Supporting immunotherapy efficacy using novel nutrition-based approaches to treat food allergy

“Teaming up with the enemy”

Marlotte Vonk

Supporting immunotherapy efficacy using novel nutrition-based approaches to treat food allergy

ISBN: 978-90-393-7004-9

Graphic design: Thomas van den Berg & Marlotte Vonk
Cover design, lay-out and printing: Guus Gijben, proefschrift-aio.nl

The research conducted in this thesis was financially supported by the NWO 'Open Technology Program' grant 12652 and embedded in the NUTRALL consortium project entitled: "Nutrition-based approach to support antigen-specific immunotherapy for food allergies".

Printing of this thesis was financially supported by Danone Nutricia Research and the Utrecht Institute for Pharmaceutical Sciences.

Supporting immunotherapy efficacy using novel nutrition-based approaches to treat food allergy

"Teaming up with the enemy"

Voedingsinterventies ter ondersteuning van immunotherapie voor de behandeling van voedselallergie

"Samenwerken met de vijand"

(*met een samenvatting in het Nederlands*)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 20 juni 2018 des middags te 2.30 uur

door

Marlotte Marianne Vonk

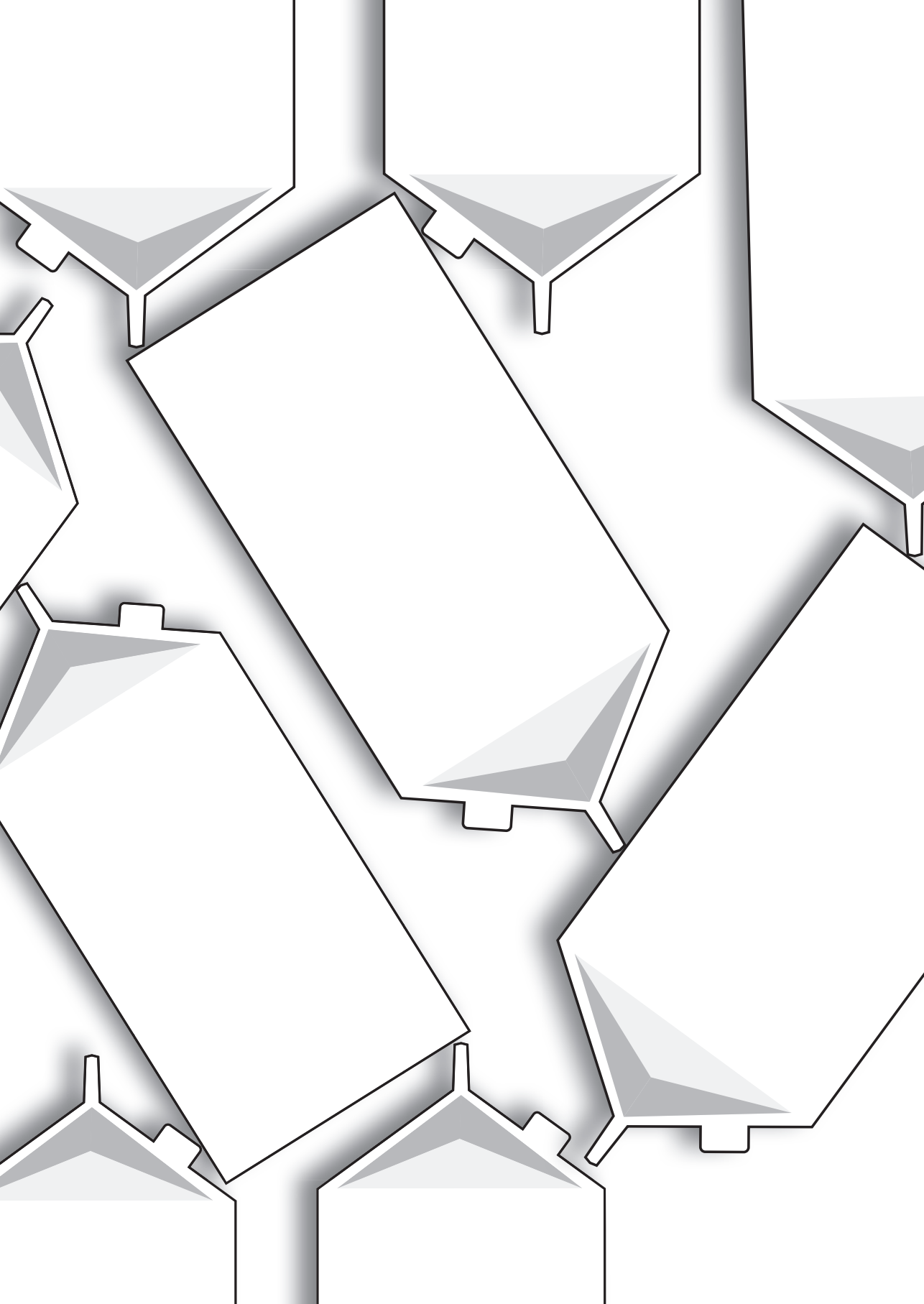
geboren op 25 mei 1989 te Gouda

Promotor:	Prof. dr. J. Garssen
Copromotoren:	Dr. B.C.A.M. van Esch Dr. L.M.J. Knippels

“Ik heb het nog nooit gedaan,
dus ik denk dat ik het wel kan”

- Pippi Langkous -

CONTENTS



CHAPTER 1

GENERAL INTRODUCTION

Atopy

The impact of environmental factors such as diet and genetic predisposition in the development of atopic disorders has been defined in the concept of the atopic march [1]. Atopy signifies an inherited hypersensitive state of the immune system leading to one or more pathological conditions (e.g., atopic dermatitis, asthma and food allergies) triggered by environmental allergens [2]. Starting with eczema and decreased skin barrier integrity, the sequential occurrence of atopic disorders in early life leads to (persistent) food allergies. A strong association between the severity and chronicity of atopic dermatitis in early life and food sensitization via antigen-presenting cells in disrupted skin has been described [3]. In addition, the absence of T helper 1 (Th1)-stimulating infections during immune development leads to a Th2-skewed response in the gastrointestinal tract of infants and excessive IgE production [4]. The epithelial barrier in the gut is exposed to a continuous high food antigen load and any disruptions in the integrity can further compromise tolerance induction toward food antigens [5].

Food allergy

Although high-quality data based on oral food challenge-proven food allergies in large populations are lacking, the general assumption is that the prevalence of food allergies in Western countries has increased over de past decades [6-8]. It has been estimated that food allergies affect up to 6% of the European population [9]. The number of hospital admissions due to food-induced anaphylaxis has doubled in children from 0-14 years of age over the last decades [10]. Moreover, increased prevalence of food sensitization and food allergies has been reported in developing countries in Asia and Africa that adapt to a Westernized life style and diet [11]. The high burden of food allergies on health, social and economic factors stress the need to intervene in the progression of food allergies and develop effective and safe treatment strategies.

In case of failure of the 'default' specific immunological unresponsiveness toward harmless food antigens recognized as oral tolerance, IgE-mediated food allergies are initiated upon a first encounter of a (dietary) protein in the intestinal lumen or skin and the uptake and processing by antigen-presenting cells (APC). Subsequently, APC present specific peptides to naïve CD4+ T cells in the lymph nodes via the MHC-T cell receptor complex. Under the influence of IL-4, IL-5 and IL-13, naïve T cells differentiate into Th2 cells. Th2 cells activate B cells in the lymphoid tissues resulting in class-switching from IgM to antigen-specific IgE production. IgE opsonizes effector cells like basophils present in the circulation and tissue-resident mast cells, a process

recognized as sensitization. A secondary encounter of the culprit food with IgE-opsonized effector cells induces cross-linking of the IgE-FcεRI complexes and initiates release of pro-inflammatory mediators into the systemic circulation and peripheral tissues [12]. The food allergic response can involve multiple organ systems (e.g., gastrointestinal tract, skin, respiratory system and oral cavity) and is characterized by various clinical symptoms: nausea, abdominal pain, constipation, diarrhea, skin rash, coughing, wheezing, edema and even systemic anaphylaxis [13].

Cow's milk allergy

Among the major food allergens (e.g., peanut, tree nuts, fish, shellfish, hen's egg, soy, wheat and seeds) are cow's milk proteins. Cow's milk contains over 25 different proteins; however, only a minority shows allergenic properties. Acidification of raw milk (pH 4.6 at 20°C) separates two main fractions: the casein proteins accounting for 80% and the whey proteins accounting for 20% of the total milk proteins [14]. The major allergenic proteins within the casein and whey fractions are αS1-casein, α-lactalbumin and β-lactoglobulin [15]. Cow's milk allergy (CMA) is recognized as one of the earliest food allergies observed in newborns. The EuroPrevall birth cohort study conducted in 9 European countries recruited 12,049 newborns and followed them until 2 years of age (77.5% of the participants). Oral food challenge-proven CMA was observed in 0.54% of the infants, with the highest rates observed in the Netherlands and the UK (both 1%) [16]. Natural resolution of food allergies typically occurs in children suffering from cow's milk, hen's egg, wheat and soy allergies, whereas allergies against peanut, tree nuts, fish and shellfish are characterized by a more persistent phenotype [17]. However, variable resolution rates have been reported in cow's milk allergic infants followed up to three to four years of age in a Danish cohort (75%) and a US cohort (19%) of children [18, 19]. It has been reported that the rate of natural outgrowth of allergies commonly known to resolve in early childhood is slowing down [20]. A Portuguese cohort of cow's milk allergic infants was followed to identify predictive factors of persistence of IgE-mediated CMA: high skin-prick-test (SPT) wheal diameter and high levels of specific IgE were associated with a reduced likelihood of natural tolerance induction [21]. After 2 years of age, none of the IgE-mediated cow's milk allergic individuals developed tolerance against cow's milk proteins compared to 34% in the non-IgE-mediated cow's milk allergic infants [21]. In addition, earlier age of first clinical signs and the severity of reactions to an oral food challenge were shown to be associated with CMA persistence in infants up to five years of age [22].

Food allergy management

Food allergies affect health-related quality of life of both patients and their caretakers due to dietary and social restrictions and anxiety for accidental exposures on a daily basis [23]. Current guidelines to manage food allergies are focused on complete allergen avoidance in the diet to prevent clinical symptoms and the administration of epinephrine in case of systemic anaphylaxis [24]. Long-term avoidance of specific food proteins can cause nutritional deficits and should be monitored carefully, especially in young infants with specific dietary requirements. Restoring oral tolerance toward food antigens by means of antigen-specific immunotherapy (AIT) has gained more interest over the past years and might provide a long-term solution for food allergic patients.

AIT consists of administration of controlled (gradually increasing) amounts of allergen on a daily basis to increase the threshold of adverse reactions during continuous exposure (i.e., 'desensitization'). Protection against allergic symptoms upon exposure observed after discontinuation of treatment is referred to as 'sustained unresponsiveness' or 'oral tolerance' [25]. Several routes of administration have been assessed, with the majority of the studies in food allergic patients focusing on oral immunotherapy (OIT) and sublingual immunotherapy (SLIT). Evidence for the clinical effectiveness of subcutaneous immunotherapy (SCIT) has been provided for respiratory allergies (e.g., allergic rhinitis, allergic rhino-conjunctivitis and allergic asthma) and insect venom hypersensitivities [26, 27]. A systematic evaluation of SCIT to treat allergic asthma indicated improved quality of life and reduced allergen-specific airway hyperreactivity (AHR); however, an increased risk of local and systemic adverse events was reported [28]. Moreover, severe systemic reactions induced by SCIT during two peanut allergy trials were common, even though clinical effectiveness was suggested [29, 30]. Research into the application of SCIT to treat food allergies has shifted to the use of recombinant hypo-allergenic proteins unable to elicit such severe adverse events [31].

Oral immunotherapy

The first reported case of OIT to treat a patient suffering from egg-induced anaphylaxis has been published in The Lancet in 1908 (A.T. Schofield, "A case of egg poisoning"). Gradual desensitization and prolonged maintenance dosing led to the recovery of tolerance toward egg proteins. To date, clinical studies provide evidence for the efficacy of OIT to treat cow's milk, peanut and hen's egg allergies; however, long-term protection in the absence of continuous allergen exposure remains a challenge [32, 33]. Evaluation of long-term safety of OIT to treat CMA was

conducted in an intervention study with 81 cow's milk allergic patients aged 5-18 years. 75% of cow's milk allergic patients subjected to OIT reacted occasionally and mild to the therapy and the treatment protocol was considered safe and efficacious for this group. However, 25% of the participants suffered from frequent, more severe and unpredictable adverse reactions upon treatment. Reaction persistence during treatment was associated with high specific IgE, increased SPT wheal size and increased symptom severity scores at baseline food challenges [34]. A systematic review and meta-analysis evaluating safety features of OIT trials showed significantly more adverse reactions (local and systemic) in the treated patients compared to the controls [35]. The current recommendation for treatment of IgE-mediated CMA states application under strict medical supervision in a specialized center with access to rescue medication in case of systemic anaphylaxis [36].

Successful clinical outcomes of OIT trials are associated with characteristic features of the antigen-specific immune response. Understanding the immunological changes induced by OIT during both desensitization and oral tolerance induction will contribute to optimization of the therapeutic approach. Initiation of OIT via frequent (low-dose) antigen exposure leads to sub-threshold activation of basophils and mast cells primed with specific IgE on surface expressed high-affinity FcεRI. Reduced release of granule content is observed despite the presence of high levels of specific IgE. Repeated stimulation of murine mast cells was shown to interfere with intracellular actin cytoskeleton arrangements with the FcεRI, thereby disabling the receptor-induced Ca^{2+} response needed to initiate degranulation [37]. Low-level allergen exposure controls responsiveness of effector cells and allows for dose escalation needed for the specific modulation of the Th2-mediated allergic response [38]. Continued high-level allergen exposure stimulates the differentiation of antigen-specific regulatory T cells and B cells (Tregs, Bregs) that target Th2 cell functionality and clonal expansion via the production of IL-10 and TGFβ [39, 40]. Treg-mediated class switching of antibody producing B cells and production of specific IgG4 by Bregs, substantially decrease the IgE/IgG4 ratio within months after initiation of OIT [41]. Specific IgG4 present in plasma samples of peanut-sensitized but tolerant individuals or peanut-allergic patients treated with OIT was shown to inhibit peanut-induced activation of sensitized basophils and mast cells *in vitro*. Depletion of IgG4 in plasma samples could partially restore effector cell activation [42]. Antigen-crosslinking of IgG subtypes bound to FcγRIIb and IgE bound to FcεRI expressed on the mast cell surface composes an inhibitory signal. In addition, IgG4 can bind and neutralize free allergens and thus prevent allergen-IgE complex formation on effector cells [43]. Long-term OIT results in a reduction in the number of tissue-resident mast cells and basophils circulating in the blood stream [44] and ensures sustained

unresponsiveness in a higher proportion of OIT-treated subjects [45]. A 6-year follow-up of OIT-treated individuals indicated that prolonged continuous allergen exposure is associated with the achievement of sustained unresponsiveness, irrespective of low (300 mg protein) or high (2 g protein) maintenance dosing [46]. Despite elaborative research into immunological mechanisms underlying OIT, knowledge gaps remain. We need an in-depth understanding of both local and systemic immune mechanisms to ensure optimal desensitization and long-term induction of tolerance next to ensured safety during and after treatment in each allergic individual.

Adjunct therapies

The use of immunomodulatory adjuvants (pre- and probiotics, Toll-like receptor ligands, modified bacteria expressing allergens, IFNγ) or anti-IgE monoclonal antibodies (omalizumab) provides new avenues in the optimization of OIT protocols [47]. Adjuvants might suppress the acute allergic response and thereby improve the safety and/or contribute to oral tolerance induction and improve the efficacy of OIT. Omalizumab was shown to prevent binding of IgE to the FcεRI and FcεRII and down-regulates receptor expression by mast cells and basophils [48]. Omalizumab treatment prior to and during a relatively rapid desensitization protocol in eleven cow's milk allergic children was accompanied by only mild side effects in 1.6% of the total doses provided (2199 doses in total). Although the results needed to be confirmed in future placebo-controlled phase II/III trials, the authors concluded that omalizumab treatment might improve the safety of OIT and allows for a more rapid dose escalation scheme [49]. Additional studies with either cow's milk, peanut or multiple food allergic subjects have found profound benefit of omalizumab treatment in rapidity of desensitization, higher maintenance dosing and reduced risk of (severe) side effects [50-52]. OIT with omalizumab treatment in three hen's egg allergic children successfully achieved desensitization; however, recurrence of allergic symptoms was observed after OIT was continued in the absence of omalizumab [53]. Questions remain with regard to the feasibility of long-term omalizumab treatment and whether patient safety is ensured upon discontinuation of the injections.

Oral administration of probiotics in combination with OIT has been studied once in a double-blind placebo-controlled randomized trial with 62 peanut allergic children. A fixed dose of *Lactobacillus rhamnosus* CGMCC and peanut protein was administered daily for a total of 18 months. Main findings of the study indicate successful achievement of sustained unresponsiveness in 82.1% of the subjects treated with OIT+probiotics compared to 3.6% in the placebo group upon a double-blind placebo-controlled food challenge (DBPCFC) performed 2 to 5 weeks after

therapy [54]. Effective desensitization was associated with reduced peanut-specific IgE levels, increased peanut-specific IgG4 levels and reduced SPT wheal size. A repeated DBPCFC after 4 years of follow-up showed sustained unresponsiveness in 58.1% in the OIT+probiotics group compared to 6.7% in the placebo group [55]. Further clarification of the additional benefit of probiotic supplementation on OIT efficacy is needed, since the study did not include a group with OIT only. Other attempts to study improved tolerance induction via oral probiotic administration included an open controlled trial in which cow's milk allergic infants were fed extensively hydrolyzed cow's milk-based infant formula (EHF) either or not combined with administration of *Lactobacillus rhamnosus* ATCC or non-cow's milk based protein formula's (rice-, soy- and amino-acid-based) which were considered as allergen avoidance groups. Tolerance induction rates after 12 months revealed significantly elevated tolerance levels in children receiving EHF+probiotics [56]. Although the data is limited, co-administration of probiotics or other nutritional compounds with immunomodulatory properties comprises a promising approach in the effort to optimize OIT protocols for food allergic patients.

Microbiome and fiber fermentation

Immune and metabolic systems are dependent on the availability of specific metabolites in the gastrointestinal tract. Human digestion of dietary components yields a substantial part of these healthy metabolites; however, an important role for the microbiome in the production of immunogenic metabolites has been recognized. Dietary fiber consists of plant-derived poly- and oligosaccharides resistant to enzymatic and chemical digestion during passage in the human digestive tract [57]. Dietary fiber is fermented in the human colon by the commensal microbes into short-chain fatty acids (SCFA), e.g., acetate, butyrate and propionate [58]. In addition to carbohydrates, SCFA can be formed after fermentation of dietary proteins and glycoproteins. Protein fermentation has suggested detrimental effects on host health, since it can simultaneously result in the production of toxic molecules (ammonia, amines, phenols and sulfides) [59]. The composition of the diet in combination with the abundance and activity of specific bacterial species resident in the colon, influence the availability of specific bioactive metabolites and thereby immune fitness. SCFA can cross the intestinal epithelial barrier and can interact with immune cells resident in the lamina propria via G-protein coupled receptors (GPCR), e.g., GPR41, GPR43 and GPR109a and were shown to support Treg differentiation in the human colon [60]. SCFA form an essential energy source for colonic epithelial cells and influence epigenetic mechanisms via inhibition of histone deacetylases in cell nuclei [61]. A limited amount of human studies has shown an association between

microbial diversity and the development or natural resolution of food allergies [62]. The gut microbiota composition in children suffering from food allergies was analyzed by means of high throughput 16S rRNA sequencing and revealed signs of microbial dysbiosis associated to gut inflammation compared to healthy controls [63]. In addition, cow's milk allergic infants were shown to have dysbiosis in their bacterial profile in faecal samples compared to healthy children [64]. Targeting the microbiome via nutritional interventions to support the growth of beneficial SCFA-producing bacteria might be a promising approach in food allergy management [65].

Biomarkers

Future application of adjunct therapies in combination with immunotherapy might benefit from the identification and validation of non-invasive and specific biomarkers. To date, immunotherapy trials rely mainly on serum levels of the gold-standard biomarker allergen-specific IgE, followed by specific IgG4, total IgE and/or the specific IgE/IgG4 ratio [66]. Immunoglobulin kinetics are used to determine patient inclusion criteria, monitor immunotherapy efficacy, determine successful (long-term) sustained unresponsiveness and were shown to be associated with safety features of highly-reactive patients [67]. Achievement of sustained unresponsiveness in egg allergic subjects treated with OIT was associated with lower baseline IgE specific for ovomucoid and egg white protein and higher post-treatment levels of IgG4 and IgA specific for egg white protein [68]. Recent advances in the biomarker field include the identification of a novel functional read-out in human basophils: intracellular fluochrome-labeled diamine oxidase expression in allergen-stimulated basophils derived from AIT-treated allergic subjects was elevated in comparison to non-treated participants with seasonal allergic rhinitis [69]. Transcription factors present in dendritic cells or surface expression of specific markers on B cells, T cells and innate lymphoid cells are currently under investigation as potential biomarkers, since mechanistic insight into both the innate and adaptive immune response during AIT rises [70]. Nevertheless, truly confirmed predictive and accessible biomarkers for the clinical responsiveness to immunotherapy are lacking to date.

Aim and outline of this thesis

A graphic outline of this thesis is depicted in **Figure 1**. The overall aim was to investigate whether supplementation with nutritional adjuvants can improve the efficacy of AIT to treat CMA. We used a pre-clinical murine model of oral food sensitization and applied OIT and SCIT to skew the allergic immune response toward a regulatory response. Dietary supplementation with non-digestible oligosaccharides or butyrate

was provided with or without OIT. Acute allergic symptoms upon food provocation tests were used as primary read-out for immunotherapy efficacy. Furthermore, we sought to gain more insight into the underlying mechanisms of desensitization and oral tolerance induction via cellular and humoral immunological parameters measured in blood, lymph organs and bone marrow and performed microbiome and bacterial metabolite analyses on faecal and cecum content samples. Murine models can provide valuable mechanistic insight into specific immunological processes taking place in non-accessible mucosal sites that are often excluded in human studies. We conducted in-depth immunological analyses in blood and tissue samples collected at several time points before, during and after the treatment protocol to study the kinetics of specific changes induced by OIT. In addition to the *in vivo* model, we performed *in vitro* experiments to investigate the epigenetic influence of dietary interventions on bone marrow-derived immune precursors. Together, the presented data of this thesis contribute to the future translation of alternative immunotherapy protocols with improved efficacy to treat food allergic patients.

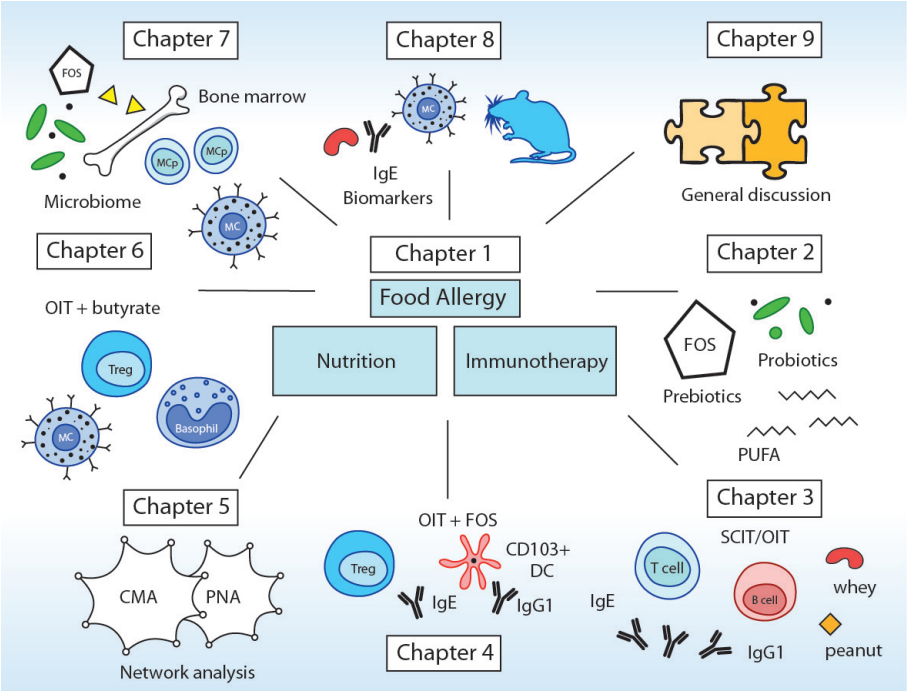


Figure 1. Graphic outline of this thesis.

In **chapter 2**, the influence of environmental factors such as maternal and infant diet, breast milk composition, exposure to pathogens, allergenicity of dietary proteins, allergen avoidance strategies and the Th2-skewed immune response in newborns was described in relation to the development of allergies in early life. Moreover, the immunomodulatory properties of probiotics, prebiotics, vitamins and poly-unsaturated fatty acids (PUFA) in the context of allergy prevention were discussed and present the rationale for the main research question of this thesis.

