintain the epithelial barrier in the gut during inflammation by activating proliferative and/or anti-apoptotic programs (63). Therefore, the increased IL-22 titers in the IgE non-responder mice could indicate repair of the epithelial barrier by IL-22 signaling. As a “leaky barrier” is one factor

contributing to the breakdown of oral tolerance (53), a protective influence of this cytokine could be hypothesized in the non-responders.

CD4⁺ T cells include Th1, Th2, Th17 cells and Tregs. Tregs modulate the immune response and suppress the immune response of other Th cells (53). When spleen cells were screened for T cell subsets in flow cytometry however, no substantial differences for CD4⁺ T cells or CD4⁺CD25⁺Foxp3⁺ Tregs could be observed between the three groups, indicating that suppression of the immune response by CD4⁺CD25⁺Foxp3⁺ Tregs is not the cause of the observed immunological distinctions between the animals. As it was repeatedly demonstrated that immunizations under acid suppression induce morphological changes in the gastrointestinal epithelium (104,106,136), histological evaluation of gastrointestinal tissue sections was performed. Interestingly, no conspicuous differences regarding inflammatory infiltrates, epithelial goblet cells, and myeloid mucosal cells in the murine gastrointestinal morphology were revealed between the three groups.

A study by Rivas et al. (137) showed that food allergic mice possess a specific gut microbiome capable of transferring susceptibility to hypersensitivity and with the ability to adapt when tolerance is enforced, which is why microbiota of allergic mice may contribute to food allergy. Although the mechanism behind changes in the microbiome that lead to sensitization has not yet been determined, components have been shown to directly influence effector T cells and/or Tregs. For example, colonization of the gut with polysaccharide A non-expressing strains of *Bacteriodes fragilis* generate IL-17-producing T cells whereas expressing strains of *B. fragilis* induce IL-10-producing Tregs (138). As receptors for these cytokines are expressed on epithelial cells, they can promote the expression of proteins important for maintaining the mucosal epithelial barrier function (137). Because the composition of the commensal microbiome seems to have an important influence on the gastrointestinal immune response (49), feces of the mice were subjected to evaluation of intestinal bacterial composition. Comparison of OTUs revealed discreet but significant differences in intestinal bacterial composition. After analysing sequence abundance data, single bacterial strain differences on OTU level were determined. Two OTUs from the *Porphyromonadaceae* family were more abundantly present only in the IgE⁺/IgG1⁺ group while significantly more abundant taxa of the

Synthrophaceae family were revealed in the non-responders. Additionally in the study by Rivas et al (137), OVA-sensitized Il4raF709 mice also revealed a microbiota signature with more abundant taxa of the *Porphyromonadaceae* family, among others, compared to allergen-sensitized wild-type control mice (137). However, in our study we looked at the gut bacterial composition shortly before sacrifice to determine factors associated with anaphylaxis in this mouse model of food allergy. Because fecal samples were collected at one time point only, we are not able to determine the changes that may have occurred over time in the bacterial flora of the gut during the development of food allergy. Hence, speculating whether or not this heterogeneity was present from the start and a cause, or a result of food allergy induction, is not possible.

In conclusion, OVA-specific IgE rather than IgG1 mediates anaphylaxis in this murine model of food allergy elicited by oral immunizations under gastric acid suppression. An increased gastric pH in all mice induced anaphylactic reactions in only the IgE-positive animals. This was underlined by mast cell degranulation, elevated Th2 cytokines and alterations in differential blood cell counts. While there were no differences noted during histological studies of the intestine and a suppression of the immune response by Tregs was also ruled-out, differences in the intestinal bacterial composition between the mice were revealed and could be a factor resulting in the heterogenic immunological responses between the animals. Certainly though, further studies need to be conducted to evaluate the contribution of time-dependent changes in the intestinal bacterial composition to the induction of food allergy in only some of the mice.

6. References

1. Bergmann K-C, Bergmann I, Schadewaldt H. *Bebilderte Geschichte der Allergologie*. München-Deisenhofen: Dustri-Verlag Dr. Karl Feistle; 2004.
2. Morgan AJ, Poland GA. The Jenner Society and the Edward Jenner Museum: tributes to a physician-scientist. *Vaccine*. Elsevier Ltd; 2011 Dec 30;29(Suppl 4):D152–4.
3. Riedel S. Edward Jenner and the history of smallpox and vaccination. *Baylor University Medical Center Proceedings*. 2005 Jan;18(1):21–5.
4. Jenner E. The Three Original Publications on Vaccination Against Smallpox. In: Eliot CW, editor. *The Harvard Classics*. New York: P.F. Collier & Son, 1909-14; 1910.
5. Fenner F, Henderson DA, Arita I, Ježek Z, Ladnyi ID. *Smallpox and its Eradication*. World Health Organization. Geneva: World Health Organization; 1988.
6. Gross CP, Sepkowitz KA. The myth of the medical breakthrough: smallpox, vaccination, and Jenner reconsidered. *International Journal of Infectious Diseases*. 1998;3(1):54–60.
7. Jackson M. *Allergy: The History of a Modern Malady*. London: Reaktion Books Ltd; 2006. p. 26.
8. Portier PG, Richet C. De l'action anaphylactique de certain venins. *C R Soc Biol*. 1902;54:170–2.
9. The Nobel Prize in Physiology or Medicine 1913- Award Ceremony Speech [Internet]. Nobelprize.org. [cited 2013 Feb 7]. Available from: http://www.nobelprize.org/nobel_prizes/medicine/laureates/1913/press.html
10. Turk JL. Von Pirquet, allergy and infectious diseases: a review. *Journal of the Royal Society of Medicine*. 1987 Jan;80(1):31–3.
11. Prausnitz C, Kuestner H. Studien ueber die Ueberempfindlichkeit. *Zbl. Bakteriол*. 1921;86:160–9.
12. Coca AF, Grove EF. Studies in hypersensitiveness: XIII. A study of atopic reagins. *The Journal of Immunology*. 1925;10:445–64.
13. Ishizaka K, Ishizaka T, Terry WD. Antigenic structure of γ E-globulin and reagenic activity. *The Journal of Immunology*. 1967;99(5).
14. Johansson SG, Bennich H. Immunological studies of an atypical (myeloma) immunoglobulin. *Immunology*. 1967;13:381–94.
15. Bennich H, Ishizaka K, Ishizaka T, Johansson SG. A comparative antigenic study of γ E-globulin and myeloma IgND. *The Journal of Immunology*. 1969;102(4):826–31.
16. Johansson SGO, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, et al. Revised nomenclature for allergy for global use: Report of the Nomenclature Review

- Committee of the World Allergy Organization, October 2003. *The Journal of Allergy and Clinical Immunology*. 2004 May;113(5):832–6.
17. Sicherer SH, Sampson HA. Food allergy. *The Journal of Allergy and Clinical Immunology*. Elsevier Ltd; 2010 Feb;125(2 Suppl 2):S116–25.
 18. Jyonouchi H. Mechanisms of IgE-mediated allergy & non-IgE-mediated immune reactions. *Expert Review of Clinical Immunology*. 2010;6(3):391–411.
 19. Burks AW, Sampson HA. Anaphylaxis and food allergy. *Clinical Reviews in Allergy & Immunology*. 1999 Jan;17(3):339–60.
 20. Sampson HA. Food anaphylaxis. *British Medical Bulletin*. 2000 May;56(4):925–35.
 21. Yunginger JW, Sweeney KG, Sturner WQ, Giannandrea LA, Teigland JD, Bray M, et al. Fatal food-induced anaphylaxis. *The Journal of the American Medical Association*. 1988 Sep 9;260(10):1450–2.
 22. Sampson HA, Mendelson L, Rosen JP. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *The New England Journal of Medicine*. 1992;327:380–4.
 23. Ben-Shoshan M, Clarke AE. Food-induced anaphylaxis: Clinical highlights and knowledge gaps. *Paediatrics & Child Health*. 2012 Jan;17(1):29–30.
 24. Wang J, Sampson HA. Food allergy. *The Journal of Clinical Investigation*. 2011;121(3):827–35.
 25. Branum AM, Lukacs SL. Food allergy among children in the United States. *Pediatrics*. 2009 Dec;124(6):1549–55.
 26. Hu Y, Chen J, Li H. Comparison of food allergy prevalence among Chinese infants in Chongqing, 2009 versus 1999. *Pediatrics International*. 2010 Oct;52(5):820–4.
 27. Savage JH, Matsui EC, Skripak JM, Wood RA. The natural history of egg allergy. *The Journal of Allergy and Clinical Immunology*. 2007 Dec;120(6):1413–7.
 28. Skripak JM, Matsui EC, Mudd K, Wood RA. The natural history of IgE-mediated cow's milk allergy. *The Journal of Allergy and Clinical Immunology*. 2007 Nov;120(5):1172–7.
 29. Wood RA. The natural history of food allergy. *Pediatrics*. 2003 Jun;111(6 Pt 3):1631–7.
 30. Hourihane JO, Roberts SA, Warner JO. Resolution of peanut allergy: case-control study. *British Medical Journal*. 1998 Apr 25;316(7140):1271–5.
 31. Dupont C. Food allergy: recent advances in pathophysiology and diagnosis. *Annals of Nutrition & Metabolism*. 2011 Jan;59(Suppl 1):8–18.
 32. Wasserman S, Watson W. Food allergy. *Allergy, Asthma & Clinical Immunology*. BioMed Central Ltd; 2011 Jan;7(Suppl 1):51–7.

33. Sicherer SH, Sampson HA. Food allergy. *The Journal of Allergy and Clinical Immunology*. 2006 Mar;117(2 Suppl Mini-Primer):S470–5.
34. Yang Y-H, Chiang B-L. Novel approaches to food allergy. *Clinical Reviews in Allergy & Immunology*. 2013 Jan 18;
35. Nowak-Wegrzyn A, Sampson HA. Future therapies for food allergies. *Journal of Allergy and Clinical Immunology*. 2011;127(3):558–73.
36. Varshney P, Jones SM, Scurlock AM, Perry TT, Kemper A, Steele P, et al. A randomized controlled study of peanut oral immunotherapy (OIT): clinical desensitization and modulation of the allergic response. *Journal of Allergy and Clinical Immunology*. 2011;127(3):654–60.
37. Kulis M, Vickery BP, Burks AW. Pioneering immunotherapy for food allergy: clinical outcomes and modulation of the immune response. *Immunologic Research*. 2011 Apr;49(1-3):216–26.
38. Leung DYM, Sampson HA, Yunginger JW, Burks AW, Schneider LC, Wortel CH, et al. Effect of Anti-IgE Therapy in Patients with Peanut Allergy. *The New England Journal of Medicine*. 2003;248(11):986–93.
39. Connors L, Wasserman S. Food allergy- the nuts and bolts [Internet]. Parkhurst Exchange. 2010 [cited 2013 Feb 6]. Available from: <http://www.parkhurstexchange.com/clinical-reviews/apr10/food-allergy>
40. Vieths S, Scheurer S, Ballmer-Weber B. Current understanding of cross-reactivity of food allergens. *Annals of the New York Academy of Sciences*. 2002;964:47–68.
41. Finkelman FD. Anaphylaxis: lessons from mouse models. *The Journal of Allergy and Clinical Immunology*. 2007 Sep;120(3):506–15; quiz 516–7.
42. Strait RT, Morris SC, Yang M, Qu X-W, Finkelman FD. Pathways of anaphylaxis in the mouse. *The Journal of Allergy and Clinical Immunology*. 2002 Apr;109(4):658–68.
43. Finkelman FD, Rothenberg ME, Brandt EB, Morris SC, Strait RT. Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *The Journal of Allergy and Clinical Immunology*. 2005 Mar;115(3):449–57; quiz 458.
44. Miyajima I, Dombrowicz D, Martin TR, Ravetch J V, Kinet J-P, Galli SJ. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc gammaRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *The Journal of Clinical Investigation*. 1997;99(5):901–14.
45. Strait RT, Morris SC, Finkelman FD. IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and FcγRIIb cross-linking. *The Journal of Clinical Investigation*. 2006;116(3):833–41.
46. Cauwels A, Janssen B, Buys E, Sips P, Brouckaert P. Anaphylactic shock depends on PI3K and eNOS-derived NO. *The Journal of Clinical Investigation*. 2006;116(8):2–9.