In order to study immunotherapy efficacy and the potential added benefit provided by nutritional interventions, we first developed a murine model of OIT and SCIT to treat CMA and peanut allergy (PNA) and investigated the dose of allergen needed to suppress acute allergic symptoms and induce immunologic changes as observed in human studies of AIT in **chapter 3**. It was shown that both OIT and SCIT effectively reduced acute allergic symptoms upon food challenges in the CMA and PNA models. Kinetics of allergen-specific immunoglobulins in serum were similar in the CMA and PNA models; however, T cell subset analysis and cytokine responses in lymph organs showed potential mechanistic differences between the two types of food allergy.

In **chapter 4**, we investigated whether supplementation with non-digestible oligosaccharides (fructo-oligosaccharides, FOS) could improve the efficacy of OIT in the CMA model. The severity of acute allergic symptoms upon local and systemic food challenges was reduced in mice treated with the combination of OIT and FOS. The challenge-induced increase in allergen-specific IgE observed in sensitized mice was absent after OIT+FOS treatment. An adoptive transfer experiment confirmed the involvement of antigen-specific CD4+CD25+ Tregs in the allergy protective effect induced by OIT+FOS.

To support future translation of the current approach to human trials, we collaborated with system biologists at TNO and performed multi-parameter analyses on the CMA and PNA immunotherapy data using Bayesian networks (BN) and topological data analysis (TDA) (**chapter 5**). This innovative approach compared mechanistic features of the CMA and PNA studies and contributes to the identification and validation of relevant biomarkers. Furthermore, TDA demonstrated a close link between *in vivo* SCFA data (e.g., butyrate, acetate and propionate) and the primary clinical outcomes in the CMA model.

We further explored the relation between the SCFA butyrate and the enhanced reduction in allergic symptoms induced by OIT+FOS in **chapter 6**. Oral butyrate administration in combination with OIT reduced acute allergic symptoms and

suppressed effector cell activation (basophils and mast cells). The combination of OIT and butyrate enhanced the suppressive activity of spleen-derived Tregs toward bone marrow-derived mast cells (BMMC) *in vitro*. It was concluded that both butyrate and FOS administration effectively improved efficacy of OIT in cow's milk allergic mice and are potential candidates for human OIT trials.

Chapter 7 presents the analysis of the microbial diversity and abundance in faecal samples in combination with bacterial metabolite (SCFA) levels in cecum content derived from mice treated with OIT+FOS. Relative abundances of specific bacterial genera were elevated in mice fed the FOS supplemented diet compared to the control diet. BMMC cultured from FOS exposed mice showed reduced expression of the FcεRI and c-Kit receptors and were less responsive in an IgE-mediated degranulation assay. We observed an increase in the abundance of the dysbiosis-associated phylum Proteobacteria in mice subjected to OIT alone and we proposed that FOS supplementation could counteract these unwanted inflammation-associated effects during treatment.

To support future application of OIT and FOS supplementation in clinical practice, we assessed the value of the gold-standard biomarker allergen-specific IgE in predicting the acute allergic response in an *in vitro* degranulation system in **chapter 8**. Effector cell degranulation observed after sensitization using experimental mouse serum and activation with allergen was shown to be associated with *in vivo* mucosal mast cell degranulation upon oral food provocation and correlated to the abundance of allergen-specific IgE in serum.

In **chapter 9**, we discussed the main findings of this thesis and proposed the potential underlying mechanisms of action of the allergy-suppressive effect induced by OIT and supplementation with FOS and butyrate. We pointed out the possible adverse effects of OIT on gut health and discussed future directions for the presented therapeutic strategies to enable translation to humans. Lastly, the thesis was finalized with the overall conclusion that the efficacy of OIT can be improved by co-administration of nutritional adjuvants with beneficial effects on immune, microbial and intestinal features, representing a promising novel approach for human food allergy trials.

REFERENCES

1. Spergel JM and Paller AS. Atopic dermatitis and the atopic march. J Allergy Clin Immunol 2003. 112(6 Suppl): p. S118-27.
2. Khan SJ, Dharmage SC, Matheson MC, and Gurrin LC. Is the atopic march related to confounding by genetics and early-life environment? A systematic review of sibship and twin data. Allergy 2018. 73(1): p. 17-28.
3. Tsakok T, Marrs T, Mohsin M, Baron S, du Toit G, Till S, and Flohr C. Does atopic dermatitis cause food allergy? A systematic review. Journal of Allergy and Clinical Immunology 2016. 137(4): p. 1071-1078.
4. Thomsen SF. Epidemiology and natural history of atopic diseases. Eur Clin Respir J 2015. 2.
5. Chahine BG and Bahna SL. The role of the gut mucosal immunity in the development of tolerance versus development of allergy to food. Curr Opin Allergy Clin Immunol 2010. 10(4): p. 394-9.
6. Sicherer SH and Sampson HA. Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention and management. J Allergy Clin Immunol 2017: p. 141(1):41-58.
7. Tang MLK and Mullins RJ. Food allergy: Is prevalence increasing? Internal Medicine Journal 2017. 47(3): p. 256-261.
8. Rona RJ, Keil T, Summers C, Gislason D, Zuidmeer L, Sodergren E, Sigurdardottir ST, Lindner T, Goldhahn K, Dahlstrom J, McBride D, and Madsen C. The prevalence of food allergy: A meta-analysis. J Allergy Clin Immunol 2007. 120(3): p. 638-46.
9. Nwaru BI, Hickstein L, Panesar SS, Roberts G, Muraro A, Sheikh A, Allergy EF, and Anaphylaxis Guidelines G. Prevalence of common food allergies in europe: A systematic review and meta-analysis. Allergy 2014. 69(8): p. 992-1007.
10. Vazquez-Ortiz M and Turner PJ. Improving the safety of oral immunotherapy for food allergy. Pediatric Allergy and Immunology 2016. 27(2): p. 117-125.
11. Leung AS, Wong GW, and Tang ML. Food allergy in the developing world. J Allergy Clin Immunol 2017: p. 141(1):76-78.e1.
12. Valenta R, Hochwallner H, Linhart B, and Pahr S. Food allergies: The basics. Gastroenterology 2015. 148(6): p. 1120-31 e4.
13. Bischoff S and Crowe SE. Gastrointestinal food allergy: New insights into pathophysiology and clinical perspectives. Gastroenterology 2005. 128(4): p. 1089-113.
14. Wal JM. Bovine milk allergenicity. Ann Allergy Asthma Immunol 2004. 93(5 Suppl 3): p. S2-11.
15. Taniuchi S, Takahashi M, Soejima K, Hatano Y, and Minami H. Immunotherapy for cow's milk allergy. Hum Vaccin Immunother 2017. 13(10): p. 2443-2451.
16. Schoemaker AA, Sprikkelman

AB, Grimshaw KE, Roberts G, Grabenhenrich L, Rosenfeld L, Siegert S, Dubakiene R, Rudzeviciene O, Reche M, Fiandor A, Papadopoulos NG, Malamitsi-Puchner A, Fiocchi A, Dahdah L, Sigurdardottir ST, Clausen M, Stanczyk-Przyluska A, Zeman K, Mills EN, McBride D, Keil T, and Beyer K. Incidence and natural history of challenge-proven cow's milk allergy in european children--europrevall birth cohort. Allergy 2015. 70(8): p. 963-72.

17. Burks AW, Tang M, Sicherer S, Muraro A, Eigenmann PA, Ebisawa M, Fiocchi A, Chiang W, Beyer K, Wood R, Hourihane J, Jones SM, Lack G, and Sampson HA. Icon: Food allergy. J Allergy Clin Immunol 2012. 129(4): p. 906-20.
18. Host A and Halken S. A prospective study of cow milk allergy in danish infants during the first 3 years of life. Clinical course in relation to clinical and immunological type of hypersensitivity reaction. Allergy 1990. 45(8): p. 587-96.
19. Skripak JM, Matsui EC, Mudd K, and Wood RA. The natural history of ige-mediated cow's milk allergy. J Allergy Clin Immunol 2007. 120(5): p. 1172-7.
20. Sicherer SH and Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. J Allergy Clin Immunol 2014. 133(2): p. 291-307; quiz 308.
21. Santos A, Dias A, and Pinheiro JA. Predictive factors for the persistence of cow's milk allergy. Pediatr Allergy Immunol 2010. 21(8): p. 1127-34.
22. Elizur A, Rajuan N, Goldberg MR, Leshno M, Cohen A, and Katz Y. Natural course and risk factors for persistence of ige-mediated cow's milk allergy. J Pediatr 2012. 161(3): p. 482-487 e1.
23. Vazquez-Ortiz M, Alvaro M, Piquer M, Dominguez O, Giner MT, Lozano J, Jimenez-Feijoo R, and Plaza AM. Impact of oral immunotherapy on quality of life in egg-allergic children. Pediatr Allergy Immunol 2015. 26(3): p. 291-294.
24. Muraro A, Werfel T, Hoffmann-Sommergruber K, Roberts G, Beyer K, Bindslev-Jensen C, Cardona V, Dubois A, duToit G, Eigenmann P, Fernandez Rivas M, Halken S, Hickstein L, Host A, Knol E, Lack G, Marchisotto MJ, Niggemann B, Nwaru BI, Papadopoulos NG, Poulsen LK, Santos AF, Skypala I, Schoepfer A, Van Ree R, Venter C, Worm M, Vlieg-Boerstra B, Panesar S, de Silva D, Soares-Weiser K, Sheikh A, Ballmer-Weber BK, Nilsson C, de Jong NW, Akdis CA, Allergy EF, and Anaphylaxis Guidelines G. Eaaci food allergy and anaphylaxis guidelines: Diagnosis and management of food allergy. Allergy 2014. 69(8): p. 1008-25.
25. Pajno GB, Fernandez-Rivas M, Arasi S, Roberts G, Akdis CA, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, Ebisawa M, Eigenmann P, Knol E, Nadeau KC, Poulsen LK, van Ree R, Santos AF, du Toit G, Dhami S, Nurmatov U, Boloh Y, Makela M, O'Mahony L, Papadopoulos N, Sackesen C, Agache I, Angier E, Halken S, Jutel M, Lau S, Pfaar O, Ryan D, Sturm G, Varga EM, van Wijk RG, Sheikh A, Muraro A, and Group

EAIG. Eaaci guidelines on allergen immunotherapy: Ige-mediated food allergy. Allergy 2017: p. 28(8):728-745.

26. Roberts G, Pfaar O, Akdis CA, Ansotegui IJ, Durham SR, Gerth van Wijk R, Halken S, Larenas-Linnemann D, Pawankar R, Pitsios C, Sheikh A, Worm M, Arasi S, Calderon MA, Cingi C, Dhami S, Fauquert JL, Hamelmann E, Hellings P, Jacobsen L, Knol EF, Lin SY, Maggina P, Mosges R, Oude Elberink JNG, Pajno GB, Pastorello EA, Penagos M, Rotiroti G, Schmidt-Weber CB, Timmermans F, Tsilochristou O, Varga EM, Wilkinson JN, Williams A, Zhang L, Agache I, Angier E, Fernandez-Rivas M, Jutel M, Lau S, van Ree R, Ryan D, Sturm GJ, and Muraro A. Eaaci guidelines on allergen immunotherapy: Allergic rhinoconjunctivitis. Allergy 2017: p. 00:1–33.
27. Rosman Y, Confino-Cohen R, and Goldberg A. Venom immunotherapy in high-risk patients: The advantage of the rush build-up protocol. Int Arch Allergy Immunol 2017. 174(1): p. 45-51.
28. Dhami S, Kakourou A, Asamoah F, Agache I, Lau S, Jutel M, Muraro A, Roberts G, Akdis CA, Bonini M, Cavkaytar O, Flood B, Gajdanowicz P, Izuhara K, Kalayci O, Mosges R, Palomares O, Pfaar O, Smolinska S, Sokolowska M, Asaria M, Netuveli G, Zaman H, Akhlaq A, and Sheikh A. Allergen immunotherapy for allergic asthma: A systematic review and meta-analysis. Allergy 2017. 72(12): p. 1825-1848.
29. Nelson HS, Lahr J, Rule R, Bock A, and Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. J Allergy Clin Immunol 1997. 99(6 Pt 1): p. 744-51.
30. Oppenheimer JJ, Nelson HS, Bock SA, Christensen F, and Leung DY. Treatment of peanut allergy with rush immunotherapy. J Allergy Clin Immunol 1992. 90(2): p. 256-62.
31. Zuidmeer-Jongejan L, Fernandez-Rivas M, Poulsen LK, Neubauer A, Asturias J, Blom L, Boye J, Bindslev-Jensen C, Clausen M, Ferrara R, Garosi P, Huber H, Jensen BM, Koppelman S, Kowalski ML, Lewandowska-Polak A, Linhart B, Maillere B, Mari A, Martinez A, Mills CE, Nicoletti C, Opstelten DJ, Papadopoulos NG, Portoles A, Rigby N, Scala E, Schnoor HJ, Sigurdardottir ST, Stavroulakis G, Stolz F, Swoboda I, Valenta R, van den Hout R, Versteeg SA, Witten M, and van Ree R. Fast: Towards safe and effective subcutaneous immunotherapy of persistent life-threatening food allergies. Clin Transl Allergy 2012. 2(1): p. 5.
32. Freeland DMH, Manohar M, Andorf S, Hobson BD, Zhang W, and Nadeau KC. Oral immunotherapy for food allergy. Semin Immunol 2017. 30: p. 36-44.
33. Chiang D and Berin MC. An examination of clinical and immunologic outcomes in food allergen immunotherapy by route of administration. Curr Allergy Asthma Rep 2015. 15(6): p. 35.
34. Vazquez-Ortiz M, Alvaro-Lozano M, Alsina L, Garcia-Paba MB, Piquer-

Gibert M, Giner-Munoz MT, Lozano J, Dominguez-Sanchez O, Jimenez R, Dias M, Martin-Mateos MA, and Plaza-Martin AM. Safety and predictors of adverse events during oral immunotherapy for milk allergy: Severity of reaction at oral challenge, specific ige and prick test. Clin Exp Allergy 2013. 43(1): p. 92-102.

35. Nurmatov U, Dhami S, Arasi S, Pajno GB, Fernandez-Rivas M, Muraro A, Roberts G, Akdis C, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, du Toit G, Ebisawa M, Eigenmann P, Knol E, Makela M, Nadeau KC, O'Mahony L, Papadopoulos N, Poulsen LK, Sackesen C, Sampson H, Santos AF, van Ree R, Timmermans F, and Sheikh A. Allergen immunotherapy for ige-mediated food allergy: A systematic review and meta-analysis. Allergy 2017. 72(8): p. 1133-1147.
36. Jutel M, Agache I, Bonini S, Burks AW, Calderon M, Canonica W, Cox L, Demoly P, Frew AJ, O'Hehir R, Kleine-Tebbe J, Muraro A, Lack G, Larenas D, Levin M, Nelson H, Pawankar R, Pfaar O, van Ree R, Sampson H, Santos AF, Du Toit G, Werfel T, Gerth van Wijk R, Zhang L, and Akdis CA. International consensus on allergy immunotherapy. J Allergy Clin Immunol 2015. 136(3): p. 556-68.
37. Ang WX, Church AM, Kulis M, Choi HW, Burks AW, and Abraham SN. Mast cell desensitization inhibits calcium flux and aberrantly remodels actin. J Clin Invest 2016. 126(11): p. 4103-4118.
38. Kulis MD, Patil SU, Wambre E, and Vickery BP. Immune mechanisms of oral immunotherapy. J Allergy Clin Immunol 2017: p. pii: S0091-6749(17)32948-2.
39. Palomares O, Akdis M, Martin-Fontecha M, and Akdis CA. Mechanisms of immune regulation in allergic diseases: The role of regulatory t and b cells. Immunol Rev 2017. 278(1): p. 219-236.
40. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, Ruckert B, Akdis CA, and Akdis M. Igg4 production is confined to human il-10-producing regulatory b cells that suppress antigen-specific immune responses. J Allergy Clin Immunol 2013. 131(4): p. 1204-12.
41. van de Veen W, Wirz OF, Globinska A, and Akdis M. Novel mechanisms in immune tolerance to allergens during natural allergen exposure and allergen-specific immunotherapy. Curr Opin Immunol 2017. 48: p. 74-81.
42. Santos AF, James LK, Bahnson HT, Shamji MH, Couto-Francisco NC, Islam S, Houghton S, Clark AT, Stephens A, Turcanu V, Durham SR, Gould HJ, and Lack G. Igg4 inhibits peanut-induced basophil and mast cell activation in peanut-tolerant children sensitized to peanut major allergens. J Allergy Clin Immunol 2015. 135(5): p. 1249-56.
43. Shamji MH and Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. J Allergy Clin Immunol 2017. 140(6): p. 1485-1498.

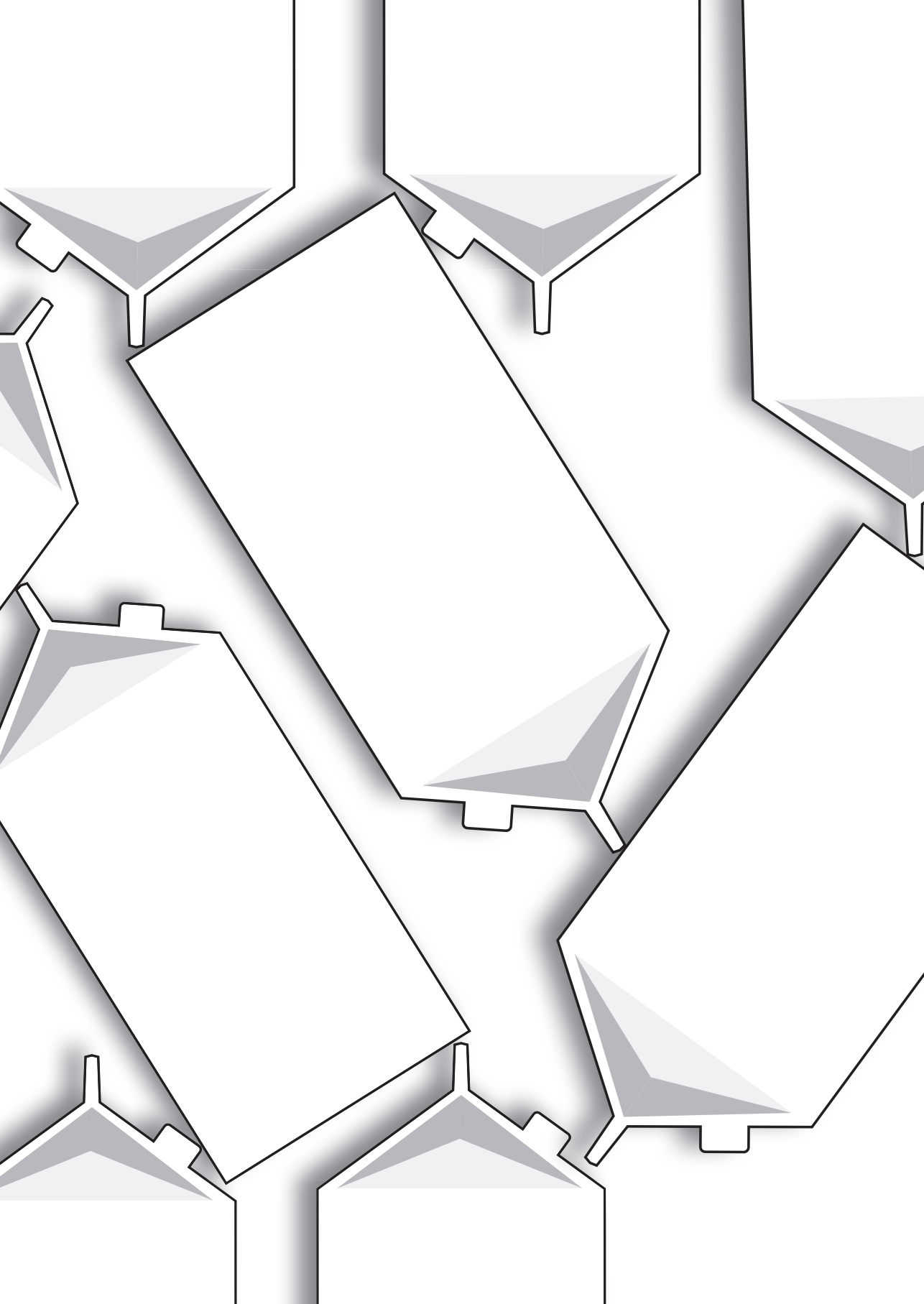
44. Akdis CA and Akdis M. Mechanisms of allergen-specific immunotherapy. J Allergy Clin Immunol 2011. 127(1): p. 18-27; quiz 28-9.
45. Jones SM, Burks AW, Keet C, Vickery BP, Scurlock AM, Wood RA, Liu AH, Sicherer SH, Henning AK, Lindblad RW, Dawson P, Berin C, Fleischer DM, Leung DYM, Plaut M, Sampson HA, and Consortium of Food Allergy R. Long-term treatment with egg oral immunotherapy enhances sustained unresponsiveness that persists after cessation of therapy. J Allergy Clin Immunol 2016. 137(4): p. 1117-1127 e10.
46. Andorf S, Manohar M, Dominguez T, Block W, Tupa D, Kshirsagar RA, Sampath V, Chinthrajah RS, and Nadeau KC. Feasibility of sustained response through long-term dosing in food allergy immunotherapy. Allergy Asthma Clin Immunol 2017. 13: p. 52.
47. Loh W and Tang M. Adjuvant therapies in food immunotherapy. Immunol Allergy Clin North Am 2018. 38(1): p. 89-101.
48. Prussin C, Griffith DT, Boesel KM, Lin H, Foster B, and Casale TB. Omalizumab treatment downregulates dendritic cell fcepsilonri expression. J Allergy Clin Immunol 2003. 112(6): p. 1147-54.
49. Nadeau KC, Schneider LC, Hoyte L, Borras I, and Umetsu DT. Rapid oral desensitization in combination with omalizumab therapy in patients with cow's milk allergy. J Allergy Clin Immunol 2011. 127(6): p. 1622-4.
50. Sampson HA, Leung DY, Burks AW, Lack G, Bahna SL, Jones SM, and Wong DA. A phase ii, randomized, doubleblind, parallelgroup, placebocontrolled oral food challenge trial of xolair (omalizumab) in peanut allergy. J Allergy Clin Immunol 2011. 127(5): p. 1309-10 e1.
51. Wood RA, Kim JS, Lindblad R, Nadeau K, Henning AK, Dawson P, Plaut M, and Sampson HA. A randomized, double-blind, placebo-controlled study of omalizumab combined with oral immunotherapy for the treatment of cow's milk allergy. J Allergy Clin Immunol 2016. 137(4): p. 1103-1110 e11.
52. Andorf S, Purington N, Block WM, Long AJ, Tupa D, Brittain E, Rudman Spergel A, Desai M, Galli SJ, Nadeau KC, and Chinthrajah RS. Anti-ige treatment with oral immunotherapy in multifood allergic participants: A double-blind, randomised, controlled trial. Lancet Gastroenterol Hepatol 2017: p. 3(2):85-94.
53. Lafuente I, Mazon A, Nieto M, Uixera S, Pina R, and Nieto A. Possible recurrence of symptoms after discontinuation of omalizumab in anti-ige-assisted desensitization to egg. Pediatr Allergy Immunol 2014. 25(7): p. 717-9.
54. Tang ML, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, and Donath S. Administration of a probiotic with peanut oral immunotherapy: A randomized trial. J Allergy Clin Immunol 2015. 135(3): p. 737-44 e8.
55. Hsiao KC, Ponsonby AL, Axelrad C,

Pitkin S, and Tang MLK. Long term effects of a probiotic and peanut oral immunotherapy (ppoit) treatment on peanut allergic children. Journal of Allergy and Clinical Immunology 2017. 139(2): p. Ab136-Ab136.