47. Brandtzaeg P. The gut as communicator between environment and host: immunological consequences. *European Journal of Pharmacology*. Elsevier B.V.; 2011 Sep;668 Suppl(0014):S16–32.
48. Ley RE, Hamady M, Lozupone C, Turnbaugh P, Roy R, Bircher JS, et al. Evolution of mammals and their gut microbes. *Science*. 2008;320(5883):1647–51.
49. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*. 2009 May;9(5):313–23.
50. Frank DN, Pace NR. Gastrointestinal microbiology enters the metagenomics era. Ouwehand AC, Vaughan EE, editors. *Current Opinion in Gastroenterology*. Taylor & Francis; 2008;24(1):4–10.
51. Abbas AK, Lichtman AH, Pillai S. *Cellular and Molecular Immunology*. 7th ed. Philadelphia: Elsevier Saunders; 2012. p. 293–309, 407–23.
52. Chehade M, Mayer L. Oral tolerance and its relation to food hypersensitivities. *The Journal of Allergy and Clinical Immunology*. 2005 Jan;115(1):3–12; quiz 13.
53. Vickery BP, Scurlock AM, Jones SM, Burks AW. Mechanisms of Immune Tolerance Relevant to Food Allergy. *Journal of Allergy and Clinical Immunology*. 2011;127(3):576–86.
54. Cahenzli J, Balmer ML, McCoy KD. Microbial-immune cross-talk and regulation of the immune system. *Immunology*. 2013 Jan;138(1):12–22.
55. Bashir MEH, Louie S, Shi HN, Nagler-Anderson C. Toll-like receptor 4 signaling by intestinal microbes influences susceptibility to food allergy. *Journal of Immunology*. 2004 Jun 1;172(11):6978–87.
56. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nature Reviews Immunology*. 2008;8(6):435–46.
57. Corthésy B. Roundtrip Ticket for Secretory IgA : Role in Mucosal Homeostasis? *The Journal of Immunology*. 2007;178:27–32.
58. Brandtzaeg P. Update on mucosal immunoglobulin A in gastrointestinal disease. *Current opinion in gastroenterology*. 2010 Nov;26(6):554–63.
59. Kim JS, Sampson HA. Food allergy: a glimpse into the inner workings of gut immunology. *Current Opinion in Gastroenterology*. 2012 Mar;28(2):99–103.
60. Karlsson MR, Johansen F-E, Kahu H, Macpherson a, Brandtzaeg P. Hypersensitivity and oral tolerance in the absence of a secretory immune system. *Allergy*. 2010 May;65(5):561–70.
61. Strait RT, Mahler A, Hogan S, Khodoun M, Shibuya A, Finkelman FD. Ingested allergens must be absorbed systemically to induce systemic anaphylaxis. *The Journal of Allergy and Clinical Immunology*. Elsevier Ltd; 2011 Apr;127(4):982–9.e1.
62. Feng T, Elson CO. Adaptive immunity in the host-microbiota dialog. *Mucosal Immunology*. Nature Publishing Group; 2011 Jan;4(1):15–21.

63. Rutz S, Eidenschenk C, Ouyang W. IL-22, not simply a Th17 cytokine. *Immunological Reviews*. 2013 Mar;252(1):116–32.
64. Vickery BP, Chin S, Burks WA. Pathophysiology of food allergy. *Pediatric Clinics of North America*. 2011;58(2):363–76.
65. Morafo V, Srivastava K, Huang C-K, Kleiner G, Lee S-Y, Sampson HA, et al. Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. *Journal of Allergy and Clinical Immunology*. 2003 May;111(5):1122–8.
66. Hogan SP, Rothenberg ME. Dietary allergenic proteins and intestinal immunity: a shift from oral tolerance to sensitization. *Clinical and Experimental Allergy*. 2008 Feb;38(2):229–32.
67. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature immunology*. 2001 Apr;2(4):361–7.
68. Rescigno M, Sabatino A Di. Dendritic cells in intestinal homeostasis and disease. *The Journal of Clinical Investigation*. 2009;119(9):2441–50.
69. Coombes JL, Siddiqui KRR, Arancibia-Cárcamo C V, Hall J, Sun C-M, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *The Journal of Experimental Medicine*. 2007 Aug 6;204(8):1757–64.
70. Chambers SJ, Bertelli E, Winterbone MS, Regoli M, Man AL, Nicoletti C. Adoptive transfer of dendritic cells from allergic mice induces specific immunoglobulin E antibody in naïve recipients in absence of antigen challenge without altering the T helper 1/T helper 2 balance. *Immunology*. 2004 May;112(1):72–9.
71. Hershberg RM, Cho DH, Youakim A, Bradley MB, Lee JS, Framson PE, et al. Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *The Journal of Clinical Investigation*. 1998 Aug 15;102(4):792–803.
72. Bland PW, Warren LG. Antigen presentation by epithelial cells of the rat small intestine. II. Selective induction of suppressor T cells. *Immunology*. 1986 May;58(1):9–14.
73. Shreedhar VK, Kelsall BL, Neutra MR. Cholera toxin induces migration of dendritic cells from the subepithelial dome region to T- and B-Cell areas of Peyer ' s patches. *Infection and Immunity*. 2003;71(1):504–9.
74. Borsutzky S, Cazac BB, Roes J, Guzmán CA. TGF-beta receptor signaling is critical for mucosal IgA responses. *Journal of Immunology*. 2004 Sep 1;173(5):3305–9.
75. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nature Immunology*. 2005 Apr;6(4):331–7.