56. Canani RB, Nocerino R, Terrin G, Frediani T, Lucarelli S, Cosenza L, Passariello A, Leone L, Granata V, Di Costanzo M, Pezzella V, and Troncone R. Formula selection for management of children with cow's milk allergy influences the rate of acquisition of tolerance: A prospective multicenter study. Journal of Pediatrics 2013. 163(3): p. 771-+.
57. McKenzie C, Tan J, Macia L, and Mackay CR. The nutrition-gut microbiome-physiology axis and allergic diseases. Immunol Rev 2017. 278(1): p. 277-295.
58. Richards LB, Li M, van Esch BCAM, Garssen J, and Folkerts G. The effects of short-chain fatty acids on the cardiovascular system. PharmaNutrition 2016. 4.
59. Windey K, De Preter V, and Verbeke K. Relevance of protein fermentation to gut health. Mol Nutr Food Res 2012. 56(1): p. 184-96.
60. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, and Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory t cells. Nature 2013. 504(7480): p. 446-50.
61. Schilderink R, Verseijden C, Seppen J, Muncan V, van den Brink GR, Lambers TT, van Tol EA, and de Jonge WJ. The scfa butyrate stimulates the epithelial production of retinoic acid via inhibition of epithelial hdac. Am J Physiol Gastrointest Liver Physiol 2016. 310(11): p. G1138-46.
62. Blazquez AB and Berin MC. Microbiome and food allergy. Transl Res 2017. 179: p. 199-203.
63. Inoue R, Sawai T, Sawai C, Nakatani M, Romero-Perez GA, Ozeki M, Nonomura K, and Tsukahara T. A preliminary study of gut dysbiosis in children with food allergy. Biosci Biotechnol Biochem 2017. 81(12): p. 2396-2399.
64. Thompson-Chagoyan OC, Fallani M, Maldonado J, Vieites JM, Khanna S, Edwards C, Dore J, and Gil A. Faecal microbiota and short-chain fatty acid levels in faeces from infants with cow's milk protein allergy. Int Arch Allergy Immunol 2011. 156(3): p. 325-32.
65. Aitoro R, Paparo L, Amoroso A, Di Costanzo M, Cosenza L, Granata V, Di Scala C, Nocerino R, Trinchese G, Montella M, Ercolini D, and Canani RB. Gut microbiota as a target for preventive and therapeutic intervention against food allergy. Nutrients 2017. 9(7).
66. Shamji MH, Kappen JH, Akdis M, Jensen-Jarolim E, Knol EF, Kleine-Tebbe J, Bohle B, Chaker AM, Till SJ, Valenta R, Poulsen LK, Calderon MA, Demoly P, Pfaar O, Jacobsen L,

Durham SR, and Schmidt-Weber CB. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: An eaaci position paper. Allergy 2017. 72(8): p. 1156-1173.

67. Savilahti EM, Kuitunen M, Valori M, Rantanen V, Bardina L, Gimenez G, Makela MJ, Hautaniemi S, Savilahti E, and Sampson HA. Use of ige and igg4 epitope binding to predict the outcome of oral immunotherapy in cow's milk allergy. Pediatric Allergy and Immunology 2014. 25(3): p. 227-235.
68. Wright BL, Kulis M, Orgel KA, Burks AW, Dawson P, Henning AK, Jones SM, Wood RA, Sicherer SH, Lindblad RW, Stablein D, Leung DY, Vickery BP, Sampson HA, and Consortium of Food Allergy R. Component-resolved analysis of iga, ige, and igg4 during egg oit identifies markers associated with sustained unresponsiveness. Allergy 2016. 71(11): p. 1552-1560.
69. Shamji MH, Layhadi JA, Scadding GW, Cheung DK, Calderon MA, Turka LA, Phippard D, and Durham SR. Basophil expression of diamine oxidase: A novel biomarker of allergen immunotherapy response. J Allergy Clin Immunol 2015. 135(4): p. 913-21 e9.
70. Kouser L, Kappen J, Walton RP, and Shamji MH. Update on biomarkers to monitor clinical efficacy response during and post treatment in allergen immunotherapy. Curr Treat Options Allergy 2017. 4(1): p. 43-53.



CHAPTER 2

DIETARY INTERVENTIONS IN INFANCY

Marlotte M. Vonk[1,2*]
Atanaska I. Kostadinova[1,2*]
Matthias V. Kopp[3]
Betty C.A.M van Esch[1,2]
Linette E.M. Willemsen[1]
Léon M.J. Knippels[1,2]
Johan Garssen[1,2]

1. Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
2. Immunology Platform, Danone Nutricia Research, Utrecht, the Netherlands
3. Department of Pediatric Pulmonology, Clinic for Pediatric and Adolescent Medicine, University of Lübeck, Lübeck, Germany

* These authors contributed equally to this work

Published in "Allergy, Immunity and Tolerance in Early Childhood: The First Steps of the Atopic March" - Pediatric Allergy and Immunology, Academic Press, Elsevier (2016)

ABSTRACT

The prevalence of food allergies in infants has increased, indicating that the maturation of the infant's immune system and the development of oral tolerance to food antigens are hampered. Exposure to an altered Western diet and hygienic living conditions appear to skew the infant's immune response toward an atopic phenotype. Allergen avoidance strategies are challenged nowadays and active tolerance induction via immunomodulatory food components and/or modified allergens is of key interest. Dietary interventions with probiotics, prebiotics, synbiotics, n-3 polyunsaturated fatty acids or antioxidants show potential in supporting tolerance induction. Clinical trials have provided promising results. However, variation in the timing, dose, duration and type of dietary intervention used delay its implementation in early life strategies for allergy prevention. Modulation of the infant's immune response might benefit from applying dietary interventions both prenatally as well as postnatally.

INTRODUCTION

Allergy is a serious health concern that might have life-threatening consequences. In recent years there has been a tendency for the number of allergic disorders to increase. One group of allergic disorders particularly prevalent in infancy and early childhood is food allergy. Estimates show that approximately 8% of children compared with 3-4% of adults have food allergies [1, 2]. Together with atopic eczema, food allergy is one of the earliest manifestations of the atopic march, which is defined as the progression from a food-allergic state in early life to inhalant-triggered hypersensitivity and/or asthma later in life [3]. An important characteristic of the allergic sensitization state is the presence of allergen-specific immunoglobulin E (IgE) levels at various stages of life, including the prenatal period [4]. About 80% of food allergies are triggered by proteins such as milk, peanuts, eggs, soybeans, tree nuts, and wheat [5]. The most common food allergy, but also the earliest, occurring within the first year of life iscow's milk allergy (CMA). Although early life food allergies are spontaneously outgrown with time, those tend to become more persistent. CMA is outgrown in about 85-90% of children by age 3 [6], and egg allergy in about 80% by age 5 [7]. However, studies show that percentages of spontaneous remission are declining to 79% for CMA and 68% for egg allergy by age 16 [8, 9]. Other food allergies, such as peanut and tree nut allergy, are more persistent (only 20% might spontaneously resolve) and are associated with most life-threatening and fatal anaphylaxis [10, 11].

Currently, there is no treatment for food allergy. Both preventive and therapeutic approaches in the clinic focus on avoiding the culprit food. In the meantime, research has been considerably influenced by the hypothesis that food allergies result from the inability to build oral tolerance or from defects in already established oral tolerance to the ingested food proteins. Therefore, scientific interest has been intensively shifting from the allergen avoidance to the tolerance induction approach. The first days of life, or maybe even the time before birth, are considered an important window of opportunity for applying preventive strategies to reduce the risk of food allergic sensitization. Preventive approaches in this period might be able to interfere with the immune system development and maturation in a beneficial way to promote the proper establishment of naturally occurring tolerance.

The underlying mechanisms and the etiology are still largely unclear; food allergy is believed to rely on a complex interaction between genetic and environmental factors. Predisposition, or high-risk status, is considered when at least one first-degree relative is diagnosed with allergy. However, many children present with atopic diseases early in life even with no family history of allergy [3]. This highlights the importance of environmental exposure in early life, especially its considerable implication in food

allergy development. One of the most significant exposures for immune maturation in early life is the diet. The increasing prevalence of atopic diseases is suggested to relate to decline in breast feeding, changes in modern Western diet (e.g., increased intake of n-6 over n-3 polyunsaturated fatty acids [PUFA]), and reduction in microbial exposure (hygiene hypothesis) [12, 13]. Furthermore, some dietary components are suggested to possess beneficial immunomodulatory effects. All of these point to the major role of environmental and dietary exposures in the pathogenesis of allergy. It is therefore of great interest to consider dietary interventions with specific dietary components or supplements with recognized beneficial effects on the immune system for prevention strategies in early life. Many dietary components such as breast milk, probiotics, prebiotics, synbiotics, n-3 long-chain PUFA (LCPUFA), and antioxidants are currently being investigated for their potential effect on altering food allergy outcome. In addition, modifying food proteins to reduce their allergenicity might contribute to food allergy prevention and oral tolerance induction. Most important, some strategies might be applicable not only postnatally, but also prenatally because of the implications that certain nutrients in the maternal diet can influence predisposition to allergies via epigenetic changes [14, 15].

The gut, the diet, and oral tolerance in early life

The gastrointestinal (GI) tract is the largest immune organ in the human body. In early life, it is the central site for natural immune tolerance evolution. Rapidly after birth, depending on the method of delivery, the neonatal GI tract is colonized by many types of microorganisms. This process takes about a week, but the number and composition of the bacteria are relatively unstable during the first year of life [16]. A complex interplay between those commensal microorganisms, the mucosal barrier, oral exposure to dietary factors, and host-related factors is responsible for maturation of the gut-associated lymphoid tissue (GALT) and mucosal immune network [13, 15]. The importance of the gut microbiota for the immune response is emphasized in studies with germ-free animals that present with abnormal development of Peyer's patches and mesenteric lymph nodes (MLN), together with an impaired resistance to infections [17]. Beyond microbiota, mice fed a balanced protein-free diet were reported to present with poorly developed GALT, reduced levels of IgG, IgA, and lymphocytes, and a predominant Th2-type immune response [18]. Such studies point to the importance of exposure to both microbes and dietary antigens for proper development and maturation of the immune system in the newborn.

Inflammatory responses to harmless antigens are undesirable and are prevented by the evolution of default homeostatic mechanisms leading to local and systemic specific unresponsiveness, also referred to as oral tolerance [19]. This phenomenon specifically occurs in the gut and develops after exposure to a dietary antigen via the oral route. This highlights the fact that the normal development of oral tolerance is strongly antigen-dependent and might require early exposure to food proteins [20]. The mechanisms underlying oral tolerance are not yet understood, but some studies indicate that they involve induction of anergy or deletion of antigen-specific T cells and differentiation of regulatory T cells (Tregs) [21]. The induction of Tregs is facilitated by professional antigen-presenting cells (APC) with regulatory properties, such as dendritic cells (DC), and suppressive soluble factors, such as interleukin-10 (IL-10) and transforming growth factor β (TGFβ) cytokines [22]. The role of Tregs in inducing and maintaining oral tolerance is considered crucial. Furthermore, Peyer's patches and MLN are important sites for tolerance induction because they accommodate the interactions between DC and naïve T cells [23]. The MLN appear to be the major site for mucosal allergen presentation and tolerance induction because lack of MLN results in no tolerance development [24]. Other factors such as the availability of the antigen, the immune microenvironment, the type and activation status of the APC, the intestinal flora, and the age and genetics of the host are essential for tolerance development [25, 26].

Although neonates have an immature gut and immunomodulatory network, their immune system is considered prone to tolerance induction. Regardless of the Th2-skewed effector response, it is suggested that any kind of immune response could be induced in the neonate if the appropriate conditions are provided [27]. Therefore, modifiable factors such as gut colonization and diet in early life can be targeted for optimizing immune system maturation and susceptibility to tolerance induction.

Allergens in the diet: from avoidance toward tolerance induction

The classical approach for preventing and reducing food allergic reactions is to avoid the offending food. Restriction diets and strict allergen avoidance have been applied not only to adults but also to infants, neonates, and even fetuses by implementing allergic protein avoidance during early life, lactation, and pregnancy [27]. However, the dogma of allergen avoidance is currently challenged by increasing interest in early life, even prenatal, allergen exposure for its crucial role in tolerance induction.

Allergens in the maternal diet

Next to early postnatal exposures, prenatal exposure to food allergens may have a crucial role in allergy development, and therefore preventive measures during pregnancy might prove effective. *In utero* exposure to food allergens is suggested to take place via transplacental transport [28] and/or the amniotic fluid [29]. Reports describe that the fetal immune system might be ready for sensitization as early as 20 weeks of gestation, based on the presence of IgM-positive B cells [30]. Around this time, allergen-specific responses to mitogenic and allergenic stimuli have been demonstrated in cord blood [31]. Furthermore, allergen-specific IgE has been detected at birth and allergic disease has been reported as early as 8 days after birth, both of which imply that *in utero* acquired antigens could initiate fetal lymphocyte proliferation and sensitization [32]. Such evidence suggests that mothers should avoid allergenic proteins in their diet during pregnancy. However, maternal restriction diets during pregnancy have not resulted in protection from food allergy in high-risk offspring. On the contrary, adverse effects on the maternal and fetal nutrition together with higher, although not significant, risk of preterm delivery and lower birth weight were documented [33]. Maternal exposure to food allergens during pregnancy, on the other hand, can benefit allergy prevention. Studies in guinea pigs have shown that oral antigen exposure not only during pregnancy, but also before conception is important for developing tolerance and can prevent postnatal IgE sensitization [32]. Mechanistically, this effect could be attributed to the transfer of maternal allergen-specific IgG, which 1) blocks IgE, 2) suppresses B cells by facilitating the cross-link between neonatal FcεRIIb and B cell receptor, or 3) helps antigenic determinants destruction by phagocytosis [32]. Results from studies in rats confirm that maternal IgG that is passively transferred to the offspring ensures long-lasting suppression against IgE sensitization [34].

After birth, there is also a risk that allergens from the maternal diet can be transferred during breast feeding. Intact dietary antigens such as bovine β-lactoglobulin (BLG), hen's egg ovalbumin (OVA), gliadin from wheat, and Ara h 1 from peanuts have been detected in human breast milk [35]. This implies that mothers should avoid allergens during lactation as well. However, maternal restriction diets during lactation have failed to prevent food allergy development [33]. Instead, evidence from animal studies suggests that maternal exposure to a dietary antigen during lactation facilitates antigen-specific tolerance induction [36]. Studies in non-atopic mice revealed that combined exposure to allergen and TGFβ in breast milk was necessary for the development of Tregs and allergen-specific tolerance in the offspring [16]. In contrast, allergen exposure of lactating allergic mice induces tolerance in the offspring via antigen-IgG complexes formed in breast milk, rather than TGFβ [37]. The intake

of allergenic proteins by pregnant or lactating mothers can influence the immune system of the newborn. However, the exact mechanism of oral tolerance induction in the child via maternal exposure to allergens or allergen alternatives alone or combined with dietary components needs to be clarified.

Allergens in the infant diet

Allergen avoidance in children at risk is an intensive topic of debate. The gold standard for primary allergy prevention in high-risk infants is avoidance of allergens. The rationale behind this approach is that delaying the introduction of complementary feeding and avoiding allergenic foods would reduce the risk of systemic allergic responses in the leaky gut of infants whose mucosal immune system is still immature [38]. In case of peanuts, this approach was applied for up to age 3 years [39]. However, later findings in the field triggered re-evaluation of the practices, and in 2008 it was concluded that there is no sufficient evidence to support specific food avoidance and complementary feeding delay [40]. In their article, Palmer *et al.* reviewed older and newer (after 2008) birth cohort studies and suggested that early allergen avoidance is associated with increased risk of sensitization [38]. This has been further confirmed in animal models in which allergen restriction in early life was linked to increased IgE sensitization [41]. Studies in humans and animals suggest the existence of alternative routes of sensitization in early life, such as via the skin, which could contribute to the disruption of normal oral tolerance development [18, 42]. By avoiding mucosal allergen exposure in early life, the normal acquisition of tolerance upon oral exposure is circumvented and as a consequence the risk of allergen sensitization via the skin is increased [18].

In past years, scientific interest has shifted toward immune tolerance induction in early life as a prospective preventive strategy. Evidence from animal studies shows that the process of oral tolerance is strongly antigen dependent and that exposure to the certain antigen during a specific time period might be essential for the process [43]. Studies that support early life allergen exposure as an important factor for tolerance induction in toddlers have observed that children from families in which a peanut snack is consumed early in life are reported to have a low incidence of peanut allergy [44]. A key study emphasizing the importance of early introduction to allergenic foods in oral tolerance reported a lower peanut allergy rate in Israeli infants fed roasted peanut butter products in their first year of life, compared with Jewish infants on a restricted diet in the United Kingdom (0.17% vs 1.85%, respectively) [45]. Animal studies further suggest that early life exposure to a large amount of allergen is more prone to promoting tolerance by means of suppressing IgE responses [46]. To further investigate

the potential effect of early allergen exposure on tolerance induction and prevention of allergic sensitization, a number of randomized controlled trials were started [47, 48].

In general, the strategy of active tolerance induction for food allergy prevention shows promising efficacy, but more insight is needed into the proper presentation of the allergen (such as form, dose, and timing) that will benefit the natural mechanisms of tolerance.

Immunomodulation via dietary interventions

The paradigm for allergen avoidance is being challenged; currently, restriction diets for both mother and child are not recommended as long as they are not already diagnosed with food allergy. Early dietary interventions for allergy prevention therefore focus on alternative protocols or dietary supplements with immunomodulatory properties (**Figure 1**). The underlying reasoning is that such dietary components might be able to modulate food allergy development by actively improving immune system maturation and supporting natural oral tolerance induction. An important determinant of the efficacy of dietary interventions aiming to prevent allergy in early life is the timing of the supplementation. Most clinical studies focus on dietary interventions after birth, but suggestions point to prenatal supplementation as a potential contributor to oral tolerance induction.

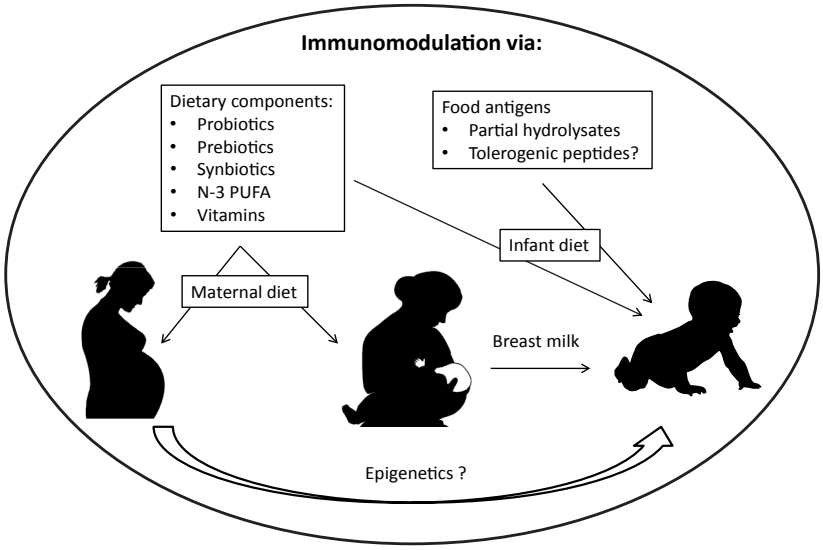


Figure 1. Early dietary interventions with immunomodulatory effects.

Breast milk

In early life, the most important dietary source that offers ideal nutritional, immunologic, and physiologic provision for healthy growth and development is breast milk. Because the developing immune system of the newborn requires not only protective factors against infections but also a suitable microenvironment for oral tolerance development, breast milk provides nutrients, growth factors, immunomodulatory molecules (e.g., immunoglobulins, lactoferrin, oligosaccharides, LCPUFA, cytokines, hormones, antioxidants), and antigens from the maternal diet and maternal immune cells [13]. Therefore, additional information on maternal diet and its influence on breast milk composition might create new horizons for the use of maternal imprinting or epigenetic modifications for allergy prevention strategies.

Hydrolysates

Because it is not always possible to feed an infant exclusively with breast milk, infant formulas based on cow's milk proteins are given in the first months of life. Cow's milk protein formulas are considered to provide infants with 106 times higher oral antigenic load compared with breast milk [49]. Modifications of the standard milk formulas are necessary to reduce the antigenic load, especially in infants at high risk of developing allergy. The most common modifications of cow's milk proteins are enzymatic hydrolysis, heat treatment, and/or ultrafiltration. The goal is to reduce the molecular weight, peptide size, and allergenicity of cow's milk proteins [50]. Lower-molecular-weight proteins are normally expected to induce immunological tolerance rather than sensitization upon uptake by GALT cells [49]. Depending on the cow's milk protein source, hydrolyzed formulas can be casein-based or whey-based. In addition, the degree of hydrolysis and the length of the remaining peptides classify hydrolysates as partial and extensive. In some cases, even amino acid-based formulas are used. Although no clear definitions exist, partially hydrolyzed formulas are considered to contain a high percentage of fractions with a molecular weight between 3 and 10 kDa, whereas extensive hydrolysates mainly contain peptides of less than 3 kDa [49]. Partially hydrolyzed formulas are often used for prevention in high-risk babies, whereas extensive and amino acid formulas are recommended for infants with a diagnosed food allergy or high risk of anaphylaxis, respectively [51]. This reflects the fact that partial hydrolysates contain larger fragments and may still induce allergic symptoms. Both partially and extensively hydrolyzed infant formulas have been investigated for food allergy prevention in the past decade, and both have shown a potential to reduce the risk of developing atopic dermatitis (AD) and CMA in high-risk infants compared with standard infant formula [12, 50, 52-55]. Most of the effects observed, regardless of their magnitude, have been reported to persist

for up to 24 months [49]. Long-term preventive effects of partial whey hydrolysates have been monitored after 5 years; they reduced the cumulative number of atopic manifestations significantly compared with standard formula [56]. A large clinical trial on the preventive effects of partial whey, extensive whey, and extensive casein hydrolysates demonstrated that the cumulative allergy-preventive effects on AD of both partial whey hydrolysate and extensive casein hydrolysate persist after 6 years [55] and 10 years [57].

Not much is known about the mechanisms by which hydrolysates might reduce the risk of allergy. In most clinical studies, hydrolyzed infant formulas have been used to avoid the sensitizing epitopes, so there is not much information about whether the clinical effects could result from active oral tolerance induction. Suggestions are that the larger fragments in partial hydrolysates account not only for higher allergenicity compared with extensive hydrolysates, but also for the higher immunogenicity that might lead to allergy prevention via tolerance induction [58]. Such hypotheses have been strongly supported by data from animal studies in which partial rather than extensive hydrolysates were demonstrated to induce tolerance to the allergen, implying the presence of tolerogenic milk protein fragments or peptides in the former but not in the latter one [59-61]. In a more recent study, Van Esch *et al.* investigated the tolerizing capacity of hydrolyzed whey formulas in an animal model of orally induced CMA. They not only demonstrated oral tolerance induction after feeding naïve mice partial hydrolysate before sensitization, but they also observed an increased frequency of Foxp3+ Tregs in the MLN. In addition, they reported that the protective effect against allergy development is transferrable to a recipient animal by means of an MLN transfer [62].

An important aspect is that most studies testing the capacity of hydrolysates to prevent allergic sensitization have been conducted in infants at high risk for allergic disease. However, atopic diseases in early life have also been observed in children without a family history of allergy. Therefore, the allergy-preventive effect of partial hydrolysate-based infant formulas might need to be investigated further in a wider group of children who are in need of breast feeding supplementation or substitution [49].

Tolerogenic peptides

Pre-exposure to intact allergen via the oral route results in oral tolerance to the protein. Previously, we described that partial hydrolysates, although having higher residual allergenicity, have better tolerance-inducing capacities than extensive hydrolysates. This suggests possible oral tolerance induction by peptide fractions

present in the partial hydrolysates. Knipping *et al.* demonstrated a specific time at which protein hydrolysis resulted in peptides possessing reduced IgE cross-linking capacity and preserved T cell-activating properties [63]. An attractive approach for oral tolerance induction is the selection of specific peptides with tolerogenic capacities to supplement protein mixtures with low allergenicity [64]. Tolerogenic peptides are relatively small (around 20 amino acids) protein fragments, comprising T cell-binding epitopes. In a preventive animal model, it was demonstrated that prior intradermal administration of a T cell-dominant αS1-casein peptide results in immunologic tolerance in both the T cell and the antibody response to the native protein [65]. Focusing on specific peptides from hydrolysates might enhance oral tolerance induction via Tregs and also direct the immune system away from Th2 phenotype and allergic sensitization. A good example of this hypothesis is the study of Bogh *et al.* which showed that co-immunization of intact BLG with BLG-derived peptide fragments can reduce the sensitizing capacity of the intact protein [66]. This emphasizes that the locations of allergenic and tolerogenic sites on proteins might be independent from each other, resulting in the possibility of separating tolerogenicity from allergenicity [67]. In a later study, it was shown that appropriate selection of peptides with tolerogenic properties after tryptic hydrolysis of BLG and their enrichment in a formula reduced the allergic response to the naïve protein in a mouse model of CMA [67]. It was indicated that this specific fraction was 50 times less allergenic compared with the total BLG hydrolysate. Both intestinal and serum antigen-specific IgE were suppressed, whereas delayed-type hypersensitivity and proliferative responses were prevented. Another study on tolerogenic peptides from BLG reported that early exposure to specific T cell epitope-containing peptides is able to prevent the allergic skin response in a murine model of orally induced CMA [68]. The tolerogenic potential of one of the peptides was remarkable because it suppressed the frequency of Th2 cells while it enhanced Foxp3+ Tregs in the MLN and tended to reduce the allergen-specific antibody response.

Regardless of the great potential of this approach to induce immunologic tolerance, there are still many questions concerning the underlying mechanisms and the various MHC class II molecules expressed on APC of different individuals. This emphasizes the need for further research that might lead to a more personalized tolerogenic approach to prevention and therapy for food allergies. Furthermore, combining this strategy with a tolerizing dietary component might result in even more powerful protection against allergy development.