76. Asseman BC, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *The Journal of Experimental Medicine*. 1999;190(7):995–1003.
77. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity*. 2007 May;26(5):579–91.
78. Tang Q, Bluestone JA. The Foxp3⁺ regulatory T cell: a jack of all trades, master of regulation. *Nature Immunology*. 2008;9(3):239–44.
79. Karlsson MR, Rugtveit J, Brandtzaeg P. Allergen-responsive CD4⁺CD25⁺ regulatory T cells in children who have outgrown cow's milk allergy. *The Journal of Experimental Medicine*. 2004 Jun 21;199(12):1679–88.
80. Beyer K, Castro R, Birnbaum A, Benkov K, Pittman N, Sampson HA. Human milk-specific mucosal lymphocytes of the gastrointestinal tract display a TH2 cytokine profile. *Journal of Allergy and Clinical Immunology*. 2002 Apr;109(4):707–13.
81. Shi HN, Walker A. Bacterial colonization and the development of intestinal defences. *Canadian Journal of Gastroenterology*. 2004 Aug;18(8):493–500.
82. Koplin J, Allen K, Gurrin L, Osborne N, Tang MLK, Dharmage S. Is caesarean delivery associated with sensitization to food allergens and IgE-mediated food allergy: a systematic review. *Pediatric Allergy and Immunology*. 2008 Dec;19(8):682–7.
83. Salzman NH. Microbiota-immune system interaction: an uneasy alliance. *Current Opinion in Microbiology*. Elsevier Ltd; 2011 Feb;14(1):99–105.
84. Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *The Journal of Immunology*. 1997;159(4):1739–45.
85. Macpherson AJ. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science*. 2000 Jun 23;288(5474):2222–6.
86. Okada H, Kuhn C, Feillet H, Bach J. The “hygiene hypothesis” for autoimmune and allergic diseases: an update. *Clinical and Experimental Immunology*. 2010 Apr;160(1):1–9.
87. Prescott SL, Björkstén B. Probiotics for the prevention or treatment of allergic diseases. *The Journal of Allergy and Clinical Immunology*. 2007;120(2):255–62.
88. Chapman JA, Bernstein IL, Lee RE, Oppenheimer J, Nicklas RA, Portnoy JM, et al. Food allergy: a practice parameter. *The American Academy of Allergy, Asthma and Immunology*. 2006;96:1–68.
89. Lehrer SB, Ayuso R, Reese G. Current understanding of food allergens. *Annals of the New York Academy of Sciences*. 2002;964:69–85.

90. Maleki SJ, Viquez O, Jacks T, Dodo H, Champagne ET, Chung S-Y, et al. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *Journal of Allergy and Clinical Immunology*. 2003 Jul;112(1):190–5.
91. Beyer K, Morrow E, Li XM, Bardina L, Bannon GA, Burks AW, et al. Effects of cooking methods on peanut allergenicity. *The Journal of Allergy and Clinical Immunology*. 2001 Jun;107(6):1077–81.
92. Commins SP, Satinover SM, Hosen J, Mozena J, Lewis BD, Woodfolk JA, et al. Delayed anaphylaxis, angioedema, or urticaria after consumption of red emat in patients with IgE antibodies specific for galactose- α -1,3-galactose. *Journal of Allergy and Clinical Immunology*. 2009;123(2):426–33.
93. Shreffler WG, Castro RR, Kucuk ZY, Charlop-powers Z, Grishina G, Yoo S, et al. The major glycoprotein allergen from *Arachis hypogaea*, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. *The Journal of Immunology*. 2006;177:3677–85.
94. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, et al. Tolerance to extensively heated milk in children with cow's milk allergy. *The Journal of Allergy and Clinical Immunology*. 2008 Aug;122(2):342–7, 347.e1–2.
95. Lemon-Mulé H, Sampson HA, Sicherer SH, Shreffler WG, Noone S, Nowak-Wegrzyn A. Immunologic changes in children with egg allergy ingesting extensively heated egg. *The Journal of allergy and clinical immunology*. 2008 Nov;122(5):977–983.e1.
96. Untersmayr E, Jensen-Jarolim E. Mechanisms of type I food allergy. *Pharmacology & Therapeutics*. 2006 Dec;112(3):787–98.
97. Hsieh K, Tsaiw C, Wuz CHH, Lin R. Epicutaneous exposure to protein antigen and food allergy. *Clinical and Experimental Allergy*. 2003;33:1067–75.
98. Lack G, Fox D, Northstone K, Golding J. Factors associated with the development of peanut allergy in childhood. *The New England Journal of Medicine*. 2003;348(11):977–85.
99. Du Toit G, Katz Y, Sasieni P, Mesher D, Maleki SJ, Fisher HR, et al. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *Journal of Allergy and Clinical Immunology*. 2008;122(5):984–91.
100. Katz Y, Rajuan N, Goldberg MR, Eisenberg E, Heyman E, Cohen A, et al. Early exposure to cow's milk protein is protective against IgE-mediated cow's milk protein allergy. *The Journal of Allergy and Clinical Immunology*. Elsevier Ltd; 2010 Jul;126(1):77–82.e1.
101. Koplin J, Osborne NJ, Wake M, Martin PE, Gurrin LC, Robinson MN, et al. Can early introduction of egg prevent egg allergy in infants? A population-based study. *Journal of Allergy and Clinical Immunology*. 2010;126(4):807–13.
102. Sicherer SH, Sampson HA. Peanut allergy: emerging concepts and approaches for an apparent epidemic. *The Journal of Allergy and Clinical Immunology*. 2007 Sep;120(3):491–503; quiz 504–5.