Probiotics

During pregnancy, the immune system of the infant is skewed toward a Th2-dominant profile to reduce the risk of uncontrolled inflammation that could lead to premature birth [69]. The balance between Th1 and Th2 cells needs to be restored after birth to avoid the development of atopic disorders. Commensal microbes present in the infant gut (such as bifidobacteria and lactobacilli) stimulate the maturation of Th1 and Treg-mediated immune responses, thereby supporting the development of immune homeostasis [70]. A strong association has been found between the development of atopic disorders in early childhood, changes in the diet, and microbial colonization patterns [15]. Studies on delayed gut colonization describe failure in oral tolerance development [71], which suggests that allergen exposure alone is not sufficient and healthy gut colonization is necessary for tolerance to both self-antigens and allergens [43]. On the other hand, disruption of the microflora by antibiotic treatment enhanced the allergic sensitization to peanut protein in a murine food allergy model [72]. In line with this, significant differences were found when intestinal microbiota composition of healthy infants and infants with atopic disorders were compared [73]. Beneficial microbes ensure mucosal integrity and are involved in maturation of the immune system of the infant by inducing oral tolerance [74]. The presence of beneficial microbes prevents colonization by exogenous pathogens and simultaneously stimulates the production of secretory IgA (sIgA) antibodies by the mucosal immune system [75]. Secretory IgA binds to microorganisms in the gut and prevents adherence to the mucosal surface, contributing to intestinal homeostasis. In allergic diseases, it is suggested that beneficial microbes induce pro-inflammatory and regulatory cytokines that favor the development of the Th1 and Treg phenotypes, skewing the immune response away from the Th2-mediated allergic phenotype. Levels of inflammatory markers were elevated in eczema-free infants receiving probiotics, which indicates that a low-grade inflammatory status may reduce the risk of developing eczema [76].

Providing a favorable microflora via probiotic supplementation in the diet might be a promising strategy to support tolerance induction and suppress the allergic response. Probiotics are defined as "live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host" [77]. Long-term outcomes of clinical trials using probiotic interventions in early childhood are inconsistent in terms of effective allergy prevention. A randomized placebo-controlled study performed by Jensen *et al.* used *Lactobacillus acidophilus* as a supplement during the first 6 months of life in a high-risk group of children [78]. After 5 years, all participants were invited to the clinic for a diagnosis of eczema, IgE-mediated food allergies, allergic rhinitis, asthma, and sensitization to common food and aeroallergens. No significant differences were

observed between the placebo group and the probiotic group regarding allergy prevalence, indicating that postnatal *L. acidophilus* supplementation does not provide long-term protection against allergic manifestations [78]. Two other clinical studies investigated the long-term effects of probiotic supplementation during the last 4 weeks of pregnancy and the first 6 months of life of the neonate [79, 80]. Kalliomaki *et al.* showed a reduction in the development of eczema during the first 7 years of life after both prenatal and postnatal *Lactobacillus rhamnosus GG* supplementation [79]. Kuitunen *et al.* used a probiotic mixture (*L. rhamnosus GG, L. rhamnosus LC705, Bifidobacterium lactis Bb12,* and *Propionibacterium*) and found a decrease in IgE-mediated allergic disorders only in the cesarean-delivered infants and not in the total cohort after 5 years of follow-up [80]. Starting probiotic supplementation before birth might enhance the efficacy of a probiotic intervention in early childhood [32, 81]. Maternal microbial transfer to the offspring might result in imprinting of the microbiota of the fetus during a critical window in immune development [82]. A review published by Kuitunen regarding clinical trials aimed to prevent eczema with probiotics stated that most studies indeed show a protective effect until age 2 [83]. However, the effect is highly dependent on the strain used, the timing of supplementation, duration and dose, mode of delivery, and allergy risk status of the infant.

Prebiotics

Human milk is composed of several immunomodulatory components that ensure optimal maturation of the infant immune system. It also provides beneficial microbes that will colonize the infant gut [84, 85]. Furthermore, the growth of beneficial microbes such as lactobacilli and bifidobacteria is supported by human milk oligosaccharides (HMOS), because they function as a substrate in fermentation by the microbiota [86]. In addition, a growing body of evidence suggests that HMOS can interact directly with cells of the immune system [86]. The prebiotic effect of HMOS is suggested to be of key importance in preventing the onset of atopic manifestations such as eczema, food allergies, allergic asthma, and allergic rhinitis in early childhood [87, 88]. Human milk contains 10-12 g/L oligosaccharides, which makes them the major component in the diet of breast-fed infants after lipids and lactose [89]. Endogenous enzymes active in the digestive tract are unable to cleave the specific linkages present in HMOS, which explains the finding that they reach the intestine intact [90].

To enhance the immunomodulatory capacity of infant formula, dietary non-digestible oligosaccharides with specific functional capacities similar to HMOS were identified. Non-digestible oligosaccharides include, among others, galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). Galacto-oligosaccharides

are β-galactosidase-linked galactose monomers derived from lactose, whereas FOS are obtained from enzymatic hydrolysis of chicory-derived inulin. The degree of polymerization is an important factor that determines functional activity; therefore, a distinction has been made between short-chain and long-chain oligosaccharides [86]. A specific combination of short-chain GOS and long-chain FOS (scGOS/lcFOS ratio 9:1) has been studied extensively in both pre-clinical and clinical setup. A large prospective randomized double-blind placebo-controlled study with formula-fed infants at risk of developing atopic manifestations investigated the effect of prebiotic supplementation on the development of allergic diseases in early childhood. Prevalence of both atopic manifestations and infections was reduced in the prebiotic group during the first 6 months of life compared with the placebo group. The protective effect was still detectable 5 years after the prebiotic intervention, indicating that immune programming in early life has a long-term protective effect [88, 91]. The addition of scGOS/lcFOS to infant formula enhanced colonization with bifidobacteria and lactobacilli, resulting in an intestinal flora more similar to the one in breast-fed infants [92]. Supplementation with scGOS/lcFOS is associated with an increase in sIgA production, which indicates that prebiotics can modulate the mucosal immune system [93]. Furthermore, an intervention with scGOS/lcFOS in formula-fed infants resulted in a metabolic profile analogous to breast milk-fed infants [94]. Fermentation of non-digestible oligosaccharides by bifidobacteria leads to the production of specific short-chain fatty acids (SCFA) and lactate and lowers the pH of the GI tract [75]. SCFA are involved in maintaining the epithelial barrier function and contribute to oral tolerance induction. Via direct interaction with cells of the immune system, SCFA are involved in regulating the inflammatory response [95].

Studies in mice using a dietary intervention with non-digestible oligosaccharides showed an enhanced Th1 and Treg response to the expense of Th2 cells in both an allergic asthma model and CMA model. The acute allergic response was suppressed and a reduction in the number of inflammatory cells was observed [96, 97]. Transferring splenocytes from oligosaccharide-treated cow's milk allergic mice to naïve recipient mice could prevent an allergic response. *Ex vivo* depletion of CD25+ Tregs before the splenic transfer inhibited the protective effect, indicating that non-digestible oligosaccharides exert systemic effects via the induction of Tregs [98, 99]. *In vitro* studies were conducted to investigate the systemic uptake of non-digestible oligosaccharides. In the gut, a monolayer of intestinal epithelial cells (IEC) forms a barrier between the luminal content and the mucosal tissue and selectively allows passage of food antigens into the lamina propria. The HMOS and dietary oligosaccharides were able to cross the intestinal epithelium via both receptor-mediated transcytosis and paracellular transfer through a Caco-2 monolayer [100,

101]. Eiwegger *et al.* showed in an *in vitro* study that HMOS shifted the Th2-dominated profile typically seen in neonates toward a more balanced Th1/Th2 profile as they suppressed Th2-type cytokine production by CD4+ T cells in cord blood [102]. Co-culture experiments using human IEC in combination with human peripheral blood mononuclear cells (PBMC) were used to elucidate the immunomodulatory mechanisms of scGOS/lcFOS. Incubation with oligosaccharides and a synthetic Toll-like receptor-9 ligand (based on bacterial CpG DNA) increased the IEC expression and secretion of galectin-9 [103]. Galectin-9 is a soluble-type lectin with sugar-binding capacities. In addition, galectin-9 is able to bind cells of the adaptive immune system, thus enabling IEC to influence the effector response [104]. Increased galectin-9 secretion was associated with skewing toward a Th1 and Treg profile and an increase in both interferon gamma (IFNγ) and IL-10 production. It could also bind to IgE and prevent antigen-IgE complex formation in a rat basophilic leukocyte assay, thus preventing degranulation *in vitro* [105]. Dietary intervention with scGOS/lcFOS increased serum galectin-9 levels in a murine model of CMA, which was shown to contribute to the suppression of allergic symptoms [106]. The immunomodulatory characteristics of non-digestible oligosaccharides, such as their interaction with cells of the GALT via galectin-9, are suggested to contribute to tolerance induction and intestinal homeostasis in a microbiota-independent manner (**Figure 2**).

Synbiotics

The beneficial effect of supplementation with either probiotics or prebiotics has led to the hypothesis that an additional or synergistic effect could be induced when both components are combined. A study performed by Schouten *et al.* showed that a dietary intervention with scGOS/lcFOS and *Bifidobacterium breve* before sensitization could more efficiently reduce the allergic response in cow's milk allergic mice compared with an intervention with either scGOS/lcFOS or *B. breve* alone [96]. Furthermore, the bifidogenic effect of the synbiotic concept was confirmed in a clinical study in which infants aged less than 7 months with AD were fed extensively hydrolyzed formula supplemented with a synbiotic mixture (scGOS/lcFOS and *B. breve*) for 12 weeks [107]. The prevalence of AD was not influenced by the dietary intervention, but the severity of symptoms in infants with IgE-mediated AD was reduced [107]. De Kivit *et al.* measured serum galectin-9 levels at baseline and after the 12-week intervention period and found a significant increase in the synbiotic group compared with the placebo group [106]. The one-year follow-up of this study showed a reduction in asthma-like symptoms and asthma medication use in the synbiotic group [108]. However, no significant difference was observed between the placebo and the synbiotic group in plasma markers of allergy, cytokine profiles after *ex vivo* restimulation of PBMC, and

percentage of Tregs present in the circulation [109]. Another randomized double-blind placebo-controlled clinical trial conducted by Kukkonen *et al.* provided high-risk neonates with a combination of four probiotic strains and GOS during the first 6 months of life. A reduction in the prevalence of eczema, but not in other allergic diseases, was observed after 2 years in the intervention group [110].

Underlying mechanisms of allergy modulation by probiotics, prebiotics, and synbiotics are not completely understood; however, both animal and clinical studies show promising results in terms of allergy prevention and suppression. Variation in protocols, probiotic strains, and type of non-digestible oligosaccharides complicates interpretation of the results. Timing of dietary interventions appears to be crucial for development of the immune system and intestinal flora of newborns. Even prenatal administration of probiotics and prebiotics might prove decisive for the development of a healthy gut flora and oral tolerance.

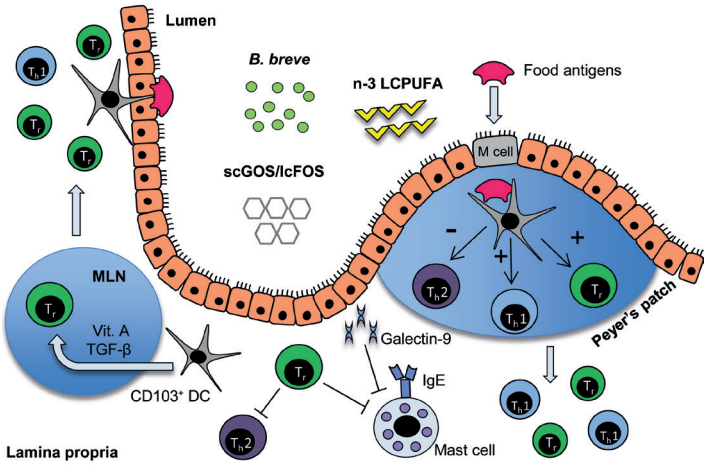


Figure 2. Effects of dietary components on the mucosal immune system. Upon sampling antigens, antigen-presenting DC present the proteins to naïve T cells and stimulate their differentiation into Th1, Th2 or Tregs. A special subtype of CD103+ DC in the lamina propria migrates to the MLN where it converts vitamin A into retinoic acid and in the presence of TGF*β* induces differentiation of Tregs. Tregs can suppress Th2 effector cells and mast cell degranulation and contribute to oral tolerance to the food antigens. Dietary components such as *B. breve* (probiotics), scGOS/lcFOS (prebiotics) and/or n-3 LCPUFA might directly interact with adaptive immune cells, induce Tregs or enhance suppression of the allergic effector response via galectin-9. MLN, mesenteric lymph nodes; DC, dendritic cells; Tregs, regulatory T cells (depicted as Tr); Th1/2, T helper 1/2 (depicted as Th1/Th2); Vit. A, vitamin A; TGFβ, transforming growth factor β; *B. breve*, *Bifidobacterium breve*; scGOS/lcFOS, short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides; LCPUFA, long-chain polyunsaturated fatty acids.

Polyunsaturated fatty acids

The change in composition of the Western diet involves a reduction of dietary fiber and antioxidant intake and an increase in the total intake of fat, all of which are suggested to contribute to the development of allergies [111]. In addition, the consumption of vegetable oils rich in n-6 PUFA has increased, leading to a rise in the n-6/n-3 PUFA dietary ratio. N-6 PUFA consists mainly of linoleic acid (LA), a precursor for the long-chain PUFA arachidonic acid (AA). Arachidonic acid can be converted into pro-inflammatory eicosanoids (e.g., prostaglandins) by cyclo-oxygenases and lipoxygenases [112]. Prostaglandins decrease the production of Th1-type cytokines (IFNγ and IL-2) and simultaneously stimulated the production of Th2-type cytokines (IL-4 and IL-5) in an *in vitro* human whole-blood assay [113]. In addition, prostaglandin E2 promotes IgE isotype switching in B cells, indicating that an increased intake of n-6 PUFA may contribute to an allergic phenotype [114]. N-3 PUFA consists mainly of α-linolenic acid (ALA), a precursor for the PUFA-eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA are able to replace AA in membrane phospholipids and change the structure and functionality of different cell types. T cell proliferation and the interaction between T cells and APC can be altered via the incorporation of n-3 PUFA into the cell membrane of lymphocytes [115]. Furthermore, the conversion of EPA into its metabolite Resolvin-E1 results in a reduced production of IL-12 and blocked transfer of inflammatory cells across the intestinal epithelium [116]. Interaction with G-protein-coupled receptor 120 present in the cell membrane is a possible mechanism of action by which EPA and DHA exert their anti-inflammatory effects [117]. In a murine CMA model, partial exchange of n-6 PUFA-rich soybean oil by n-3 PUFA-rich tuna oil in the diet largely prevented allergic sensitization in mice and enhanced Foxp3+ Tregs in the spleen [118]. Transferring splenocytes or isolated CD25+ Tregs from these mice to naïve recipients protected the latter from developing CMA [119].

During pregnancy, epigenetic factors such as nutrition and microbial exposure influence the immune status of the mother and have an indirect effect on fetal immune development [120]. Maternal dietary changes, resulting in an increased n-6/n-3 PUFA ratio, are proposed to contribute to a disturbed maturation of the neonatal immune system by inducing Th2-type cytokines. Dietary supplementation with fish oil rich in EPA and DHA might be involved in restoring immune balance. Maternal n-3 PUFA supplementation during pregnancy decreased cord blood levels of the Th2 cytokines IL-4 and IL-13 [121, 122]. In another intervention trial with prenatal n-3 PUFA supplementation from 20 weeks of gestation until birth, higher proportions of n-3 PUFA were found in neonatal erythrocyte membranes combined with reduced levels of IL-5, IL-13, IL-10, and IFNγ [123]. However, at 1 year of age no difference in the

frequency of AD was found between the n-3 PUFA and the placebo group, although symptoms appeared to be less severe [123]. In another randomized, placebo-controlled intervention study, n-3 PUFA supplementation during both pregnancy and lactation significantly decreased the prevalence of food allergies and eczema at 1 year of age [124]. The preventive effect of fish oil against IgE-mediated disorders was sustained even after 2 years of follow-up [125]. Furthermore, allergy prevention by postnatal n-3 PUFA supplementation in newborns until age 6 months resulted in higher DHA and EPA levels in plasma and reduced AA levels in erythrocyte membranes [126]. However, clinical outcomes of allergy were not significantly different between the placebo and the fish oil group, which indicated that postnatal supplementation with n-3 PUFA may not be sufficient to reduce the risk of developing allergies in early childhood. The same finding was observed in other postnatal fish oil supplementation studies: supplementation to the infant or to lactating mothers did not result in a reduced prevalence of allergies later in life [120]. The allergy-preventive effect of n-3 PUFA supplementation might depend on the presence of maternal factors involved in the development of the immune system in early life.

Antioxidants

Observational studies have linked the intake of dietary antioxidants such as vitamin A and its metabolites, vitamin C, D, E, zinc, selenium, and folate, to the prevalence of asthma and allergic disorders in early childhood [127]. Allergic disorders are known to cause oxidative stress, and it is difficult to define whether a reduced antioxidant status is a cause or a consequence of the allergic diseases in infants [128]. A systematic review published in 2011 by Patelarou *et al.* found an association between inadequate antioxidant status *in utero* and during early childhood and the development of allergic disorders [129]. Elevated antioxidant concentrations in maternal, child, or cord blood serum were related to a decreased prevalence of asthma, wheezing, or eczema during early life. A Danish birth cohort included 44,594 mother-infant pairs and examined the relationship between maternal intake of vitamins A, E, and K during pregnancy and the development of asthma and allergic rhinitis in the offspring during the first 7 years of life. A weak beneficial effect of vitamin A and E intake during pregnancy on the prevalence of allergic rhinitis at a young age was found. In contrast, high vitamin K intake was linked to an increased prevalence of asthma [130].

To date, evidence from well-conducted randomized controlled trials is lacking and the exact mechanism of action of antioxidants in the development of a balanced immune system in neonates has not been clarified. Some studies point to CD103+ DC present in the lamina propria that convert vitamin A into retinoic acid, which is

essential for the development of Tregs in the MLN and the promotion of a sIgA switch of B cells in the presence of IL-10 and TGFβ [131, 132]. The influence of vitamin A and its derivatives, the retinoids, on postnatal lymphocyte proliferation and differentiation was determined in an *in vivo* OVA-induced postnatal allergy model in mice. Dams were fed a regular diet, a vitamin A-elimination diet, or vitamin A-supplemented diet during lactation, and similar diets were fed directly to the pups after weaning. Allergic sensitization was induced via a single intraperitoneal OVAinjection in 28-day-old pups. Vitamin A supplementation was associated with a decrease in the amount of CD4+ and CD8+ splenic lymphocytes and significantly enhanced IL-4 and OVA-specific IgE concentrations after allergic sensitization. The authors concluded that despite its suggested role in Treg induction, vitamin A supplementation of the maternal diet during lactation and of the pups' diet after weaning increased the severity of allergic sensitization in the offspring [133].

Animal studies found a positive effect of vitamin E on IgE-mediated atopic disorders, and a similar association was found in a human cohort in which the relation between vitamin E intake, serum IgE concentrations, and allergic skin sensitization was investigated [134]. Vitamin E suppressed the production of IL-4 in peripheral blood T cells by blocking transcription factor NF-κB and thus inhibiting activation of the promoter sites [135]. An inverse association between maternal vitamin D status during pregnancy and the development of asthma and wheezing in early childhood was found in several birth cohort studies with more than 750 mother-infant pairs [136]. To test the hypothesis whether maternal supplementation with vitamin Dfrom 27 weeks of gestation could reduce the prevalence of wheezing at age 3 years in the offspring, a randomized controlled trial was conducted. No significant differences were found between the vitamin D and control groups for infant wheezing, atopy, or eczema at age 3 [137]. On the other hand, high maternal and cord blood vitamin D status was positively associated with a risk for the development of food allergies during the first 2 years of life in infants enrolled in the German LINA birth cohort [138]. These studies show conflicting results on allergic outcomes upon vitamin D supplementation, revealing both inhibitory and stimulatory properties, depending on the timing and/or dosing and duration of supplementation [139-141].

Clear evidence from randomized controlled trials is needed to determine the role of antioxidants during postnatal immune development and to learn whether supplementation during pregnancy and lactation decreases or increases the risk of atopic disorders in early childhood. Until safe recommendations can be made, a healthy balanced diet is the best way to control the development of allergic diseases in infants.

CONCLUSION

The increasing prevalence of allergic disorders among infants might reflect changes in Western diet composition and reduced exposure to microorganisms in early childhood. Food allergy is one of the first allergic outcomes developing from a Th2-prone state that can progress to respiratory allergies and asthma later in life. It is important to intervene as early as possible, even prenatally, to actively prevent sensitization to foods and stop the development of the atopic march. Early introduction to larger protein fragments or small peptides with tolerogenic capacities appears to be an attractive approach for oral tolerance induction and allergy prevention. The exposure to food proteins with reduced allergenicity and tolerogenic properties might prevent allergic disease development by supporting maturation of the neonatal gut and/or by stimulating normal tolerance establishment. Clinical trials are needed to further clarify the preventive effect of this approach, and might resolve the dilemma of early life exposure to allergenic proteins. Using dietary components with immunomodulatory properties as adjuvants is another approach to skew the Th2-dominated immune response toward a more tolerogenic response in early childhood. Although evidence from clinical trials points to the beneficial effects of supplementation with dietary components on prevention of allergic sensitization, this approach has not been implemented as an allergy-preventive strategy yet. Supplementing the diet with probiotics, prebiotics, n-3 PUFA, or specific antioxidants is suggested to support oral tolerance induction and prevent allergy development in early childhood. Contradictory findings on some dietary components, such as vitamins, emphasize the importance of clarifying the exact window of opportunity in early life, before specific recommendations on this approach can be made. Recent evidence suggests that dietary interventions should start during gestation because maternal factors may influence the development of both the microflora and the immune system of the offspring. Epigenetic changes induced by maternal (dietary) factors might be needed for lifelong protection against allergic disorders in the infant. More clinical trials are needed to confirm the beneficial effects of dietary interventions and to promote their use as a strategy for (food) allergy prevention.

REFERENCES

1. Gupta RS, Springston EE, Warrier MR, Smith B, Kumar R, Pongracic J, and Holl JL. The prevalence, severity, and distribution of childhood food allergy in the United States. Pediatrics 2011. 128(1): p. e9-17.
2. Liu AH, Jaramillo R, Sicherer SH, Wood RA, Bock SA, Burks AW, Massing M, Cohn RD, and Zeldin DC. National prevalence and risk factors for food allergy and relationship to asthma: results from the National Health and Nutrition Examination Survey 2005-2006. J Allergy Clin Immunol 2010. 126(4): p. 798-806 e13.
3. Joneja JM. Infant food allergy: where are we now? JPEN J Parenter Enteral Nutr 2012. 36(1 Suppl): p. 49S-55S.
4. Depner M, Ege MJ, Genuneit J, Pekkanen J, Roponen M, Hirvonen MR, Dalphin JC, Kaulek V, Krauss-Etschmann S, Riedler J, Braun-Fahrlander C, Roduit C, Lauener R, Pfefferle PI, Weber J, von Mutius E, and Group PS. Atopic sensitization in the first year of life. J Allergy Clin Immunol 2013. 131(3): p. 781-8.
5. Lee LA and Burks AW. Food allergies: prevalence, molecular characterization, and treatment/prevention strategies. Annu Rev Nutr 2006. 26: p. 539-65.
6. Host A and Halken S. A prospective study of cow milk allergy in Danish infants during the first 3 years of life. Clinical course in relation to clinical and immunological type of hypersensitivity reaction. Allergy 1990. 45(8): p. 587-96.
7. Sampson HA and McCaskill CC. Food hypersensitivity and atopic dermatitis: evaluation of 113 patients. J Pediatr 1985. 107(5): p. 669-75.
8. Savage JH, Matsui EC, Skripak JM, and Wood RA. The natural history of egg allergy. J Allergy Clin Immunol 2007. 120(6): p. 1413-7.
9. Skripak JM, Matsui EC, Mudd K, and Wood RA. The natural history of IgE-mediated cow's milk allergy. J Allergy Clin Immunol 2007. 120(5): p. 1172-7.
10. Bock SA, Munoz-Furlong A, and Sampson HA. Fatalities due to anaphylactic reactions to foods. J Allergy Clin Immunol 2001. 107(1): p. 191-3.
11. Bock SA, Munoz-Furlong A, and Sampson HA. Further fatalities caused by anaphylactic reactions to food, 2001-2006. J Allergy Clin Immunol 2007. 119(4): p. 1016-8.
12. Muraro A, Halken S, Arshad SH, Beyer K, Dubois AE, Du Toit G, Eigenmann PA, Grimshaw KE, Hoest A, Lack G, O'Mahony L, Papadopoulos NG, Panesar S, Prescott S, Roberts G, de Silva D, Venter C, Verhasselt V, Akdis AC, Sheikh A, Allergy EF, and Anaphylaxis Guidelines G. EAACI food allergy and anaphylaxis guidelines. Primary prevention of food allergy. Allergy 2014. 69(5): p. 590-601.
13. West CE, D'Vaz N, and Prescott SL. Dietary immunomodulatory factors in the development of immune tolerance. Curr Allergy Asthma Rep

2011. 11(4): p. 325-33.
14. Martino DJ and Prescott SL. Silent mysteries: epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease. Allergy 2010. 65(1): p. 7-15.
15. West CE, Videky DJ, and Prescott SL. Role of diet in the development of immune tolerance in the context of allergic disease. Curr Opin Pediatr 2010. 22(5): p. 635-41.
16. Verhasselt V. Neonatal tolerance under breastfeeding influence: the presence of allergen and transforming growth factor-beta in breast milk protects the progeny from allergic asthma. J Pediatr 2010. 156(2 Suppl): p. S16-20.
17. Round JL and Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 2009. 9(5): p. 313-23.
18. Scurlock AM, Vickery BP, Hourihane JO, and Burks AW. Pediatric food allergy and mucosal tolerance. Mucosal Immunol 2010. 3(4): p. 345-54.
19. Canani RB and Di Costanzo M. Gut Microbiota as Potential Therapeutic Target for the Treatment of Cow's Milk Allergy. Nutrients 2013. 5(3): p. 651-662.
20. Calligaris L, Longo G, Badina L, Berti I, and Barbi E. Cow's milk allergy in children, from avoidance to tolerance. Endocr Metab Immune Disord Drug Targets 2014. 14(1): p. 47-53.
21. Chehade M and Mayer L. Oral tolerance and its relation to food hypersensitivities. J Allergy Clin Immunol 2005. 115(1): p. 3-12; quiz 13.
22. van Esch BC, Willemsen LE, Garssen J, and Knippels LM. Prevention strategies for food allergy might intervene with the development of atopic dermatitis and the atopic march, chapter in Food allergies: symptoms, diagnosis, and treatment. Editor P. Rodgers. 2011. Nova Science Publishers Inc. United States.
23. Adel-Patient K, Nutten S, Bernard H, Fritsche R, Ah-Leung S, Meziti N, Prioult G, Mercenier A, and Wal JM. Immunomodulatory potential of partially hydrolyzed beta-lactoglobulin and large synthetic peptides. J Agric Food Chem 2012. 60(43): p. 10858-66.
24. Spahn TW, Weiner HL, Rennert PD, Lugering N, Fontana A, Domschke W, and Kucharzik T. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. Eur J Immunol 2002. 32(4): p. 1109-13.
25. Burks AW, Laubach S, and Jones SM. Oral tolerance, food allergy, and immunotherapy: implications for future treatment. J Allergy Clin Immunol 2008. 121(6): p. 1344-50.
26. Mousallem T and Burks AW. Immunology in the Clinic Review Series; focus on allergies: immunotherapy for food allergy. Clin Exp Immunol 2012. 167(1): p. 26-31.
27. Verhasselt V. Oral tolerance in neonates: from basics to potential prevention of allergic disease. Mucosal Immunol 2010. 3(4): p. 326-33.
28. Halken S and Host A. Prevention of

allergic disease. Exposure to food allergens and dietetic intervention. Pediatr Allergy Immunol 1996. 7(9 Suppl): p. 102-7.