103. Sicherer SH, Furlong TJ, Maes HH, Desnick R, Sampson HA, Gelb BD. Genetics of peanut allergy: a twin study. *Journal of Allergy and Clinical Immunology*. 2000;106(1 (Part 1)):53–6.
104. Untersmayr E, Schöll I, Swoboda I, Beil WJ, Förster-Waldl E, Walter F, et al. Antacid medication inhibits digestion of dietary proteins and causes food allergy: a fish allergy model in BALB/c mice. *Journal of Allergy and Clinical Immunology*. 2003;112(3):616–23.
105. Untersmayr E, Bakos N, Schöll I, Kundi M, Roth-Walter F, Szalai K, et al. Anti-ulcer drugs promote IgE formation toward dietary antigens in adult patients. *The FASEB Journal*. 2005 Jan 25;6:656–8.
106. Pali-Schöll I, Yildirim A, Ackermann U, Knauer T, Becker C, Garn H, et al. Anti-acids lead to immunological and morphological changes in the intestine of BALB/c mice similar to human food allergy. *Experimental and Toxological Pathology*. 2008;60(4-5):337–45.
107. Untersmayr E, Jensen-Jarolim E. The role of protein digestibility and antacids on food allergy outcomes. *The Journal of Allergy and Clinical Immunology*. 2008;121(6):1301–10.
108. Pali-Schöll I, Jensen-Jarolim E. Anti-acid medication as a risk factor for food allergy. *Allergy*. 2011 Apr;66(4):469–77.
109. Takagi K, Teshima R, Okunuki H, Itoh S, Kawasaki N, Kawanishi T, et al. Kinetic analysis of pepsin digestion of chicken egg white ovomucoid and allergenic potential of pepsin fragments. *International Archives of Allergy and Immunology*. 2005 Jan;136(1):23–32.
110. Diesner SC, Knittelfelder R, Krishnamurthy D, Gajdzik L, Jensen-Jarolim E, Untersmayr E. Dose-dependent food allergy induction against ovalbumin under acid-suppression: a murine food allergy model. *Immunology Letters*. 2008;121(1):45–51.
111. Berin MC, Mayer L. Immunophysiology of experimental food allergy. *Mucosal Immunology*. 2009 Jan;2(1):24–32.
112. Schöll I, Untersmayr E, Bakos N, Roth-Walter F, Gleiss A, Boltz-Nitulescu G, et al. Antiulcer drugs promote oral sensitization and hypersensitivity to hazelnut allergens in BALB/c mice and humans. *The American Journal of Clinical Nutrition*. 2005 Jan;81(1):154–60.
113. Untersmayr E, Diesner SC, Brämwig KH, Knittelfelder R, Bakos N, Gundacker C, et al. Characterization of intrinsic and extrinsic risk factors for celery allergy in immunosenescence. *Mechanisms of Ageing and Development*. 2007;129(3):120–8.
114. Untersmayr E, Vestergaard H, Malling H-J, Jensen LB, Platzer MH, Boltz-Nitulescu G, et al. Incomplete digestion of codfish represents a risk factor for anaphylaxis in patients with allergy. *The Journal of Allergy and Clinical Immunology*. 2007;119(3):711–7.

115. Diesner SC, Olivera A, Dillahun S, Schultz C, Watzlawek T, Förster-Waldl E, et al. Sphingosine-kinase 1 and 2 contribute to oral sensitization and effector phase in a mouse model of food allergy. *Immunology Letters*. 2012;141(2):210–9.
116. Brunner R, Wallmann J, Szalai K, Karagiannis P, Kopp T, Scheiner O, et al. The impact of aluminium in acid-suppressing drugs on the immune response of BALB/c mice. *Clinical and Experimental Allergy*. 2007 Oct;37(10):1566–73.
117. Untersmayr E, Bakos N, Schöll I, Kundi M, Roth-Walter F, Szalai K, et al. Anti-ulcer Drugs Promote IgE Formation Toward Dietary Antigens in Adult Patients. *The FASEB Journal*. 2005;6:656–8.
118. Diesner SC, Schultz C, Pfitzner B, Assmann V, Krishnamurthy D, Weichselbaumer M, et al. Characteristics associated with anaphylaxis in an experimental food allergy model under gastric acid suppression. Manuscript submitted for publication.
119. Lane D. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. *Nucleic acid techniques in bacterial systematics*. Chichester: John Wiley & Sons; 1991. p. 115–75.
120. Juretschko S, Timmermann G, Schmid M, Schleifer K-H, Pommerening-Röser A, Koops H-P, et al. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Applied and Environmental Microbiology*. 1998;64(6):3042–51.
121. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*. 2009 Dec;75(23):7537–41.
122. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*. 2011 Aug 15;27(16):2194–200.
123. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*. 2007 Aug;73(16):5261–7.
124. Moolenbeek C, Ruitenberg EJ. The “Swiss roll”: a simple technique for histological studies of the rodent intestine. *Laboratory Animals*. 1981 Jan;15(1):57–9.
125. Dixon P. VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science*. 2003;14(6):927–30.
126. Dray S, Dufour A-B. The ade4 package: implementing the duality diagram from ecologists. *Journal of Statistical Software*. 2003;22(4):1–20.
127. Benjamini Y, Yekutieli D. The control of the false discovery rate in multiple testing under dependency. *Annals of Statistics*. 2001;29(4):1165–88.
128. Riemer AB, Gruber S, Pali-Schöll I, Kinaciyan T, Untersmayr E, Jensen-Jarolim E. Suppression of gastric acid increases the risk of developing immunoglobulin E-