29. Holloway JA, Warner JO, Vance GH, Diaper ND, Warner JA, and Jones CA. Detection of house-dust-mite allergen in amniotic fluid and umbilical-cord blood. Lancet 2000. 356(9245): p. 1900-2.
30. Jeurink PV, Meulenbroek LA, Garssen J, and Knippels LM. Allergen avoidance versus tolerance induction, chapter in Pharma-Nutrition; An Overview. Editors G. Folkerts and J. Garssen. 2014. Springer. Switzerland. p. 373-91.
31. Jones AC, Miles EA, Warner JO, Colwell BM, Bryant TN, and Warner JA. Fetal peripheral blood mononuclear cell proliferative responses to mitogenic and allergenic stimuli during gestation. Pediatr Allergy Immunol 1996. 7(3): p. 109-16.
32. Boyle RJ and Tang ML. Can allergic diseases be prevented prenatally? Allergy 2006. 61(12): p. 1423-31.
33. Kramer MS and Kakuma R. Maternal dietary antigen avoidance during pregnancy or lactation, or both, for preventing or treating atopic disease in the child. Cochrane Database Syst Rev 2012(9): p. CD000133.
34. Jarrett EE and Hall E. IgE suppression by maternal IgG. Immunology 1983. 48(1): p. 49-58.
35. Palmer DJ and Makrides M. Diet of lactating women and allergic reactions in their infants. Curr Opin Clin Nutr Metab Care 2006. 9(3): p. 284-8.
36. Verhasselt V, Milcent V, Cazareth J, Kanda A, Fleury S, Dombrowicz D, Glaichenhaus N, and Julia V. Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. Nat Med 2008. 14(2): p. 170-5.
37. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, Monteiro R, Dombrowicz DD, Julia V, Glaichenhaus N, and Verhasselt V. Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. Mucosal Immunol 2010. 3(5): p. 461-74.
38. Palmer DJ and Prescott SL. Does early feeding promote development of oral tolerance? Curr Allergy Asthma Rep 2012. 12(4): p. 321-31.
39. American Academy of Pediatrics. Committee on Nutrition. Hypoallergenic infant formulas. Pediatrics 2000. 106(2 Pt 1): p. 346-9.
40. Agostoni C, Decsi T, Fewtrell M, Goulet O, Kolacek S, Koletzko B, Michaelsen KF, Moreno L, Puntis J, Rigo J, Shamir R, Szajewska H, Turck D, van Goudoever J, and Nutrition ECo. Complementary feeding: a commentary by the ESPGHAN Committee on Nutrition. J Pediatr Gastroenterol Nutr 2008. 46(1): p. 99-110.
41. Allen CW, Campbell DE, and Kemp AS. Food allergy: is strict avoidance the only answer? Pediatr Allergy Immunol 2009. 20(5): p. 415-22.
42. Fox AT, Sasieni P, du Toit G, Syed H, and Lack G. Household peanut

consumption as a risk factor for the development of peanut allergy. J Allergy Clin Immunol 2009. 123(2): p. 417-23.

43. Prescott SL, Smith P, Tang M, Palmer DJ, Sinn J, Huntley SJ, Cormack B, Heine RG, Gibson RA, and Makrides M. The importance of early complementary feeding in the development of oral tolerance: concerns and controversies. Pediatr Allergy Immunol 2008. 19(5): p. 375-80.
44. Levy Y, Broides A, Segal N, and Danon YL. Peanut and tree nut allergy in children: role of peanut snacks in Israel? Allergy 2003. 58(11): p. 1206-7.
45. Du Toit G, Katz Y, Sasieni P, Mesher D, Maleki SJ, Fisher HR, Fox AT, Turcanu V, Amir T, Zadik-Mnuhin G, Cohen A, Livne I, and Lack G. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. J Allergy Clin Immunol 2008. 122(5): p. 984-91.
46. Jarrett EE. Perinatal influences on IgE responses. Lancet 1984. 2(8406): p. 797-9.
47. LEAP study (Induction of Tolerance Through Early Introduction of Peanut in High-Risk Children). http://www.clinicaltrials.gov/ct2/show/NCT00329784, accessed on August 11, 2014.
48. EAT study (Enquiring About Tolerance). http://www.eatstudy.co.uk, accessed on August 11, 2014.
49. von Berg A. Modified proteins in allergy prevention. Nestle Nutr Workshop Ser Pediatr Program 2009. 64: p. 239-47; discussion 247-57.
50. Hays T and Wood RA. A systematic review of the role of hydrolyzed infant formulas in allergy prevention. Arch Pediatr Adolesc Med 2005. 159(9): p. 810-6.
51. Fiocchi A, Brozek J, Schunemann H, Bahna SL, von Berg A, Beyer K, Bozzola M, Bradsher J, Compalati E, Ebisawa M, Guzman MA, Li H, Heine RG, Keith P, Lack G, Landi M, Martelli A, Rance F, Sampson H, Stein A, Terracciano L, Vieths S, and World Allergy Organization Special Committee on Food A. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. Pediatr Allergy Immunol 2010. 21 Suppl 21: p. 1-125.
52. Alexander DD and Cabana MD. Partially hydrolyzed 100% whey protein infant formula and reduced risk of atopic dermatitis: a meta-analysis. J Pediatr Gastroenterol Nutr 2010. 50(4): p. 422-30.
53. Halken S, Hansen KS, Jacobsen HP, Estmann A, Faelling AE, Hansen LG, Kier SR, Lassen K, Lintrup M, Mortensen S, Ibsen KK, Osterballe O, and Host A. Comparison of a partially hydrolyzed infant formula with two extensively hydrolyzed formulas for allergy prevention: a prospective, randomized study. Pediatr Allergy Immunol 2000. 11(3): p. 149-61.
54. Szajewska H and Horvath A. Meta-analysis of the evidence for a partially hydrolyzed 100% whey formula for the prevention of allergic diseases.

Curr Med Res Opin 2010. 26(2): p. 423-37.

55. von Berg A, Filipiak-Pittroff B, Kramer U, Link E, Bollrath C, Brockow I, Koletzko S, Grubl A, Heinrich J, Wichmann HE, Bauer CP, Reinhardt D, Berdel D, and group GIs. Preventive effect of hydrolyzed infant formulas persists until age 6 years: long-term results from the German Infant Nutritional Intervention Study (GINI). J Allergy Clin Immunol 2008. 121(6): p. 1442-7.
56. Vandenplas Y, Hauser B, Van den Borre C, Clybouw C, Mahler T, Hachimi-Idrissi S, Deraeve L, Malfroot A, and Dab I. The long-term effect of a partial whey hydrolysate formula on the prophylaxis of atopic disease. Eur J Pediatr 1995. 154(6): p. 488-94.
57. von Berg A, Filipiak-Pittroff B, Kramer U, Hoffmann B, Link E, Beckmann C, Hoffmann U, Reinhardt D, Grubl A, Heinrich J, Wichmann HE, Bauer CP, Koletzko S, Berdel D, and group GIs. Allergies in high-risk schoolchildren after early intervention with cow's milk protein hydrolysates: 10-year results from the German Infant Nutritional Intervention (GINI) study. J Allergy Clin Immunol 2013. 131(6): p. 1565-73.
58. Exl BM and Fritsche R. Cow's milk protein allergy and possible means for its prevention. Nutrition 2001. 17(7-8): p. 642-51.
59. Fritsche R. Induction of oral tolerance to cow's milk proteins in rats fed with a whey protein hydrolysate. Nutrition Research 1998. 18(8): p. 1335-1341.
60. Fritsche R, Pahud JJ, Pecquet S, and Pfeifer A. Induction of systemic immunologic tolerance to beta-lactoglobulin by oral administration of a whey protein hydrolysate. J Allergy Clin Immunol 1997. 100(2): p. 266-73.
61. Peng HJ, Su SN, Tsai JJ, Tsai LC, Kuo HL, and Kuo SW. Effect of ingestion of cow's milk hydrolysed formulas on whey protein-specific Th2 immune responses in naive and sensitized mice. Clin Exp Allergy 2004. 34(4): p. 663-70.
62. van Esch BC, Schouten B, de Kivit S, Hofman GA, Knippels LM, Willemsen LE, and Garssen J. Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3+ regulatory T-cells in the mesenteric lymph nodes. Pediatr Allergy Immunol 2011. 22(8): p. 820-6.
63. Knipping K, van Esch BC, van Ieperen-van Dijk AG, van Hoffen E, van Baalen T, Knippels LM, van der Heide S, Dubois AE, Garssen J, and Knol EF. Enzymatic treatment of whey proteins in cow's milk results in differential inhibition of IgE-mediated mast cell activation compared to T-cell activation. Int Arch Allergy Immunol 2012. 159(3): p. 263-70.
64. Lo CW and Kleinman RE. Infant formula, past and future: opportunities for improvement. Am J Clin Nutr 1996. 63(4): p. 646S-50S.
65. Hirahara K, Hisatsune T, Choi CY, and Kaminogawa S. Profound immunological tolerance in the antibody response against

bovine alpha s1-casein induced by intradermal administration of a dominant T cell determinant. Clin Immunol Immunopathol 1995. 76(1 Pt 1): p. 12-8.

66. Bogh KL, Barkholt V, and Madsen CB. The sensitising capacity of intact beta-lactoglobulin is reduced by co-administration with digested beta-lactoglobulin. Int Arch Allergy Immunol 2013. 161(1): p. 21-36.
67. Pecquet S, Bovetto L, Maynard F, and Fritsche R. Peptides obtained by tryptic hydrolysis of bovine beta-lactoglobulin induce specific oral tolerance in mice. J Allergy Clin Immunol 2000. 105(3): p. 514-21.
68. Meulenbroek LA, van Esch BC, Hofman GA, den Hartog Jager CF, Nauta AJ, Willemsen LE, Bruijnzeel-Koomen CA, Garssen J, van Hoffen E, and Knippels LM. Oral treatment with beta-lactoglobulin peptides prevents clinical symptoms in a mouse model for cow's milk allergy. Pediatr Allergy Immunol 2013. 24(7): p. 656-64.
69. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol 2007. 7(5): p. 379-90.
70. Castellazzi AM, Valsecchi C, Caimmi S, Licari A, Marseglia A, Leoni MC, Caimmi D, Miraglia del Giudice M, Leonardi S, La Rosa M, and Marseglia GL. Probiotics and food allergy. Ital J Pediatr 2013. 39: p. 47.
71. Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, and Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol 1997. 159(4): p. 1739-45.
72. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, Tjota MY, Seo GY, Cao S, Theriault BR, Antonopoulos DA, Zhou L, Chang EB, Fu YX, and Nagler CR. Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci U S A 2014. 111(36): p. 13145-50
73. Noverr MC and Huffnagle GB. Does the microbiota regulate immune responses outside the gut? Trends Microbiol 2004. 12(12): p. 562-8.
74. Sansonetti PJ and Medzhitov R. Learning tolerance while fighting ignorance. Cell 2009. 138(3): p. 416-20.
75. Oozeer R, van Limpt K, Ludwig T, Ben Amor K, Martin R, Wind RD, Boehm G, and Knol J. Intestinal microbiology in early life: specific prebiotics can have similar functionalities as human-milk oligosaccharides. Am J Clin Nutr 2013. 98(2): p. 561S-71S.
76. Marschan E, Kuitunen M, Kukkonen K, Poussa T, Sarnesto A, Haahtela T, Korpela R, Savilahti E, and Vaarala O. Probiotics in infancy induce protective immune profiles that are characteristic for chronic low-grade inflammation. Clin Exp Allergy 2008. 38(4): p. 611-8.
77. Gourbeyre P, Denery S, and Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 2011. 89(5): p. 685-95.
78. Jensen MP, Meldrum S, Taylor AL,

Dunstan JA, and Prescott SL. Early probiotic supplementation for allergy prevention: long-term outcomes. J Allergy Clin Immunol 2012. 130(5): p. 1209-1211 e5.

79. Kalliomaki M, Salminen S, Poussa T, and Isolauri E. Probiotics during the first 7 years of life: a cumulative risk reduction of eczema in a randomized, placebo-controlled trial. J Allergy Clin Immunol 2007. 119(4): p. 1019-21.
80. Kuitunen M, Kukkonen K, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Haahtela T, and Savilahti E. Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. J Allergy Clin Immunol 2009. 123(2): p. 335-41.
81. Bertelsen RJ, Brantsaeter AL, Magnus MC, Haugen M, Myhre R, Jacobsson B, Longnecker MP, Meltzer HM, and London SJ. Probiotic milk consumption in pregnancy and infancy and subsequent childhood allergic diseases. J Allergy Clin Immunol 2014. 133(1): p. 165-71 e1-8.
82. West CE, Jenmalm MC, and Prescott SL. The gut microbiota and its role in the development of allergic disease: a wider perspective. Clin Exp Allergy 2014. 45(1): p. 43-53
83. Kuitunen M. Probiotics and prebiotics in preventing food allergy and eczema. Curr Opin Allergy Clin Immunol 2013. 13(3): p. 280-6.
84. Jeurink PV, van Esch BC, Rijnierse A, Garssen J, and Knippels LM. Mechanisms underlying immune effects of dietary oligosaccharides. Am J Clin Nutr 2013. 98(2): p. 572S-7S.
85. Martin R, Langa S, Reviriego C, Jiminez E, Marin ML, Xaus J, Fernandez L, and Rodriguez JM. Human milk is a source of lactic acid bacteria for the infant gut. J Pediatr 2003. 143(6): p. 754-8.
86. Boehm G and Moro G. Structural and functional aspects of prebiotics used in infant nutrition. J Nutr 2008. 138(9): p. 1818S-1828S.
87. Bjorksten B, Sepp E, Julge K, Voor T, and Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol 2001. 108(4): p. 516-20.
88. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, and Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child 2006. 91(10): p. 814-9.
89. Boehm G, Fanaro S, Jelinek J, Stahl B, and Marini A. Prebiotic concept for infant nutrition. Acta Paediatr Suppl 2003. 91(441): p. 64-7.
90. Nauta AJ and Garssen J. Evidence-based benefits of specific mixtures of non-digestible oligosaccharides on the immune system. Carbohydr Polym 2013. 93(1): p. 263-5.
91. Arslanoglu S, Moro GE, Boehm G, Wienz F, Stahl B, and Bertino E. Early neutral prebiotic oligosaccharide supplementation reduces the incidence of some allergic manifestations in the first 5 years of life. J Biol Regul Homeost Agents 2012. 26(3 Suppl): p. 49-59.

92. Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B, and Boehm G. Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. J Pediatr Gastroenterol Nutr 2002. 34(3): p. 291-5.
93. Scholtens PA, Alliet P, Raes M, Alles MS, Kroes H, Boehm G, Knippels LM, Knol J, and Vandenplas Y. Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. J Nutr 2008. 138(6): p. 1141-7.
94. Knol J, Scholtens P, Kafka C, Steenbakkers J, Gro S, Helm K, Klarczyk M, Schopfer H, Bockler HM, and Wells J. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. J Pediatr Gastroenterol Nutr 2005. 40(1): p. 36-42.
95. Lepski S and Brockmeyer J. Impact of dietary factors and food processing on food allergy. Mol Nutr Food Res 2013. 57(1): p. 145-52.
96. Schouten B, van Esch BC, Hofman GA, van Doorn SA, Knol J, Nauta AJ, Garssen J, Willemsen LE, and Knippels LM. Cow milk allergy symptoms are reduced in mice fed dietary synbiotics during oral sensitization with whey. J Nutr 2009. 139(7): p. 1398-403.
97. Vos AP, van Esch BC, Stahl B, M'Rabet L, Folkerts G, Nijkamp FP, and Garssen J. Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. Int Immunopharmacol 2007. 7(12): p. 1582-7.
98. Schouten B, van Esch BC, Hofman GA, Boon L, Knippels LM, Willemsen LE, and Garssen J. Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. J Nutr 2010. 140(4): p. 835-41.
99. Schouten B, van Esch BC, Hofman GA, de Kivit S, Boon L, Knippels LM, Garssen J, and Willemsen LE. A potential role for CD25+ regulatory T-cells in the protection against casein allergy by dietary non-digestible carbohydrates. Br J Nutr 2012. 107(1): p. 96-105.
100. Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, Urbanek R, and Szepfalusi Z. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatr Allergy Immunol 2010. 21(8): p. 1179-88.
101. Gnoth MJ, Rudloff S, Kunz C, and Kinne RK. Investigations of the in vitro transport of human milk oligosaccharides by a Caco-2 monolayer using a novel high performance liquid chromatography-mass spectrometry technique. J Biol Chem 2001. 276(37): p. 34363-70.
102. Eiwegger T, Stahl B, Schmitt J, Boehm G, Gerstmayr M, Pichler J, Dehlink E, Loibichler C, Urbanek R, and Szepfalusi Z. Human milk--derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine

production of cord blood T-cells in vitro. Pediatr Res 2004. 56(4): p. 536-40.

103. de Kivit S, Kraneveld AD, Knippels LM, van Kooyk Y, Garssen J, and Willemsen LE. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. J Innate Immun 2013. 5(6): p. 625-38.
104. Rijnierse A, Jeurink PV, van Esch BC, Garssen J, and Knippels LM. Food-derived oligosaccharides exhibit pharmaceutical properties. Eur J Pharmacol 2011. 668 Suppl 1: p. S117-23.
105. Niki T, Tsutsui S, Hirose S, Aradono S, Sugimoto Y, Takeshita K, Nishi N, and Hirashima M. Galectin-9 is a high affinity IgE-binding lectin with anti-allergic effect by blocking IgE-antigen complex formation. J Biol Chem 2009. 284(47): p. 32344-52.
106. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BC, Knol J, Sprikkelman AB, van der Aa LB, Knippels LM, Garssen J, van Kooyk Y, and Willemsen LE. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012. 67(3): p. 343-52.
107. van der Aa LB, Heymans HS, van Aalderen WM, Sillevis Smitt JH, Knol J, Ben Amor K, Goossens DA, Sprikkelman AB, and Synbad Study G. Effect of a new synbiotic mixture on atopic dermatitis in infants: a randomized-controlled trial. Clin Exp Allergy 2010. 40(5): p. 795-804.
108. van der Aa LB, van Aalderen WM, Heymans HS, Henk Sillevis Smitt J, Nauta AJ, Knippels LM, Ben Amor K, Sprikkelman AB, and Synbad Study G. Synbiotics prevent asthma-like symptoms in infants with atopic dermatitis. Allergy 2011. 66(2): p. 170-7.
109. van der Aa LB, Lutter R, Heymans HS, Smids BS, Dekker T, van Aalderen WM, Sillevis Smitt JH, Knippels LM, Garssen J, Nauta AJ, Sprikkelman AB, and Synbad Study G. No detectable beneficial systemic immunomodulatory effects of a specific synbiotic mixture in infants with atopic dermatitis. Clin Exp Allergy 2012. 42(4): p. 531-9.
110. Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, and Kuitunen M. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. J Allergy Clin Immunol 2007. 119(1): p. 192-8.
111. van den Elsen L, Garssen J, and Willemsen L. Long chain N-3 polyunsaturated fatty acids in the prevention of allergic and cardiovascular disease. Curr Pharm Des 2012. 18(16): p. 2375-92.
112. Stulnig TM. Immunomodulation by poly unsaturated fatty acids: mechanisms and effects. Int Arch Allergy Immunol 2003. 132(4): p. 310-21.
113. Miles EA, Aston L, and Calder PC. In

vitro effects of eicosanoids derived from different 20-carbon fatty acids on T helper type 1 and T helper type 2 cytokine production in human whole-blood cultures. Clin Exp Allergy 2003. 33(5): p. 624-32.