- mediated drug hypersensitivity: human diclofenac sensitization and a murine sensitization model. *Clinical and Experimental Allergy*. 2010 Mar;40(3):486–93.
129. Smit JJ, Willemsen K, Hassing I, Fiechter D, Storm G, Van Bloois L, et al. Contribution of classic and alternative effector pathways in peanut-induced anaphylactic responses. *PloS one*. 2011 Jan;6(12):e28917.
 130. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, et al. Mast cells are required for experimental oral allergen – induced diarrhea. *Journal of Clinical Investigation*. 2003;112(11):1666–77.
 131. Simons FER, Frew AJ, Ansotegui IJ, Bochner BS, Golden DBK, Finkelman FD, et al. Risk assessment in anaphylaxis: current and future approaches. *The Journal of Allergy and Clinical Immunology*. 2007 Jul;120(1 Suppl):S2–24.
 132. Krishnamurthy D, Starkl P, Szalai K, Roth-Walter F, Diesner SC, Mittlboeck M, et al. Monitoring neutrophils and platelets during casein-induced anaphylaxis in an experimental BALB/c mouse model. *Clinical and Experimental Allergy*. 2012 Jul;42(7):1119–28.
 133. Agata H, Kondo N, Fukutomi O, Shinoda S, Orii T. Interleukin-2 production of lymphocytes in food sensitive atopic dermatitis. *Archives of Disease in Childhood*. 1992 Mar;67(3):280–4.
 134. Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA. Anti- and pro-inflammatory roles of TGF- β , IL-10, and IL-22 in immunity and autoimmunity. *Current Opinion in Pharmacology*. 2009;9(4):447–53.
 135. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser L a, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *The Journal of Experimental Medicine*. 2010 Jun 7;207(6):1293–305.
 136. Untersmayr E, Ellinger A, Beil WJ, Jenson-Jarolim E. Eosinophils accumulate in the gastric mucosa of food-allergic mice. *International Archives of Allergy and Immunology*. 2004;135(1):1–2.
 137. Noval Rivas M, Burton OT, Wise P, Zhang Y-Q, Hobson SA, Garcia Lloret M, et al. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. *The Journal of Allergy and Clinical Immunology*. Elsevier Ltd; 2013 Jan;131(1):201–12.
 138. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor pathways establishes commensal gut colonization. *Science*. 2011;332(6032):974–7.
 139. Lorentz A, Schwengberg S, Sellge G, Manns MP, Bischoff SC. Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4. *Journal of Immunology*. 2000 Jan 1;164(1):43–8.
 140. Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity*. 1996 Mar;4(3):313–9.

7. List of Tables

<i>Table 1: Information regarding chemicals used.</i>	<i>27</i>
<i>Table 2: List of medications used for anti-acid treatment of mice.</i>	<i>28</i>
<i>Table 3: Content and catalogue information of kits used.</i>	<i>28</i>
<i>Table 4: Antibody catalogue information.</i>	<i>29</i>
<i>Table 5: Cell media composition.</i>	<i>29</i>
<i>Table 6: Solutions for buffer preparation.</i>	<i>30</i>
<i>Table 7: Antibody concentrations for T cell count and cytokine production analysis using flow cytometry.</i>	<i>40</i>

8. List of Figures

Figure 1: Classification of food adverse reactions. Adverse reactions to food can be classified as toxic or non-toxic reactions. Toxic reactions include reactions to food borne pathogens or pharmacologically active food components. Non-toxic reactions can then be further classified as food intolerances or food allergies. Food allergic reactions can be either IgE-mediated or non-IgE-mediated.7

Figure 2: Pathophysiological events of type I food allergic reactions. First, the food allergen crosses the epithelial barrier. It then stimulates a Th2 response, which signals B cells to produce IgE by promoting isotype switching. Subsequently, IgE can bind to FcεRI on mast cells and allergy effector cells. If the patient is exposed to the offending food a second time, it comes to cross-linking of the IgE antibodies and the release of mediators such as histamine, prostaglandins and leukotrienes leading to the clinical symptoms of food allergy. 10

Figure 3: Mechanisms of anaphylaxis in murine models. Systemic anaphylaxis in mice may follow either the classic or alternative pathway. In classic IgE-dependent anaphylaxis, exposure to antigen causes cross-linking of IgE bound to FcεRI on mast cells, inducing the release of the primary mediator histamine and sometimes PAF. In the IgG-dependent mechanism, the antigen-IgG complex stimulates cross-linking of FcγRIII on macrophages and triggers the release of PAF. Both histamine and PAF enhance smooth muscle contractions and increase vascular permeability, leading to the clinical symptoms of anaphylaxis. If more IgG than IgE is present then antigen-specific IgG can block the classic pathway. This figure is adapted from Finkelman (41). 11

Figure 4: Immunity in the gastrointestinal system. A huge number of commensal bacteria can be found in the intestinal lumen. A monolayer of epithelial cells spans the intestinal tract. A mucus layer on top of the epithelium contains antimicrobial substances and factors that trap pathogenic organisms. Together, they comprise the mucosal epithelium, which serves as a primary barrier against microbial invasion. The underlying supportive tissue is called the lamina propria. Specialized epithelial cells such as dendritic cells, M-cells and intestinal epithelial cells sample antigens and deliver them to the MALT within the lamina propria or lymph nodes, where components of the innate (macrophages, epithelial cells) and adaptive (T cells, IgA-producing B cells and cytokines) immune system must either induce tolerance to non-harmful foreign substances or recognize and respond to invaders..... 13

Figure 5: Subsets of CD4⁺ T cells. Naïve T cells can differentiate to CD4⁺ T cell subsets. They can become Th cells (Th1, Th17 and Th2 cells) or Tregs. Depending on their cytokine profile, they may have different effector functions. Th1 cells are associated with the production of pro-inflammatory cytokines such as TNF-α and IFN-γ. Th17 cells, on the other hand, produce IL-17 and IL-22. Th2 cells produce cytokines IL-4, IL-5 and IL-13. Tregs can either be natural or inducible Tregs. Natural Tregs (T_R) express CD25 as well as Foxp3. Inducible Tregs can be further classified as TGF-β-secreting Th3 cells and IL-10-secreting Tr1 cells. Tregs can block Th17 and Th2 responses. This figure is adapted from Brandtzaeg (47). 18