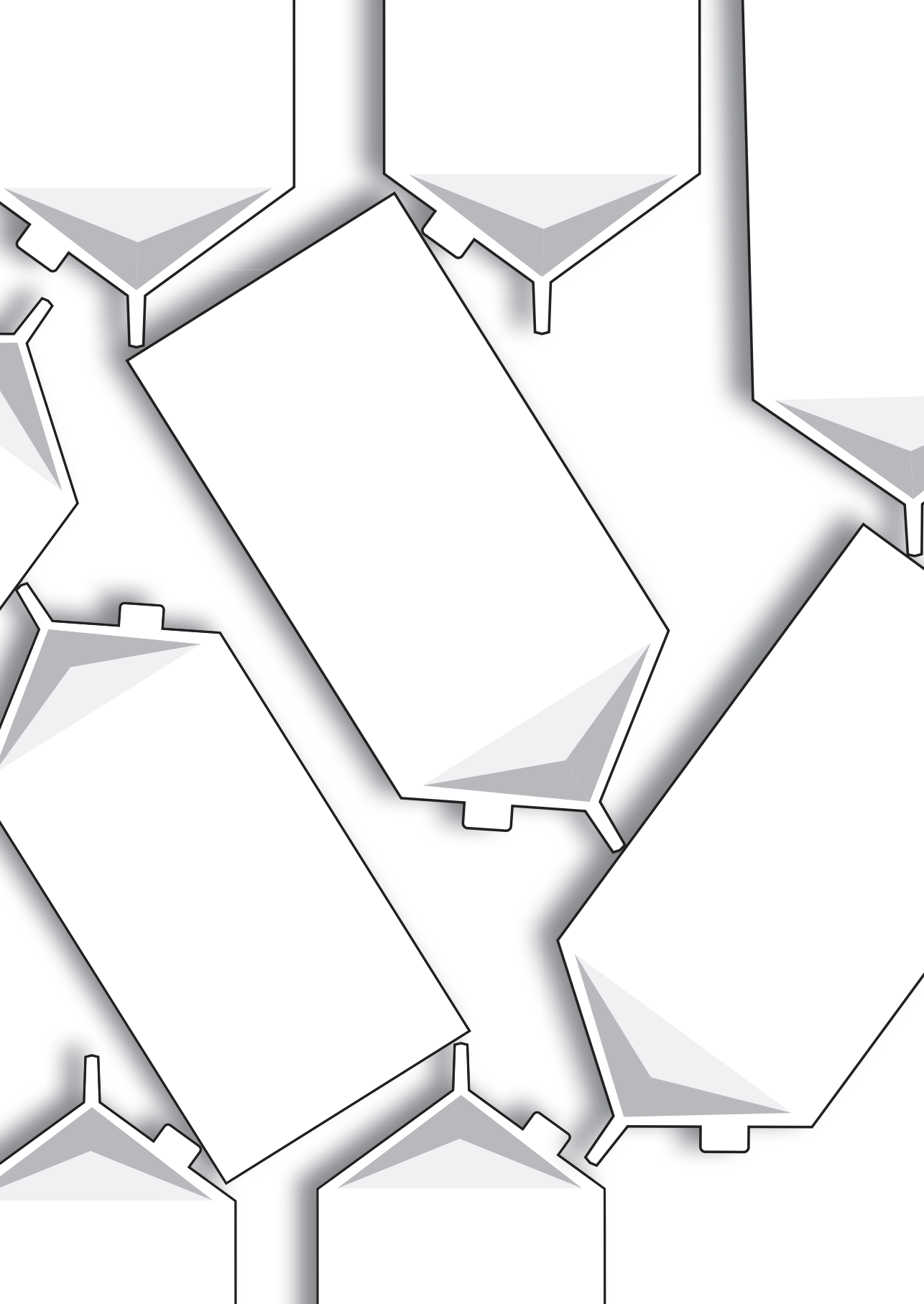
114. Prescott SL and Calder PC. N-3 polyunsaturated fatty acids and allergic disease. Curr Opin Clin Nutr Metab Care 2004. 7(2): p. 123-9.
115. Gottrand F. Long-chain polyunsaturated fatty acids influence the immune system of infants. J Nutr 2008. 138(9): p. 1807S-1812S.
116. Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, Yang R, Petasis NA, and Serhan CN. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. J Exp Med 2005. 201(5): p. 713-22.
117. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, and Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 2010. 142(5): p. 687-98.
118. van den Elsen LW, van Esch BC, Hofman GA, Kant J, van de Heijning BJ, Garssen J, and Willemsen LE. Dietary long chain n-3 polyunsaturated fatty acids prevent allergic sensitization to cow's milk protein in mice. Clin Exp Allergy 2013. 43(7): p. 798-810.
119. van den Elsen LW, Meulenbroek LA, van Esch BC, Hofman GA, Boon L, Garssen J, and Willemsen LE. CD25+ regulatory T cells transfer n-3 long chain polyunsaturated fatty acids-induced tolerance in mice allergic to cow's milk protein. Allergy 2013. 68(12): p. 1562-70.
120. Jenmalm MC and Duchen K. Timing of allergy-preventive and immunomodulatory dietary interventions - are prenatal, perinatal or postnatal strategies optimal? Clin Exp Allergy 2013. 43(3): p. 273-8.
121. Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, and Prescott SL. Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. Clin Exp Allergy 2003. 33(4): p. 442-8.
122. Krauss-Etschmann S, Hartl D, Rzehak P, Heinrich J, Shadid R, Del Carmen Ramirez-Tortosa M, Campoy C, Pardillo S, Schendel DJ, Decsi T, Demmelmair H, Koletzko BV, and Nutraceuticals for Healthier Life Study G. Decreased cord blood IL-4, IL-13, and CCR4 and increased TGF-beta levels after fish oil supplementation of pregnant women. J Allergy Clin Immunol 2008. 121(2): p. 464-470 e6.
123. Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, and Prescott SL. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. J Allergy Clin Immunol 2003. 112(6): p. 1178-84.
124. Furuhjelm C, Warstedt K, Larsson J, Fredriksson M, Bottcher MF, Falth-

Magnusson K, and Duchen K. Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy. Acta Paediatr 2009. 98(9): p. 1461-7.

125. Furuhjelm C, Warstedt K, Fageras M, Falth-Magnusson K, Larsson J, Fredriksson M, and Duchen K. Allergic disease in infants up to 2 years of age in relation to plasma omega-3 fatty acids and maternal fish oil supplementation in pregnancy and lactation. Pediatr Allergy Immunol 2011. 22(5): p. 505-14.
126. D'Vaz N, Meldrum SJ, Dunstan JA, Martino D, McCarthy S, Metcalfe J, Tulic MK, Mori TA, and Prescott SL. Postnatal fish oil supplementation in high-risk infants to prevent allergy: randomized controlled trial. Pediatrics 2012. 130(4): p. 674-82.
127. Prescott S and Nowak-Wegrzyn A. Strategies to prevent or reduce allergic disease. Ann Nutr Metab 2011. 59 Suppl 1: p. 28-42.
128. Allan K, Kelly FJ, and Devereux G. Antioxidants and allergic disease: a case of too little or too much? Clin Exp Allergy 2010. 40(3): p. 370-80.
129. Patelarou E, Giourgouli G, Lykeridou A, Vrioni E, Fotos N, Siamaga E, Vivilaki V, and Brokalaki H. Association between biomarker-quantified antioxidant status during pregnancy and infancy and allergic disease during early childhood: a systematic review. Nutr Rev 2011. 69(11): p. 627-41.
130. Maslova E, Hansen S, Strom M, Halldorsson TI, and Olsen SF. Maternal intake of vitamins A, E and K in pregnancy and child allergic disease: a longitudinal study from the Danish National Birth Cohort. Br J Nutr 2014. 111(6): p. 1096-108.
131. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, and Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007. 204(8): p. 1757-64.
132. Prescott D, Lee J, and Philpott DJ. An epithelial armamentarium to sense the microbiota. Semin Immunol 2013. 25(5): p. 323-33.
133. Ruhl R, Hanel A, Garcia AL, Dahten A, Herz U, Schweigert FJ, and Worm M. Role of vitamin A elimination or supplementation diets during postnatal development on the allergic sensitisation in mice. Mol Nutr Food Res 2007. 51(9): p. 1173-81.
134. Fogarty A, Lewis S, Weiss S, and Britton J. Dietary vitamin E, IgE concentrations, and atopy. Lancet 2000. 356(9241): p. 1573-4.
135. Li-Weber M, Giaisi M, Treiber MK, and Krammer PH. Vitamin E inhibits IL-4 gene expression in peripheral blood T cells. Eur J Immunol 2002. 32(9): p. 2401-8.
136. Han YY, Blatter J, Brehm JM, Forno E, Litonjua AA, and Celedon JC. Diet and asthma: vitamins and methyl donors. Lancet Respir Med 2013. 1(10): p. 813-22.
137. Goldring ST, Griffiths CJ, Martineau AR,

Robinson S, Yu C, Poulton S, Kirkby JC, Stocks J, Hooper R, Shaheen SO, Warner JO, and Boyle RJ. Prenatal vitamin d supplementation and child respiratory health: a randomised controlled trial. PLoS One 2013. 8(6): p. e66627.

138. Weisse K, Winkler S, Hirche F, Herberth G, Hinz D, Bauer M, Roder S, Rolle-Kampczyk U, von Bergen M, Olek S, Sack U, Richter T, Diez U, Borte M, Stangl GI, and Lehmann I. Maternal and newborn vitamin D status and its impact on food allergy development in the German LINA cohort study. Allergy 2013. 68(2): p. 220-8.
139. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, and O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. J Immunol 2001. 167(9): p. 4974-80.
140. Lange NE, Litonjua A, Hawrylowicz CM, and Weiss S. Vitamin D, the immune system and asthma. Expert Rev Clin Immunol 2009. 5(6): p. 693-702.
141. Staeva-Vieira TP and Freedman LP. 1,25-dihydroxyvitamin D3 inhibits IFN-gamma and IL-4 levels during in vitro polarization of primary murine CD4+ T cells. J Immunol 2002. 168(3): p. 1181-9.



CHAPTER 3

THE EFFICACY OF ORAL AND SUBCUTANEOUS ANTIGEN-SPECIFIC IMMUNOTHERAPY IN MURINE COW'S MILK- AND PEANUT ALLERGY MODELS

Marlotte M. Vonk[1,2*]
Laura Wagenaar[3*]
Raymond H.H. Pieters[3]
Léon M.J. Knippels[1,2]
Linette E.M. Willemsen[1]
Joost J. Smit[3]
Betty C.A.M. van Esch[1,2]
Johan Garssen[1,2$]

1. Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
2. Immunology Platform, Danone Nutricia Research, Utrecht, the Netherlands
3. Department of Immunotoxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

* These authors contributed equally to this work
$ All authors are part of the NUTRALL research consortium

Published in Clinical and Translational Allergy (2017)

ABSTRACT

Background: Antigen-specific immunotherapy (AIT) is a promising therapeutic approach for both cow's milk allergy (CMA) and peanut allergy (PNA), but needs optimization in terms of efficacy and safety.

Aim: Compare oral immunotherapy (OIT) and subcutaneous immunotherapy (SCIT) in murine models for CMA and PNA and determine the dose of allergen needed to effectively modify parameters of allergy.

Methods: Female C3H/HeOuJ mice were sensitized intragastrically (i.g.) to whey or peanut extract with cholera toxin. Mice were treated orally (5 times/week) or subcutaneously (3 times/week) for three consecutive weeks. Hereafter, the acute allergic skin response, anaphylactic shock symptoms and body temperature were measured upon intradermal (i.d.) and intraperitoneal (i.p.) challenge, and mast cell degranulation was measured upon i.g. challenge. Allergen-specific IgE, IgG1 and IgG2a were measured in serum at different time points. Single cell suspensions derived from lymph organs were stimulated with allergen to induce cytokine production and T cell phenotypes were assessed using flow cytometry.

Results: Both OIT and SCIT decreased clinically related signs upon challenge in the CMA and PNA model. Interestingly, a rise in allergen-specific IgE was observed during immunotherapy, hereafter, treated mice were protected against the increase in IgE caused by allergen challenge. Allergen-specific IgG1 and IgG2a increased due to both types of AIT. In the CMA model, SCIT and OIT reduced the percentage of activated Th2 cells and increased the percentage of activated Th1 cells in the spleen. OIT increased the percentage of regulatory T cells (Tregs) and activated Th2 cells in the MLN. Th2 cytokines IL-5, IL-13 and IL-10 were reduced after OIT, but not after SCIT. In the PNA model, no differences were observed in percentages of T cell subsets. SCIT induced Th2 cytokines IL-5 and IL-10, whereas OIT had no effect.

Conclusion: We have shown clinical protection against allergic manifestations after OIT and SCIT in a CMA and PNA model. Although similar allergen-specific antibody patterns were observed, differences in T cell and cytokine responses were shown. Whether these findings are related to a different mechanism of AIT in CMA and PNA needs to be elucidated.

INTRODUCTION

Food allergy is an important socio-economic health problem estimated to occur in 10% of pre-school children (Westernized countries) and 1-2% of adult individuals (USA) [1, 2]. Two of the major allergenic foods, peanut- and cow's milk protein, show different disease patterns. Cow's milk allergy (CMA) is most prevalent during early childhood, but is often outgrown [3] while peanut allergy (PNA) is more persistent and is the most frequent cause of life-threatening allergic reactions in adults [4]. Unfortunately, current treatment options for food allergies are limited, being a strict elimination diet and self-administration of epinephrine in case of an anaphylactic response. The need for effective and safe therapeutic options has elicited intensive research into antigen-specific immunotherapy (AIT) as an active tolerance-inducing strategy.

One form of AIT, subcutaneous immunotherapy (SCIT), is effective and safe in respiratory allergies and insect venom hypersensitivities [5-7] and has been recognized as the gold-standard immunotherapy method for decades [8]. However, to date, SCIT has not been used to treat food allergies due to the high incidence of severe side effects in two conducted peanut allergy trials [9, 10]. The less invasive alternative, oral immunotherapy (OIT), has been shown to increase the threshold of food tolerated in a double-blind placebo-controlled food challenge (DBPCFC) in a majority of the subjects in several randomized placebo-controlled clinical trials when on therapy [11]. Nonetheless, OIT for food allergy is still an experimental therapeutic strategy because of the risk of side effects and accidental symptoms toward a previously tolerated dose. OIT in cow's milk- and peanut allergic children was accompanied by persistent adverse reactions during treatment [12, 13]. In addition, sustained unresponsiveness to a food challenge after discontinuation of OIT has only been demonstrated in a minority of the subjects [14]. This clearly leaves OIT open for improvement in both therapy safety and efficacy.

Specific immunological aspects have been suggested to be involved in desensitization and the development of clinical tolerance, including a suppressed T helper 2 (Th2) cell response [11] and the induction of regulatory T cells (Tregs) [15-17], decreased antigen-specific IgE and increased antigen-specific IgG4 levels [18] and effector cell unresponsiveness [19] in mice and/or human. Further attempts to link immunologic changes induced by AIT to clinical protection have been made using murine models of egg allergy. The induction of long-term tolerance was unsuccessful; however, significant changes in intestinal gene expression were observed in clinically protected mice [20, 21]. In humans, clinical tolerance was associated with hypomethylation of the forkhead box protein 3 (Foxp3) locus in Tregs [22].

The goal of this study was to compare the efficacy of OIT and SCIT and to determine the dose of allergen needed to effectively modify parameters of allergy in a murine CMA and PNA model.

MATERIALS AND METHODS

Mice

All animal procedures were performed according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, the Netherlands. Specific-pathogen free six-week-old female C3H/HeOuJ mice (n=6-8/group) were purchased from Charles River Laboratories (L'Arbresle Cedex, France) and were fed a peanut- and cow's milk protein-free standard mouse chow (AIN-93G soya, Special Diets Services, Witham, UK). The animals were housed at the animal facility of Utrecht University on a 12 h light/dark cycle with unlimited access to food and water.

Reagents

Peanut protein extract (PE) was prepared from raw peanuts (provided by Intersnack Nederland BV, the Netherlands) as described previously [23]. Concisely, protein was extracted from ground peanut by mixing 150 g of ground peanut with 750 ml of 20 mM Tris buffer (pH 7.2). After stirring every 10 min for 2 h at room temperature (RT), the aqueous fraction was collected after centrifugation (3000 × g for 30 min) and subsequently centrifuged at 10,000 × g for 30 min to remove residual traces of fat and insoluble particles. The extract contained 30 mg/ml protein as determined by Bradford analysis with Bovine Serum Albumin (BSA) as a standard. Whey protein powder was provided by Nutricia Research (Utrecht, the Netherlands). Cholera toxin (CT) was purchased from List Biological Laboratories Inc. (Campbell, CA, USA).

Experimental design: oral sensitization, immunotherapy and challenges

Mice were sensitized intragastrically (i.g.) to whey (20 mg in 0.5 ml PBS) or PE (6 mg in 200 µl PBS) using CT (15 µg/mouse) as an adjuvant [23, 24] (day 0, 7, 14, 21 and 28 for whey and day 0, 1, 2, 7, 14, 21 and 28 for PE) (**Figure 1**). Sham-sensitized mice received CT in PBS alone. One week after the last sensitization (day 42), the mice were treated orally (OIT: 0.1, 1, 10 and 100 mg whey or 0.15, 1.5 and 15 mg PE in 500 µl PBS) for 5 times/week or subcutaneously (SCIT: 2.5, 10 and 25 µg whey or 1, 10 and 100 µg PE in 200 µl PBS) for 3 times/week, for three consecutive weeks (day

42-60). Sham-sensitized and allergen-sensitized control mice were treated i.g. with PBS alone. On day 65, all mice were challenged intradermally (i.d.) in both ear pinnae with 10 µg whey or 1 µg PE in 20 µl PBS to determine the acute allergic skin response, anaphylactic shock symptom scores and body temperature levels. On day 70 and 84, i.g. challenges (using 50 mg whey or 15 mg PE in 500 µl PBS) were performed to measure mucosal mast cell degranulation in blood samples collected after 30 min. After intraperitoneal (i.p.) challenges on day 77 and 91 (using 100 µg whey or 100 µg PE in 200 µl PBS) anaphylactic shock symptom scores and body temperature levels were measured. At day 92, mice were killed with cervical dislocation and blood and organs were collected.

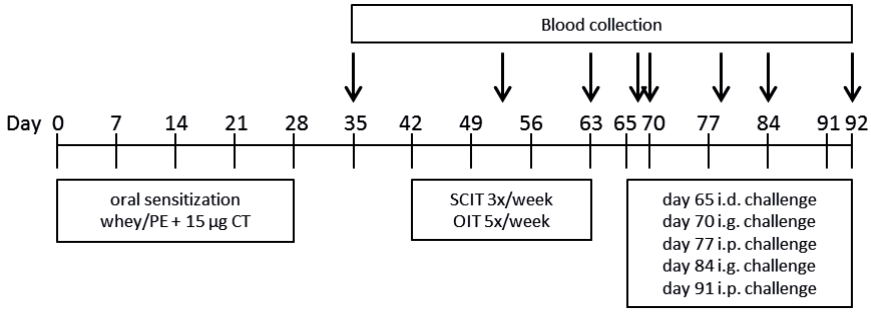


Figure 1. Experimental set-up of PNA and CMA model. Mice were sensitized i.g. to whey, PE or PBS alone in combination with CT (day 0, 7, 14, 21 and 28 for CMA and day 0, 1, 2, 7, 14, 21 and 28 for PNA). From day 42, the mice were treated orally for 5 times/week or subcutaneously for 3 times/week with allergen or PBS alone, for three consecutive weeks (day 42-60). On day 65, all mice were challenged i.d. to determine the acute allergic skin response, anaphylactic shock symptom scores and body temperature levels. On day 70 and 84, i.g. challenges were performed to measure mucosal mast cell degranulation. After i.p. challenges on day 77 and 91, anaphylactic shock symptom scores and body temperature levels were measured. At day 92, the mice were killed with cervical dislocation and blood and organs were collected. PE, peanut extract; CT, cholera toxin; SCIT, subcutaneous immunotherapy; OIT, oral immunotherapy; i.d., intradermal; i.g., intragastric; i.p., intraperitoneal.

Acute allergic skin response, anaphylaxis symptom score and body temperature after challenge

After AIT, on day 65, all mice were anesthetized using inhalation of isoflurane to measure ear thickness in duplicate prior to and 1 h after an i.d. injection with allergen in both ear pinnae. Basal ear thickness (µm) was subtracted from the ear thickness 1 h post-challenge to determine ear swelling as a measure for the acute allergic skin response. Body temperature was measured 30 min after the i.d. challenge using a rectal thermometer and signs of anaphylaxis were scored according to the method described by Li *et al.* [25]. The anaphylaxis-associated drop in body temperature reaches a maximum at time point 30 min after i.d. challenge. In addition, body temperature was measured every 10 min after the i.p. challenge on day 77, using a rectal thermometer and anaphylaxis was scored at time point 40 min after challenge [25].

Levels of mMCP-1 and allergen-specific IgE, IgG1, IgG2a in serum

Blood samples were collected at nine specific time points during the animal experiment (day 35, 50, 63, 65, 70, 78, 84, 92) via cheek puncture and after centrifugation (10,000 rpm for 10 min) sera were stored at -20°C until further analysis.

Levels of whey-specific immunoglobulin (Ig) E, IgG1 and IgG2a were determined by means of ELISA as described previously [24]. Briefly, 96-wells high-binding plates (Costar 3590, Corning Incorporated, Corning, NY, USA) were coated overnight at 4°C with 100 µl (20 µg/ml) whey in coating buffer (carbonate-bicarbonate buffer, 0.05 M, pH 9.6; Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands). The plates were washed (PBS with 0.05% Tween20) and blocked for 1 h (RT) in ELISA buffer (50 mM TRIS, 137 mM NaCl, 2 mM EDTA and 0.05% Tween20) with 0.5% BSA. Serum samples were diluted and incubated on the plates for 2 h (RT). After washing, 100 µl biotin-labeled rat anti-mouse IgE, IgG1 and IgG2a (1 µg/ml; BD Biosciences, Alphen a/d Rijn, the Netherlands) was incubated for 1.5 h (RT). Subsequently, plates were washed and incubated with streptavidin poly horseradish peroxidase (Sanquin, Amsterdam, the Netherlands) for 1 h (RT). After washing, a color reaction was initiated by adding o-phenylendiamine (Sigma). The reaction was stopped using 4 M H_2SO_4 and optical density was measured with a Benchmark microplate reader (BioRad, Hercules, CA, USA) at 490 nm.

PE-specific IgE, IgG1 and IgG2a levels in serum were detected by ELISA as previously described [23]. Briefly, for IgG1 and IgG2a, 96-wells high-binding plates (Costar 3590, Corning Incorporated, Corning, NY, USA) were coated overnight at 4°C with 10 µg/ml PE in PBS followed by blocking 1 h (RT) with 0.5% BSA-ELISA buffer. Serum samples were diluted and incubated for 2 h (RT). For detection, AP-coupled anti-IgG1 and anti-IgG2a were added for 1 h (RT). Subsequently, 1 mg/ml p-nitrofenylphosphate in diethanolamine buffer was used for the color reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm using an Asys expert 96 plate reader (Biochrom, Cambourne, UK).

To measure PE-specific IgE, 96-wells high-binding plates (Costar 3590, Corning Incorporated, Corning, NY, USA) were coated overnight at 4°C with 1 µg/ml rat anti-mouse IgE (BD Biosciences) followed by blocking for 1 h (RT) with 0.5% BSA-ELISA buffer. Serum samples were diluted and incubated for 2 h (RT). Subsequently, PE-DIG conjugate solution was added for 1 h (RT). The coupling of DIG to PE was performed according to the manufacturer's instructions. Briefly, the coupled proteins were separated on a sephadex G-25 column and labeling efficiency was determined by means of spectrophotometry at 280 nm. After incubation for 1 h (RT) with peroxidase-conjugated anti-DIG fragments, a tetramethylbenzidine substrate (0.1 mg/ml) solution was used and the color reaction was stopped with 2 M H_2SO_4. Absorbance was measured at 450 nm. Concentrations of IgE, IgG1 and IgG2a were calculated in arbitrary units (AU) using a standard curve of pooled sera from alum-i.p. whey- or PE-sensitized mice.

Mucosal mast cell protease-1 (mMCP-1) was determined by using a mMCP-1 Sandwich ELISA kit (Mouse MCPT-1 Ready-SET-Go!® ELISA, eBioscience, Breda, the Netherlands) according to the manufacturer's instructions. Levels of mMCP-1 were determined in serum samples obtained 30 min after i.g. challenge.

Analysis of T cell populations using flow cytometry

After collection and homogenization of the spleen (incl. red blood cell lysis) and the mesenteric lymph nodes (MLN; only in the CMA model), single cell suspensions were used to analyze T cell subsets by flow cytometry. 5-10x10^5 cells per well were collected in fluorescence activated cell sorting (FACS) buffer (PBS containing 0.25% BSA, 0.05% NaN_3 and 0.5 mM EDTA) and plated. The cells were blocked for 20 min using PBS containing 1% BSA and 5% fetal calf serum (FCS) in the CMA experiment and Fc block (anti-mouse CD16/32 clone 93, eBioscience) in the PNA experiment. Subsequently, cells were stained with the following antibodies in FACS buffer for

30 min at 4°C: anti-CD4-PerCpCy5.5 (1:100, clone RM4-5), anti-CD25-AlexaFluor 488 (1:100, clone PC61.5), anti-Foxp3-APC (1:50, clone FJK-16s), anti-CD69-APC (1:100, clone H1.2F3), anti-CXCR3-PE (1:50, clone CXCR3-173), anti-CD3e-PerCpCy5.5 (1:100, clone 145-2C11), anti-CD8α-PE (1:100, clone 53-6.7), anti-CD4-FITC (1:200, clone RM4-5), anti-CD25-PE (1:200, clone PC61.5), anti-CD3e-FITC (1:200, clone 145-2C11) from eBioscience, anti-T1St2-FITC (1:50, clone DJ8) from mdbioproducts, anti-CD4-FITC (1:100, clone RM4-5), anti-CD4-PerCp (1:200, clone RM4-5), anti-CD8α-PerCp (1:100, clone 53-6.7), anti-CD4-APC (1:200, clone RM4-5), anti-CD69-PE (1:200, clone H1.2F3) from BD Biosciences. Antibody concentrations were individually titrated beforehand and isotype controls were used. Dead and/or aggregated cells were excluded based on forward/sideward scatter properties. Cut-off gates for positivity were established using the fluorescence-minus-one (FMO) technique. Cells stained for extracellular markers were fixed using 1% paraformaldehyde and cells stained for intracellular Foxp3-APC were permeabilized and fixed using the buffer set purchased from eBioscience according to the manufacturer's protocol. Analysis of the CMA samples was performed on the FACS Canto II (BD Biosciences) and Flowlogic software (Inivai Technologies, Mentone, Australia). Analysis of the PNA samples was performed on the BD Accuri™ C6 flow cytometer and BD sampler software (BD Biosciences).

Cytokine release after *ex vivo* stimulation with whey or PE

$8x10^5$ cells per well in 200 µl derived from spleen and MLN (in CMA model) were cultured in U-bottom culture plates (Greiner, Frickenhausen, Germany) using RPMI 1640 medium (Lonza, Verviers, Belgium) with 10% FCS, penicillin (100 U/ml)/ streptomycin (100 µg/ml) (Sigma) and β-mercaptoethanol (CMA model, 20 µM). All cells received either stimulation with culture medium as a negative control, a polyclonal stimulation with anti-CD3 (CMA model; 1 µg/ml, clone 17A2, eBioscience) or anti-CD3/CD28 (PNA model; 1 µg/ml, clone 145-2C11 and clone 37.51, eBioscience) or antigen-specific stimulation with whey (50 µg/ml) or PE (100 µg/ml). Plates were incubated for 48 h (anti-CD3 or anti-CD3/CD28) or 96 h (whey or PE) to assess production of interleukin (IL)-5, IL-10, IL-13 and Interferon γ (IFNγ) by T cells. Culture supernatants were collected and stored at -20°C until further analysis with the Ready-SET-Go!® ELISA (eBioscience) according to the manufacturer's instructions.