Figure 6: Immunization protocol in a murine model under gastric acid suppression. PIS were taken from tail veins of the mice before immunizations. The mice were then immunized seven times (PPI + oral immunization). They were treated with the PPI omeprazole (i.v. on three consecutive days and i.v. a second time one hour later on days 2 and 3) and then fed OVA with sucralfate on days 2 and 3 during each three-day immunization cycle. MIS was subsequently collected from tail veins for analysis of IgE, IgG1, IgA and IgG2a titers. Mice were orally challenged with PBS (PBS OC) and ten days later with OVA (OC 1). On the day of the sacrifice, mice were again orally challenged (OC 2) and after one hour i.v. challenged with OVA for evaluation of anaphylactic responses.31

Figure 7: Inclusion criteria and allocation to the three groups of interest. 64 BALB/c mice were subjected to our immunization protocol under concomitant gastric acid suppression. After preliminary ELISAs, 25 mice were allocated to the three groups of interest according to their serological IgE and IgG1 titers and continued.32

Figure 8: Oral provocation and sacrifice. Mice were first orally challenged with PBS as a negative control. On day 136, mice were orally challenged with OVA. The rectal temperature was taken and blood was collected by the tail vein after each oral provocation for evaluation of the anaphylactic responses. On the day of the sacrifice, mice were orally and i.v. challenged. After the sacrifice, blood was collected by cardiac puncture for differential blood cell count analysis.37

Figure 9: OVA-specific antibody titers of mice subjected to the oral immunization protocol under gastric acid suppression. Ten mice showed high OVA-specific IgE (A) and IgG1 (B) levels and were rendered IgE responders (IgE+/IgG1+). Five were only IgG1 positive (IgE-/IgG1+). Ten more were neither IgE nor IgG1 positive and classified as IgE non-responders (IgE-/IgG1-). OVA-specific IgG2a (C) and IgA (D) were also increased in the IgE responder group. ***P<0.001, **P<0.01, *P<0.05..... 49

Figure 10: Evaluation of anaphylactic symptoms. Mouse mast cell protease-1 (mMCP-1) levels (A), measured after the first oral challenge (OC1), were increased significantly in the IgE responder group. This was accompanied by a drop in rectal body temperature (B) measured 5, 10 and 15 minutes after intravenously (i.v.) challenging mice with OVA. The IgE+/IgG1+ group is represented by dots, the IgE-/IgG1+ group by squares and the IgE-/IgG1- by triangles. ***P<0.001, §P<0.001. 50

Figure 11: PH levels of the stomach in mice one hour after i.v. injection with a PPI. No significant differences were measured between the three groups. 51

Figure 12: Total IgA (A) and OVA-specific IgA (B) titers in the intestinal lavages. IgE responders showed significantly increased levels of total IgA. Both the IgE responders and the IgG1-positive group showed significantly increased levels of OVA-specific IgA. **P<0.01, *P<0.05..... 52

Figure 13: Cytokine production of the three groups of interest (IgE+/IgG+, IgE-/IgG+, IgE-/IgG-). IL-4 (A), IL-5 (B), IL-13 (C), IFN- γ (D), IL-2 (E), IL-22 (F), IL-17 (G) and IL-10 (H) levels were measured using the mouse Th1/Th2/Th17/Th22 13plex Flowcytometry kit by stimulating spleen cells of mice with OVA and full medium as a control (ctrl) for 72 hours. Th2 cytokine production (IL-4, IL-5, IL-13) was increased in the IgE responder group. IL-2 and IL-17 were also elevated in this group. IL-22 was additionally higher in the IgE non-responder group. **P<0.01, *P<0.05..... 52

Figure 14: T cell count determined in spleens by flow cytometry. No significant differences between CD4+ T cell (A) and CD4+CD25+Foxp3+ Treg (B) counts were detected between the three groups of interest. 53

Figure 15: Differential blood cell count analysis during anaphylaxis. Levels of lymphocytes (A), monocytes (B), neutrophils (C), red blood cells (D), hematocrit (E) and platelets (F) were determined in blood samples taken before provocation (pre-challenge) and 15 minutes after i.v. challenging mice (post-challenge). Lymphocytes were decreased while red blood cells, hematocrit and monocytes were increased in the IgE responders. Neutrophils and platelets were decreased in the IgE-/IgG+ group. The cells before and after the OVA challenge were determined using the two-way ANOVA and Bonferroni post-test; #####P<0.0001, ##<0.01. Differences within the groups were determined by one-way ANOVA and Tukey's multiple comparison test; **P<0.01, *P<0.05. 54

Figure 16: Bacterial community composition analysis in fecal samples of mice. Murine fecal samples of the three groups of interest were examined by PCA (A). 19% of variances in data are described by principle component (PC) 1 and 12% by PC 2. (B) shows the Bray-Curtis distance matrix heat map on OTU data, in which each mouse is depicted (1=IgE+/IgG1+; 2=IgE-/IgG1+; 3=IgE-/IgG1-). ((C) adj. P=0.04) shows the relative abundance of Smithella (OTU 253) for the IgE+/IgG1+ and IgE-/IgG1+ groups (IgG-positive) and the IgE-/IgG1- group (IgG-negative). The boxplot diagram in (D) illustrates the relative abundances of Barnesiella (OTU 185) and Tennerella (OTU 213) between the three groups. The median is shown as a black line while the boxes depict the inner quartile value range. 56

9. Appendix

9.1. Zusammenfassung

In der westlichen Welt sind ungefähr 3 bis 6% der Population von einer allergischen Reaktion auf Nahrungsmittel betroffen. Da die Prävalenz der Nahrungsmittelallergien in der westlichen Welt zunimmt, sind sie zu einem wichtigen Anliegen des öffentlichen Gesundheitswesens geworden. Eine kausale Behandlung wurde noch nicht beschrieben. Daher ist heutzutage die Vermeidung der oralen Aufnahme von Allergieauslösern die einzige therapeutische Option, um eine Überempfindlichkeit auf Lebensmittel, die mitunter zu einem lebensbedrohlichen anaphylaktischen Schock führen kann, zu verhindern. Um neue Therapieoptionen zu finden, ist es erforderlich die Sensibilisierungs- und Effektorphase allergischer Reaktionen zu untersuchen.