Statistics

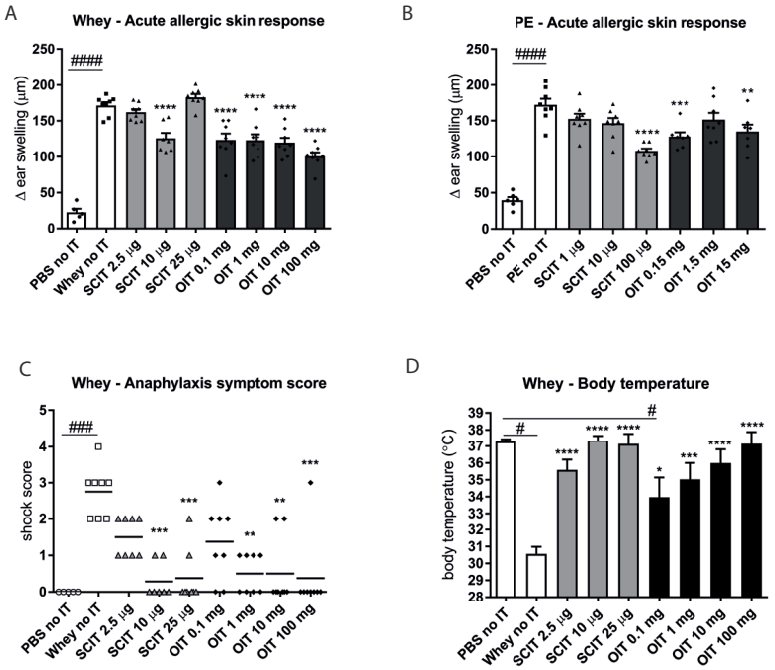
The acute allergic skin response, body temperature levels, flow cytometry data, cytokine concentrations and serum mMCP-1 and immunoglobulin levels are depicted as mean ± SEM and were statistically analyzed with GraphPad Prism software version 6.00 (GraphPad software, La Jolla, CA, USA) using one-way ANOVA and Dunnett's post-hoc test for multiple comparisons to compare the treatment groups with the sensitized control animals within each individual experiment. Body temperature curves were statistically analyzed using a repeated measures two-way ANOVA and Dunnett's post hoc test for multiple comparisons with matched values. Anaphylaxis symptom scores were analyzed using Kruskal-Wallis test for nonparametric data with Dunn's post hoc test. Results were considered statistically significant when $p<0.05$.

RESULTS

Reduction in allergic manifestations upon challenge with whey or PE in OIT and SCIT mice

The acute allergic skin response, measured as ear swelling after i.d. injection with whey or PE, was increased in sensitized mice (whey/PE no IT) compared to sham-sensitized mice (PBS no IT) **(Figures 2A,B)**. In the CMA model, SCIT reduced the acute allergic skin response only at a dose of 10 µg, whereas OIT reduced acute allergic skin responses at all dosages **(Figure 2A)**. Furthermore, both SCIT (10, 25 µg) and OIT (1, 10, 100 mg) reduced anaphylactic shock symptom scores **(Figure 2C)**. SCIT and OIT prevented the characteristic drop in body temperature observed during anaphylaxis and this effect appeared to be dose-related **(Figure 2D)**. In the PNA model, SCIT (100 µg) and OIT (0.15, 15 mg) reduced the acute allergic skin response **(Figure 2B)**. No anaphylactic response was induced after i.d. administration of the used PE dosage, therefore no body temperature and anaphylactic shock symptom score data were included. The i.p. challenge performed in PE-sensitized mice on day 77 indicated protection against clinical responses in a dose-related manner after both SCIT and OIT. Both the drop in body temperature **(Figures 2G,H)** and the anaphylactic shock symptom scores **(Figure 2I)** were significantly reduced in all AIT groups. The i.p. challenge performed in the whey-sensitized mice on day 77 did not show protection against clinical signs, since the used dose of 100 µg induced severe anaphylaxis in all groups (data not shown). In addition, a second i.p. challenge performed on day 91 in both food allergy models did not induce an anaphylactic response in allergen-sensitized control animals (data not shown).

To determine the effect of OIT and SCIT on the local effector response in the gastrointestinal tract, mMCP-1 concentrations were measured in serum collected 30 min after i.g. challenge (day 70) (**Figures 2E,F**). Mast cell degranulation was reduced in all treatment groups in the CMA model, except OIT with 0.1 mg whey (**Figure 2E**). A second i.g. challenge (day 84) did not induce detectable mMCP-1 levels in serum (data not shown). In PE-sensitized animals, a reduction in mMCP-1 was observed in all SCIT groups and the 0.15 mg and 1.5 mg OIT groups (**Figure 2F**). The second i.g. challenge (day 84) showed a similar induction of mMCP-1 in sensitized animals and this increase was absent in SCIT (100 µg) and OIT (0.15, 15 mg, data not shown). In short, SCIT and OIT induced clinical protection against food challenges in both the CMA and PNA model.



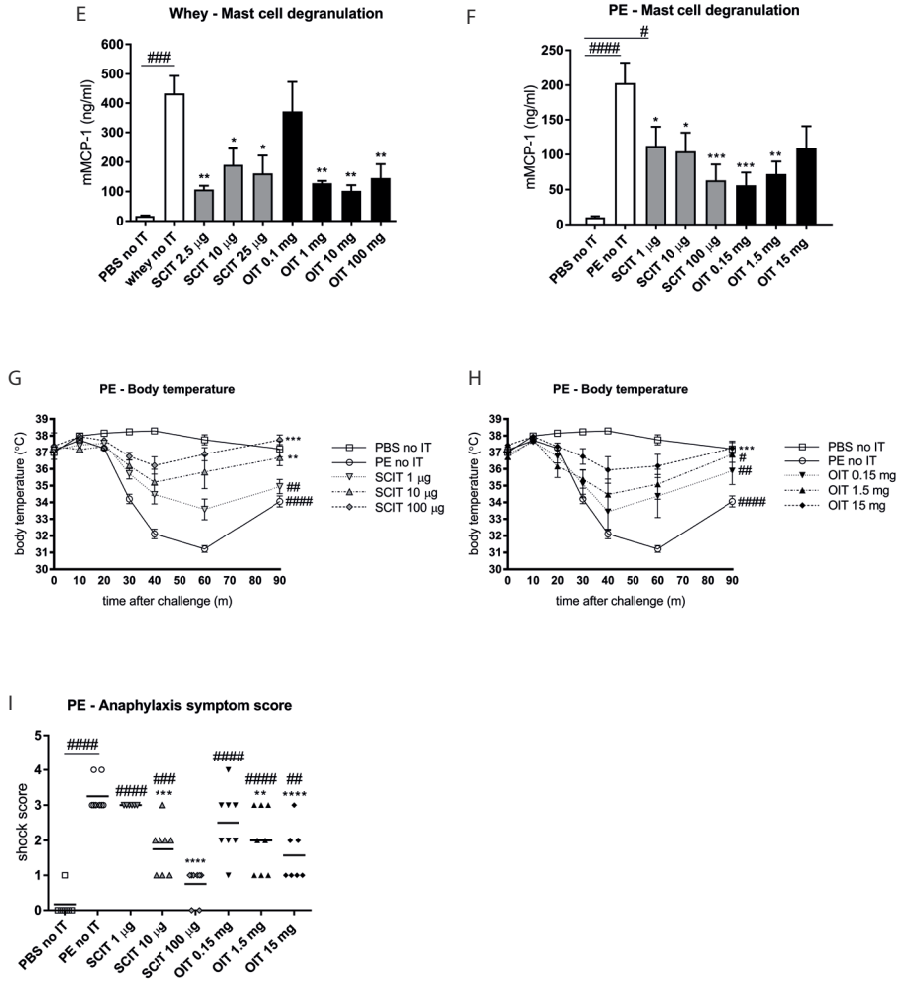
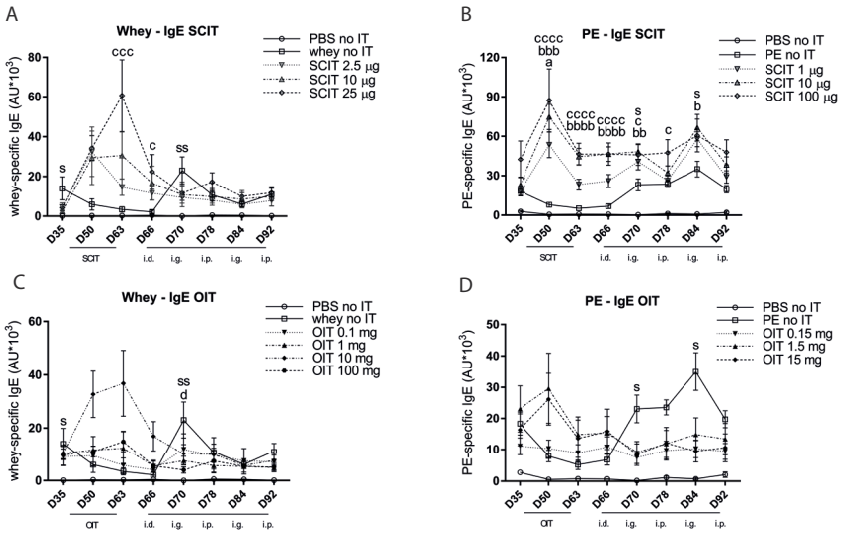


Figure 2. Allergic manifestations evaluated in whey- or PE-sensitized mice after receiving SCIT and OIT. (A,B) Acute allergic skin response measured as Δ ear swelling 1 h after i.d. challenge. **(C)** Anaphylactic shock symptom scores determined 30 min after i.d. challenge in CMA model. **(D)** Body temperature measured 30 min after i.d. challenge in CMA model. **(E,F)** Concentrations of mMCP-1 in serum collected 30 min after i.g. challenge. **(G,H)** Change in body temperature after i.p. challenge in PNA model. **(I)** Anaphylactic shock symptom scores determined 40 min after i.p. challenge in PNA model. Data are represented as mean ± SEM n=6-8 mice/group. Statistical analysis was performed using one-way ANOVA and Dunnett's post hoc test for multiple comparisons or a repeated measures two-way ANOVA and Dunnett's post hoc test for multiple comparisons with matched values for the temperature curve in **(G,H)**. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ compared to sham control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to whey- or PE-sensitized control. OIT, oral immunotherapy; SCIT, subcutaneous immunotherapy; PE, peanut extract; CT, cholera toxin; mMCP-1, mucosal mast cell protease-1; IT, immunotherapy.

Induction of allergen-specific IgE upon challenge absent in OIT and SCIT mice

Allergen-specific IgE, IgG1 and IgG2a levels in serum were measured in particular to investigate whether OIT and SCIT modulated the humoral response. During and after AIT (day 50-63), SCIT increased allergen-specific IgE levels in the PNA model (**Figure 3B**) and in the CMA model with a dose of 25 µg (**Figure 3A**). At day 70, five days after the i.d. challenge, the rise in allergen-specific IgE observed in sensitized control animals compared to sham-sensitized control animals was absent in the OIT and SCIT mice (**Figures 3A-D**). In addition, OIT with 100 mg whey showed significantly lower whey-specific IgE levels compared to the whey-sensitized control group (**Figure 3C**). High dose SCIT and OIT induced IgG1 and IgG2a in the CMA and PNA model (day 63, **Figures 3E-L**). The induction of allergen-specific IgG1 and IgG2a was delayed in sensitized control mice; an increase was observed after the i.d. challenge (day 70) and levels appeared to continuously rise upon repeated challenges (day 70, 78, 84 and 92). However, allergen-specific IgG1 levels in the SCIT and OIT mice did not further increase after day 70/78 (**Figures 3E-H**) despite the challenges. In summary, the data demonstrate that for both allergens OIT and SCIT protect against a challenge-induced rise in allergen-specific IgE and induced allergen-specific IgG1 and IgG2a during immunotherapy.



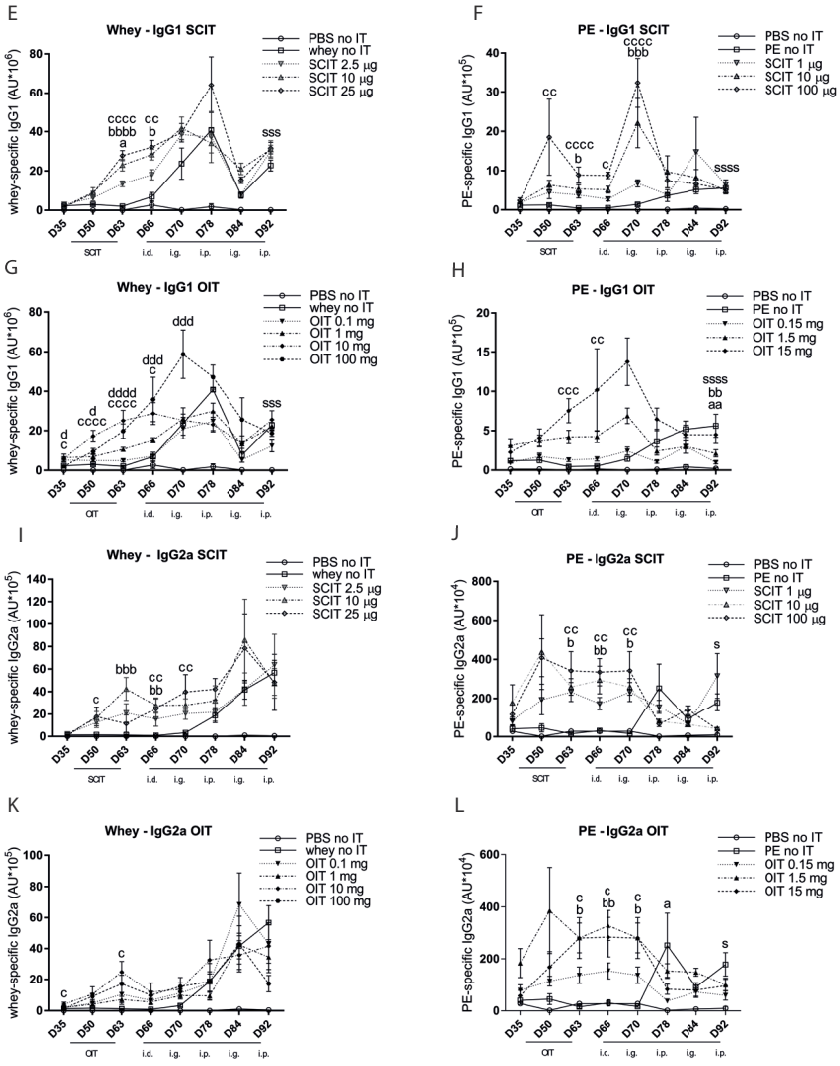


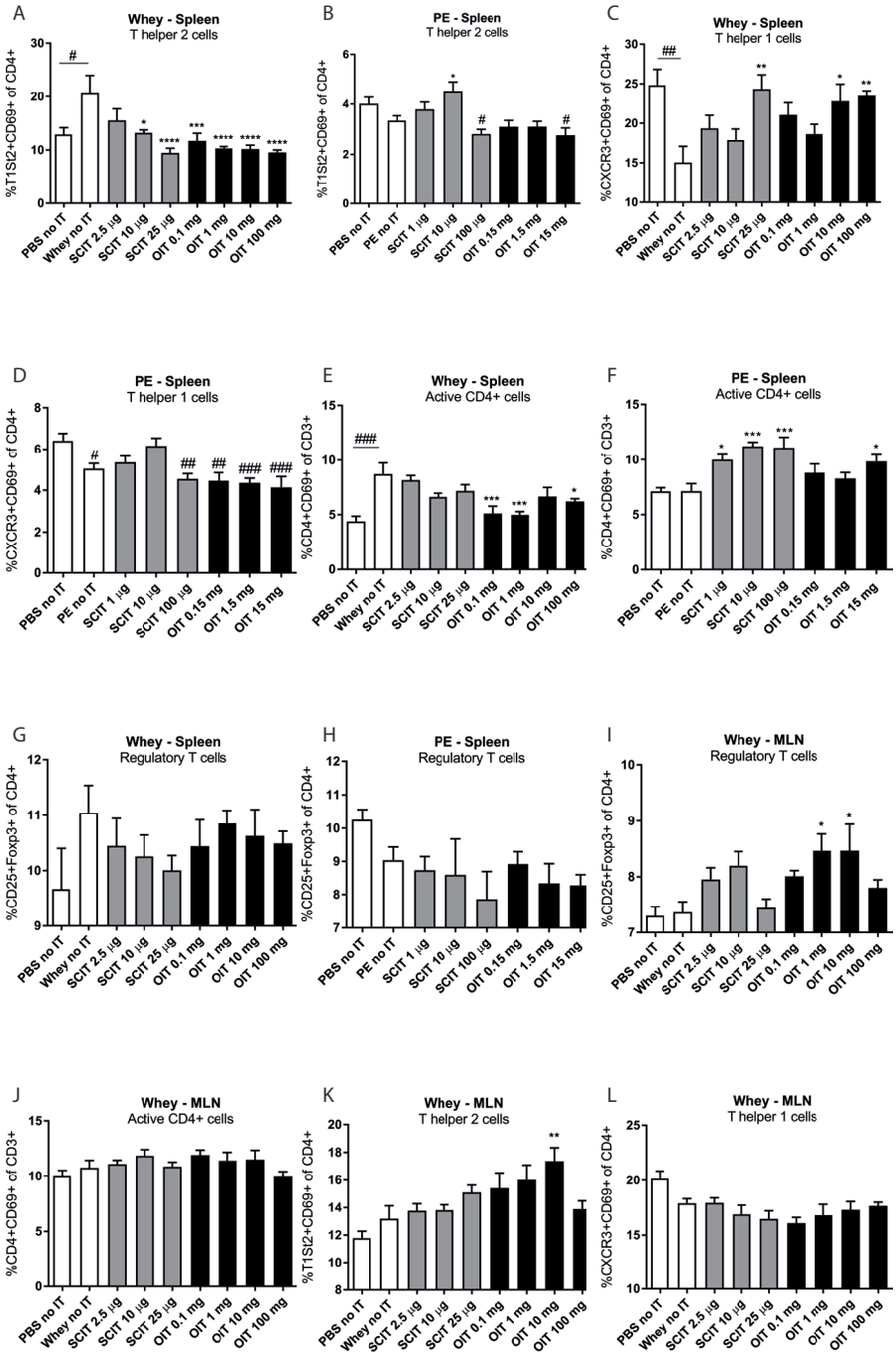
Figure 3. Allergen-specific IgE, IgG1 and IgG2a levels in serum determined by ELISA. (A,B) Allergen-specific IgE in SCIT groups. **(C,D)** Allergen-specific IgE in OIT groups. **(E,F)** Allergen-specific IgG1 in SCIT groups. **(G,H)** Allergen-specific IgG1 in OIT groups. **(I,J)** Allergen-specific IgG2a in SCIT groups. **(K,L)** Allergen-specific IgG2a in OIT groups. Data are represented as mean ± SEM n=6-8 mice/group. Statistical analysis was performed on each individual time point using one-way ANOVA and Dunnett's post hoc test for multiple comparisons. All treatment groups were compared to the sensitized control group and significant differences were indicated with letters e.g., $^{a}p<0.05$, $^{aa}p<0.01$, $^{aaa}p<0.001$, $^{aaaa}p<0.0001$. In CMA figures: a for SCIT 2.5 µg and OIT 0.1 mg, b for SCIT 10 µg and OIT 1 mg, c for SCIT 25 µg and OIT 10 mg, d for OIT 100 mg and s for sham control. In PNA figures: a for SCIT 1 µg and OIT 0.15 mg, b for SCIT 10 µg and OIT 1.5 mg and c for SCIT 100 µg and OIT 15 mg and s for sham control. OIT, oral immunotherapy; SCIT, subcutaneous immunotherapy; IT, immunotherapy; PE, peanut extract; i.d., intradermal challenge; i.g., intragastric challenge; i.p., intraperitoneal challenge.

Shifted T cell profile in lymph organs after OIT and SCIT

In the spleen, the percentage of activated Th2 cells (T1St2+CD69+ of CD4+ cells) was elevated in whey-sensitized control animals compared to sham-sensitized control animals (**Figure 4A**). OIT (all dosages) and SCIT (10, 25 µg) reduced the percentage of activated Th2 cells. This reduction coincided with an increase in the percentage of activated Th1 cells (CXCR3+CD69+ of CD4+ cells) in the 25 µg SCIT and 10 mg and 100 mg OIT groups (**Figure 4C**) compared to the whey-sensitized control animals. In contrast to the CMA model, no difference in the percentage of activated Th2 cells was observed in PE-sensitized control animals compared to sham-sensitized control animals (**Figure 4B**). The percentage of activated Th1 cells was decreased in PE-sensitized mice compared to sham-sensitized mice (**Figure 4D**). In addition, SCIT and OIT with PE (15 mg) increased the percentage of activated CD4+ T cells (CD4+CD69+ of CD3+ cells) compared to the PE-sensitized control animals (**Figure 4F**).

In the MLN collected in the CMA model, the percentage of CD4+CD25+Foxp3+ Tregs was elevated in OIT mice (1, 10 mg) (**Figure 4I**) compared to whey-sensitized control animals. In addition, an increase in percentage of activated Th2 cells in the 10 mg OIT group was observed. No effect of OIT and SCIT on the induction of Tregs was found in spleen in either the CMA or the PNA model (**Figures 4G,H**). Briefly, we observed differences in the percentages of T cells in the lymph organs of the cow's milk and peanut allergic mice in response to therapy.

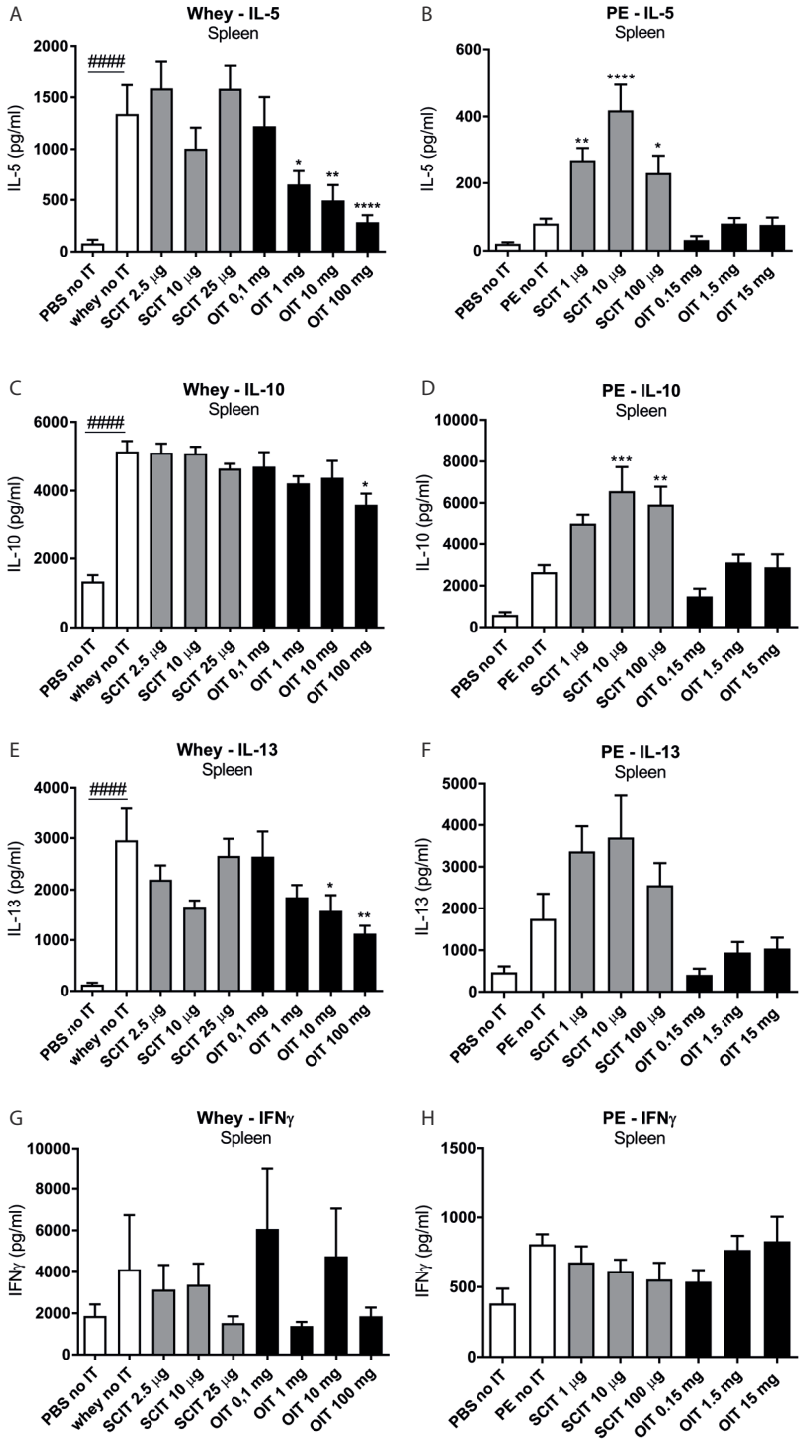
Figure 4. Flow cytometric analysis of T cell populations in the spleen and MLN. Cells were gated based on FSC–SSC properties and the Fluorescence-minus-one (FMO) technique. **(A,B)** Percentage of activated Th2 cells (T1St2+CD69+ of CD4+) in spleen. **(C,D)** Percentage of activated Th1 cells (CXCR3+CD69+ of CD4+) in spleen. **(E,F)** Percentage of activated CD4+ cells (CD4+CD69+ of CD3+) in spleen. **(G,H)** Percentage of Tregs (CD25+Foxp3+ of CD4+) in spleen. **(I)** Percentage of Tregs (CD25+Foxp3+ of CD4+) in MLN of CMA animals. **(J)** Percentage of activated CD4+ cells (CD4+CD69+ of CD3+) in MLN of CMA animals. **(K)** Percentage of activated Th2 cells (T1St2+CD69+ of CD4+) in MLN of CMA animals. **(L)** Percentage of activated Th1 cells (CXCR3+CD69+ of CD4+) in MLN of CMA animals. All data are represented as mean ± SEM n=6-8 mice/group. Statistical analysis was performed using one-way ANOVA and Dunnett's post hoc test for multiple comparisons. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to sham control. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ compared to whey- or PE-sensitized control. PE, peanut extract; OIT, oral immunotherapy; SCIT, subcutaneous immunotherapy; IT, immunotherapy.