Das gastrointestinale Immunsystem muss sich täglich mit einer großen Anzahl von Nahrungsantigenen auseinandersetzen, jedoch führt nur eine begrenzte Fraktion dieser oral aufgenommenen Antigene zu einer allergischen Reaktion. Der Grund hierfür ist die Entwicklung immunologischer Toleranz, die aus Interaktionen zwischen den angeborenen und adaptiven Immunsystemen im gastrointestinalen Trakt resultiert. Man ist der Ansicht, dass die Fehlentwicklung oder Zusammenbruch der oralen Toleranz ein Th2 Ungleichgewicht zur Folge hat und somit zur Nahrungsmittelallergie führt. Darüber hinaus sind Charakteristika von Nahrungsmittelallergenen und von betroffenen Patienten untersucht worden, um die Faktoren verstehen zu können, die zum Zusammenbruch der oralen Toleranz beitragen.

Schon zuvor wurde die Beeinträchtigung der Proteinverdauung im gastrointestinalen Trakt durch die Einnahme von magensäurehemmenden Medikamenten als Risiko gesehen, um an einer Nahrungsmittelunverträglichkeit zu erkranken. Die Verdauung und enzymatische Spaltung eiweißhaltiger Nahrungsbestandteile wird durch die Neutralisierung der Magensäure beeinträchtigt und verdauungslabile Proteine können so in Wechselwirkung mit dem mukosalen Immunsystem treten. Wir haben unser bestehendes Nahrungsmittelallergie-Mausmodell bei den BALB/c Inzucht-Mäusen verwendet, die mit OVA oral unter gleichzeitiger Säuresuppression immunisiert

wurden, um allergische Reaktionen auf Nahrungsmittel zu induzieren. Jedoch haben sich ähnlich wie beim Menschen nicht bei allen Tieren Unverträglichkeitsreaktionen gezeigt. Das Ziel der Studie war daher die Untersuchung der Faktoren, die zu den unterschiedlichen Immunreaktionen der Mäuse trotz identer Immunisierungsmethoden geführt haben.

Zusammenfassend führte eine Erhöhung des pH-Wertes im Magen aller beobachteter Mäuse nur in den allergischen Tieren zu einer anaphylaktischen Reaktion. Hier wurde die Anaphylaxis durch OVA-spezifisches IgE und nicht durch IgG1 ausgelöst. Mastzelldegranulation, erhöhte Th2 Zytokine im Serum der Mäuse und Veränderungen des Differentialblutbildes lassen auf IgE-vermittelte Anaphylaxis schließen. Wir konnten weder entscheidende Unterschiede betreffend histologischer Auffälligkeiten der murinen Darmschleimhaut, noch Unterschiede in der Anzahl der T-Zellen oder der T-regulatorischen Zellen feststellen. Allerdings konnten wir signifikante Unterschiede auf Ebene einzelner bakterieller Stämme in der mikrobiellen Zusammensetzung des Darmes der allergischen Mäuse erkennen. Daher kommt dies als ein beitragender Faktor der unterschiedlichen Immunreaktion der Tiere in Frage. Es ist zweifelsohne erforderlich, dass weitere Studien durchgeführt werden müssen, um die zeitabhängige Veränderung der Mikrobiome, während der Sensibilisierung und dessen Beitrag zur Induktion von Nahrungsmittelallergien in manchen, aber nicht allen Mäusen, besser zu verstehen.

9.2. Curriculum Vitae

Personal Information

Name	Vera Elisabeth Assmann
Sex	Female
Nationality	Austrian and Canadian

Education

As of 10/2006	Molecular Biology (Diploma degree program) University of Vienna, Vienna (Austria) Focus: Immunology/Microbiology, Molecular Medicine, Genetics
01/2006 - 06/2006	Enriched High School Education Ontario Science Centre Science School, Toronto (Canada)
09/2002 - 01/2006	High School Education St. Augustine Catholic High School, Markham (Canada)

Training

09/2011 - 10/2011	Medical University of Vienna, Vienna - Department of Pathophysiology and Allergy Research Topic: „ <i>Evaluation of targeted PGLA microparticles as a novel oral treatment strategy for type I food allergy.</i> ”
04/2011 - 05/2011	Medical University of Vienna, Vienna - Department of Pathophysiology and Allergy Research, in cooperation with Paris-Lodron University of Salzburg, Salzburg - Institute for Molecular Biology Topic: “ <i>The nitration of ovalbumin in H.pylori infection.</i> ”
07/2009 - 08/2009	Concordia University, Montreal - Centre for Structural and Functional Genomics Topic: “ <i>Transcriptome and secretome analysis of biomass-degrading fungi Thielavia Terrestris.</i> ”
07/2008	University of Vienna, Vienna - Institute of Inorganic Chemistry Topics: <i>Bioinorganic chemistry, Complex chemistry</i>

01/2005 - 04/2005 **The Hospital for Sick Children, Toronto (Sanofi-Aventis Biotech Challenge)**
 - Molecular Structure and Function Program
 Topic: *“The effects of bird’s nest glycoprotein extract on immune system cells.”*

Language Skills

German (bilingual), English (bilingual), French (basic)

Computer Skills

Microsoft Office

Additional Information

09/2011	ÖGAI Conference 2011, Graz Austrian Society for Allergology and Immunology (Member)
09/2011	Center symposium of the Center for Pathophysiology, Infectiology und Immunology (CePII), Vienna; Poster presentation
07/2010	Workplace Hazardous Materials Information System (WHMIS) Training, Concordia University, Montreal