A
Whey - Spleen
T helper 2 cells
%T1St2+CD69+ of CD4+
B
PE - Spleen
T helper 2 cells
%T1St2+CD69+ of CD4+
C
Whey - Spleen
T helper 1 cells
%CXCR3+CD69+ of CD4+
D
PE - Spleen
T helper 1 cells
%CXCR3+CD69+ of CD4+
E
Whey - Spleen
Active CD4+ cells
%CD4+CD69+ of CD3+
F
PE - Spleen
Active CD4+ cells
%CD4+CD69+ of CD3+
G
Whey - Spleen
Regulatory T cells
%CD25+Foxp3+ of CD4+
H
PE - Spleen
Regulatory T cells
%CD25+Foxp3+ of CD4+
I
Whey - MLN
Regulatory T cells
%CD25+Foxp3+ of CD4+
J
Whey - MLN
Active CD4+ cells
%CD4+CD69+ of CD3+
K
Whey - MLN
T helper 2 cells
%T1St2+CD69+ of CD4+
L
Whey - MLN
T helper 1 cells
%CXCR3+CD69+ of CD4+
PBS no IT
Whey no IT
PE no IT
SCIT 2.5 µg
SCIT 1 µg
SCIT 10 µg
SCIT 25 µg
SCIT 100 µg
OIT 0.1 mg
OIT 0.15 mg
OIT 1 mg
OIT 1.5 mg
OIT 10 mg
OIT 15 mg
OIT 100 mg

Altered cytokine production after *ex vivo* stimulation of lymphocytes with whey or PE

Cellular activation was confirmed in the control conditions of the *ex vivo* stimulation assay; a significant increase in IL-5, IL-10, IL-13 and IFNγ production was observed in all groups after polyclonal stimulation of cultured cells using anti-CD3 (CMA model) or anti-CD3/CD28 (PNA model) compared to stimulation with only medium in both food allergy models (data not shown). *Ex vivo* stimulation with whey increased the IL-5, IL-10 and IL-13 concentration in supernatants of splenocyte cultures derived from whey-sensitized control animals (**Figures 5A,C,E**). OIT (1, 10 and 100 mg) reduced the release of IL-5 and IL-13. Except for 100 mg OIT, IL-10 levels remained high in the OIT groups. SCIT did not change cytokine levels. The Th1-related IFNγ release upon stimulation did not differ among the groups (**Figure 5G**). In the PNA model, cytokine production was affected by SCIT but not by OIT (**Figures 5B,D,F,H**). Compared to PE-sensitized control mice, SCIT increased antigen-induced release of IL-5 at 1 and 10 µg (**Figure 5B**) and IL-10 at 10 and 100 µg (**Figure 5D**). No change in IL-13 production was observed in the SCIT groups (**Figure 5F**). Again, IFNγ release upon stimulation did not differ between groups (**Figure 5H**). Overall, SCIT induced an increase (IL-5, IL-10) in the PNA model, whereas SCIT in the CMA model did not affect cytokine levels. OIT did not affect cytokine production in the PNA model, whereas it reduced Th2-associated cytokine production in the CMA model.

Figure 5. Cytokine concentrations after *ex vivo* stimulation of splenocytes with whey or PE determined by ELISA. Splenocytes were cultured for 96 h in the presence of PE, whey or medium (medium data not shown). **(A,B)** IL-5 concentration, **(C,D)** IL-10 concentration, **(E,F)** IL-13 concentration and **(G,H)** IFNγ concentration. Data are represented as mean ± SEM n=6-8 mice/group. Statistical analysis was performed using one-way ANOVA and Dunnett's post hoc test for multiple comparisons. ####p<0.0001 compared to sham control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to whey- or PE-sensitized control. PE, peanut extract; OIT, oral immunotherapy; SCIT, subcutaneous immunotherapy; IT, immunotherapy.


A
Whey - IL-5
Spleen
IL-5 (pg/ml)
2000
1500
1000
500
0
####
*
**
****
PBS no IT
whey no IT
SCIT 2.5 µg
SCIT 10 µg
SCIT 25 µg
OIT 0,1 mg
OIT 1 mg
OIT 10 mg
OIT 100 mg
B
PE - IL-5
Spleen
IL-5 (pg/ml)
600
400
200
0
**
****
*
PBS no IT
PE no IT
SCIT 1 µg
SCIT 10 µg
SCIT 100 µg
OIT 0.15 mg
OIT 1.5 mg
OIT 15 mg
C
Whey - IL-10
Spleen
IL-10 (pg/ml)
6000
4000
2000
0
####
*
PBS no IT
whey no IT
SCIT 2.5 µg
SCIT 10 µg
SCIT 25 µg
OIT 0,1 mg
OIT 1 mg
OIT 10 mg
OIT 100 mg
D
PE - IL-10
Spleen
IL-10 (pg/ml)
10000
8000
6000
4000
2000
0
***
**
PBS no IT
PE no IT
SCIT 1 µg
SCIT 10 µg
SCIT 100 µg
OIT 0.15 mg
OIT 1.5 mg
OIT 15 mg
E
Whey - IL-13
Spleen
IL-13 (pg/ml)
4000
3000
2000
1000
0
####
*
**
PBS no IT
whey no IT
SCIT 2.5 µg
SCIT 10 µg
SCIT 25 µg
OIT 0,1 mg
OIT 1 mg
OIT 10 mg
OIT 100 mg
F
PE - IL-13
Spleen
IL-13 (pg/ml)
5000
4000
3000
2000
1000
0
PBS no IT
PE no IT
SCIT 1 µg
SCIT 10 µg
SCIT 100 µg
OIT 0.15 mg
OIT 1.5 mg
OIT 15 mg
G
Whey - IFNγ
Spleen
IFNγ (pg/ml)
10000
8000
6000
4000
2000
0
PBS no IT
whey no IT
SCIT 2.5 µg
SCIT 10 µg
SCIT 25 µg
OIT 0,1 mg
OIT 1 mg
OIT 10 mg
OIT 100 mg
H
PE - IFNγ
Spleen
IFNγ (pg/ml)
1500
1000
500
0
PBS no IT
PE no IT
SCIT 1 µg
SCIT 10 µg
SCIT 100 µg
OIT 0.15 mg
OIT 1.5 mg
OIT 15 mg

DISCUSSION

We aimed to compare the efficacy of OIT and SCIT in models of PNA and CMA and to determine the dose of allergen needed to effectively modify parameters of allergy. We have shown in both models that OIT and SCIT reduced clinical manifestations of food allergy and resulted in comparable changes in serum levels of allergen-specific IgE and IgG subtypes. Differences in T cell populations and cytokine profiles suggest a potential difference in the mechanism of AIT for PNA and CMA.

While both types of immunotherapy were able to decrease allergic manifestations upon challenge, the effective therapeutic dose differed per allergen. OIT using 0.1 mg whey did not decrease signs of anaphylaxis and only mildly prevented the drop in body temperature. The efficacy of OIT was evident in the groups receiving 1 mg whey or higher. In SCIT, 10 µg whey was the most effective dose. In the PNA model, the intermediate and high dosages (1.5, 15 mg OIT and 10, 100 µg SCIT) were the most effective in modulation of disease parameters.

To investigate the effect of OIT and SCIT on mucosal mast cell degranulation, all mice were challenged per oral gavage. Although OIT with 0.1 mg whey was ineffective, SCIT and OIT effectively reduced mMCP-1 levels in both food allergy models. This finding indicates that regular administration of an allergen dose above a certain threshold influences responsiveness of effector cells along the gastrointestinal tract. This is a known effect of AIT and reflects desensitization. Repeated stimulation of the FcεRI present on basophils during OIT in peanut allergic individuals reduces basophil activation as shown by down-regulation of the activation marker CD63 [26]. Another possible explanation for the reduced release of mediators by effector cells might be a reduced number of basophils and mast cells in the early phase of immunotherapy [19]. In addition, repeated exposure to the allergen can contribute to exhaustion of effector cells [27]. In the CMA model, a second i.g. challenge after two weeks resulted in a low mMCP-1 concentration in the serum of the whey-sensitized control group. This observation might be associated with the low levels of allergen-specific IgE in serum of the whey-sensitized control animals on day 84, while whey-specific IgG2a levels were increased. Such low IgE levels may be insufficient to re-sensitize mucosal mast cells [28]. *In vitro* studies showed that mast cells are able to refill their granules and respond again to an allergen challenge after 24 h to 48 h [29]. In accordance, PE-specific IgE levels in serum of the sensitized control animals were not decreased and IgG2a levels were not elevated at day 84 and similar mMCP-1 levels were found upon a second i.g. challenge when compared to the first i.g. challenge. Although protection against i.p. challenge-induced anaphylaxis was shown in OIT and SCIT mice in the PNA model (day 77), a second i.p. challenge did not induce anaphylaxis

in allergen-sensitized animals (both CMA and PNA model, day 91). We hypothesized that repeated systemic (i.p.) challenges, as conducted in the current models to ensure a detectable Th2 cell-mediated effector response, leads to exhaustion of the effector cells present in the peritoneum and this unintentionally affects allergic outcomes.
In humans, AIT is known to increase antigen-specific IgE in serum. However, if the treatment is prolonged for a period of months or even years, IgE levels tend to decline [30]. Peanut allergic individuals subjected to OIT showed an initial increase in IgE, but levels were stabilized despite an oral food challenge [31]. In accordance, our findings indicate an increase in allergen-specific IgE in mice receiving OIT and SCIT, followed by a return to baseline when immunotherapy was discontinued. Remarkably, IgE levels failed to increase after the allergen challenges in the SCIT and OIT groups. The induction of allergen-specific IgG1 and IgG2a by OIT and SCIT appeared to be dose-dependent. In humans, IgG4 levels are elevated during immunotherapy and are associated with protection against clinical symptoms [31, 32]. IgG subtypes are proposed to capture the antigen and thereby inhibit binding to IgE present on mast cells and basophils and thus prevent degranulation [19]. The reduced mMCP-1 release measured after i.g. challenge might be explained by the elevated IgG subtype levels in serum. Furthermore, IgG levels were increased by the challenge protocol, including in the serum of the allergen-sensitized control animals. This finding might explain the absence of clinical signs in the follow-up challenges in the CMA model.

The effect of OIT and SCIT on the humoral response can be linked to the percentages of T helper cell subsets in the lymphoid organs in the CMA model, but not in the PNA model. Specific IgE production by plasma cells is sustained by a Th2 cell dominated immune response in the presence of IL-4, IL-5 and IL-13 [33]. Clinical protection after OIT in peanut allergic subjects in a randomized controlled study was accompanied by a reduction in IL-5 and IL-13 concentration [31]. Skewing of the immune response from a Th2 profile toward a more regulatory profile is associated with a modified cytokine milieu [34]. The reduced percentage of activated Th2 cells in the spleen of OIT mice (CMA model) was accompanied by a dose-dependent reduction in the IL-5 and IL-13 concentration in stimulated cultures. This observation is consistent with the fact that exposure to a high allergen dose leads to anergy in specific T cells [35]. On the contrary, IL-5 and IL-13 production was not decreased in the SCIT groups, although the percentage of activated Th2 cells was decreased in the 10 µg and 25 µg groups. These findings suggest that the route of antigen administration is important in the modulation of specific T cell responsiveness during immunotherapy. Th2-associated cytokine IL-4 was not detected in the stimulated cell cultures, as was previously described for the current food allergy model with the C3H/HeOuJ strain [36]. A similar pattern of IL-5 and IL-13 levels as described for spleen was

observed in MLN culture supernatants in the CMA model, indicating a suppressed Th2 responsiveness after OIT but not after SCIT. Nevertheless, a tendency toward an increase in activated Th2 cells was observed in the MLN of the OIT groups, with a significant difference in the OIT 10 mg group. In the PNA model, SCIT increased levels of IL-5 and IL-10 compared to the PE-sensitized control animals, whereas OIT did not have an effect on cytokine production. Earlier studies show that tolerance induction by SCIT is accompanied by a shift from a Th2 cytokine profile toward a Th1 cytokine profile, but there are discrepancies in the literature [37]. The observed differences in Th2 cytokine production between OIT and SCIT and between the CMA and PNA models might be explained by the induction of antigen-specific Tregs that can exert a suppressive function toward effector T cells [38, 39].

High dose SCIT and OIT induced a shift in the percentages of activated splenic Th1 and Th2 cells after the final i.p. challenge in the CMA model but not in the PNA model. The observation in the CMA model is in accordance with the hypothesis that oral tolerance induction is characterized by a shift from a Th2 response toward a Th1 response [33]. Furthermore, low-dose induction of tolerance is accompanied by increased numbers of CD4+CD25+Foxp3+ Tregs [33]. Indeed, this was observed in the CMA model where OIT (1, 10 mg) increased the percentage of CD4+CD25+Foxp3+ Tregs in the MLN. The fact that no difference in percentage of Foxp3+ Tregs was observed in spleen (PNA and CMA), is contradictory to the results published by Dioszeghy et al, who have shown an increase in Foxp3+ Tregs in the spleen of peanut-allergic mice subjected to OIT [17]. This dissimilarity might be explained by the different mouse strains used in both studies; C3H/HeOuJ and BALB/c show differences in allergic responses [36]. In addition, we could not link splenic *ex vivo* IL-10 levels to the presence of Tregs in both food allergy models. An increase in the IL-10 concentration was found in allergen-stimulated cultures derived from lymph organs of allergic mice, indicating the contribution of Th2-derived IL-10 [40, 41]. Hence, clinical protection observed after AIT and allergen challenge in both the CMA and PNA models might partially be explained by the induced IgG1 and IgG2a levels in combination with low IgE levels. Given the fact that IgG can also drive an alternative food-induced anaphylaxis pathway [42], the potential protective effect of IgG1 and IgG2a needs to be confirmed with a more mechanistic approach.

Despite differences between the CMA and PNA models, overall, the reported clinical, cellular and humoral data can be linked to our current understanding of oral tolerance and immunotherapy mechanisms in humans [19]. However, future use of both models would require further investigation of the exact role of immunoregulatory mechanisms, such as regulatory T cells or antibody-mediated protection, and of long-term effects of the therapeutic strategies.

CONCLUSION

In conclusion, the murine CMA and PNA studies showed that clinical protection can be achieved via OIT and SCIT. Although similar allergen-specific immunoglobulin patterns were observed, differences in T cell populations and cytokine responses were shown. More insight into the mechanism of (long- term) tolerance induction is needed; nonetheless, our findings contribute to the development of effective AIT protocols. In the future, the current OIT models will be used to study the possible benefit of using immunomodulatory food components (e.g., non-digestible oligosaccharides) as adjunct therapy to support antigen-specific immunotherapy in terms of efficacy and safety.

ACKNOWLEDGEMENTS

We would like to thank M. van Roest, L. Kruijssen, M.W.H.C. Bol-Schoenmakers, M.A.P. Diks, G.M. Dingjan and G.A. Hofman for their technical assistance.

REFERENCES

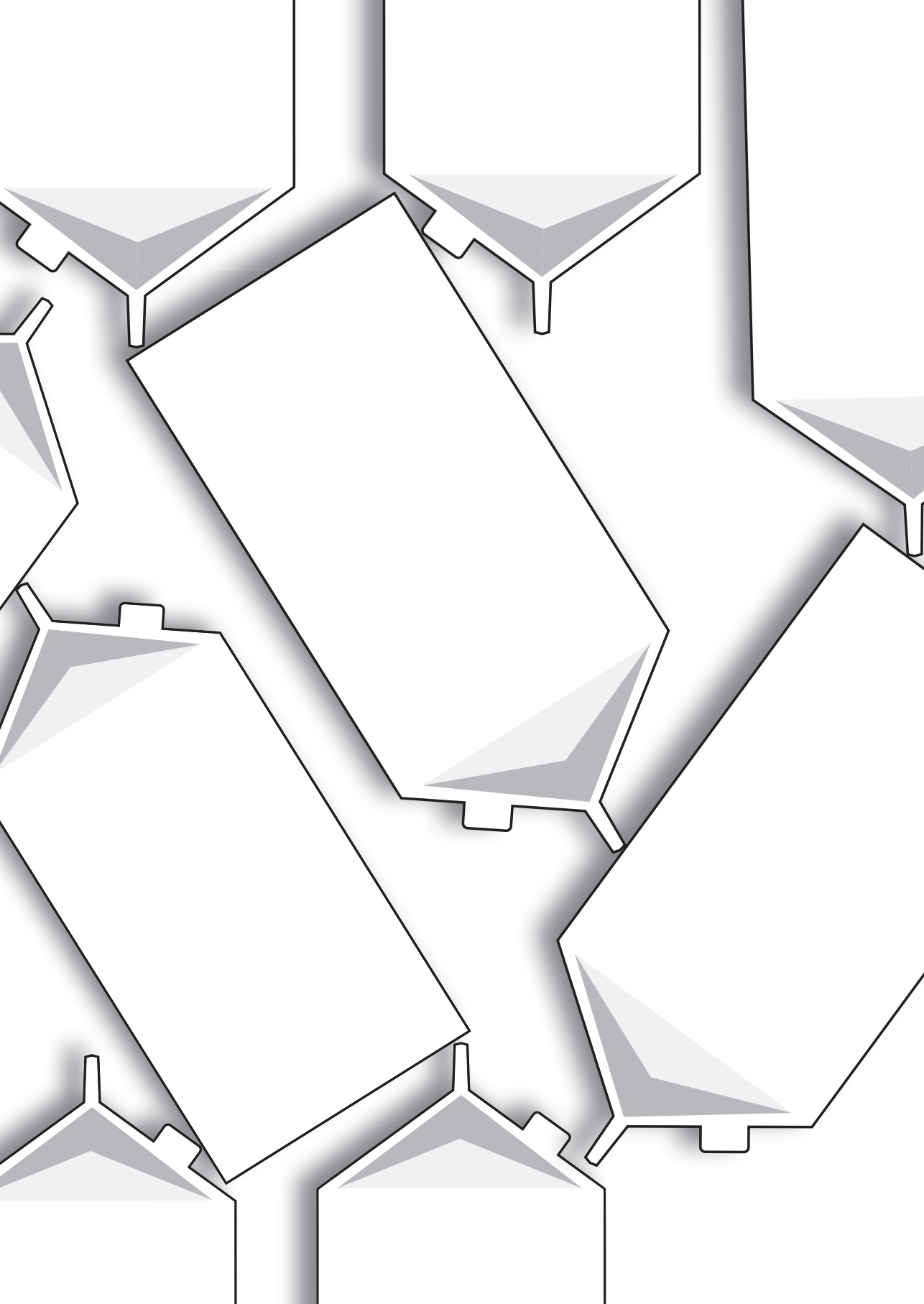
1. Prescott SL, Pawankar R, Allen KJ, Campbell DE, Sinn J, Fiocchi A, Ebisawa M, Sampson HA, Beyer K, and Lee BW. A global survey of changing patterns of food allergy burden in children. World Allergy Organ J 2013. 6(1): p. 21.
2. Sampson HA. Update on food allergy. J Allergy Clin Immunol 2004. 113(5): p. 805-19; quiz 820.
3. Ludman S, Shah N, and Fox AT. Managing cows' milk allergy in children. BMJ 2013. 347: p. f5424.
4. Al-Ahmed N, Alsowaidi S, and Vadas P. Peanut allergy: An overview. Allergy Asthma Clin Immunol 2008. 4(4): p. 139-43.
5. Erekosima N, Suarez-Cuervo C, Ramanathan M, Kim JM, Chelladurai Y, Segal JB, and Lin SY. Effectiveness of subcutaneous immunotherapy for allergic rhinoconjunctivitis and asthma: A systematic review. Laryngoscope 2014. 124(3): p. 616-27.
6. Roberts G, Hurley C, Turcanu V, and Lack G. Grass pollen immunotherapy as an effective therapy for childhood seasonal allergic asthma. J Allergy Clin Immunol 2006. 117(2): p. 263-8.
7. Antolin-Amerigo D, Moreno Aguilar C, Vega A, and Alvarez-Mon M. Venom immunotherapy: An updated review. Curr Allergy Asthma Rep 2014. 14(7): p. 449.
8. Mondoulet L, Dioszeghy V, Ligouis M, Dhelft V, Dupont C, and Benhamou PH. Epicutaneous immunotherapy on intact skin using a new delivery system in a murine model of allergy. Clin Exp Allergy 2010. 40(4): p. 659-67.
9. Oppenheimer JJ, Nelson HS, Bock SA, Christensen F, and Leung DY. Treatment of peanut allergy with rush immunotherapy. J Allergy Clin Immunol 1992. 90(2): p. 256-62.
10. Nelson HS, Lahr J, Rule R, Bock A, and Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. J Allergy Clin Immunol 1997. 99(6 Pt 1): p. 744-51.
11. Chiang D and Berin MC. An examination of clinical and immunologic outcomes in food allergen immunotherapy by route of administration. Curr Allergy Asthma Rep 2015. 15(6): p. 536.
12. Vazquez-Ortiz M, Alvaro-Lozano M, Alsina L, Garcia-Paba MB, Piquer-Gibert M, Giner-Munoz MT, Lozano J, Dominguez-Sanchez O, Jimenez R, Dias M, Martin-Mateos MA, and Plaza-Martin AM. Safety and predictors of adverse events during oral immunotherapy for milk allergy: Severity of reaction at oral challenge, specific ige and prick test. Clin Exp Allergy 2013. 43(1): p. 92-102.
13. Virkud YV, Burks AW, Steele PH, Edwards LJ, Berglund JP, Jones SM, Scurlock AM, Perry TT, Pesek RD, and Vickery BP. Novel baseline predictors of allergic side effects during peanut oral immunotherapy. J Allergy Clin Immunol 2016. p. 139
14. Berin MC and Mayer L. Can we produce true tolerance in patients with food allergy? J Allergy Clin Immunol 2013.

131(1): p. 14-22.
15. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, Shreffler WG, Steele P, Henry KA, Adair M, Francis JM, Durham S, Vickery BP, Zhong X, and Burks AW. Clinical efficacy and immune regulation with peanut oral immunotherapy. J Allergy Clin Immunol 2009. 124(2): p. 292-300, 300 e1-97.
16. Cavkaytar O, Akdis CA, and Akdis M. Modulation of immune responses by immunotherapy in allergic diseases. Curr Opin Pharmacol 2014. 17: p. 30-7.
17. Dioszeghy V, Mondoulet L, Puteaux E, Dhelft V, Ligouis M, Plaquet C, Dupont C, and Benhamou PH. Differences in phenotype, homing properties and suppressive activities of regulatory t cells induced by epicutaneous, oral or sublingual immunotherapy in mice sensitized to peanut. Cell Mol Immunol 2016. 14(9): p. 770-782
18. Vickery BP, Lin J, Kulis M, Fu Z, Steele PH, Jones SM, Scurlock AM, Gimenez G, Bardina L, Sampson HA, and Burks AW. Peanut oral immunotherapy modifies ige and igg4 responses to major peanut allergens. J Allergy Clin Immunol 2013. 131(1): p. 128-34 e1-3.
19. Akdis CA and Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. World Allergy Organ J 2015. 8(1): p. 17.
20. Leonard SA, Martos G, Wang W, Nowak-Wegrzyn A, and Berin MC. Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa. J Allergy Clin Immunol 2012. 129(6): p. 1579-1587 e1.
21. Berin C and Wang W. Oral immunotherapy in the sensitized mouse: Barriers to tolerance induction. Journal of Allergy and Clinical Immunology 2010. 125(2): p. Ab25-Ab25.
22. Syed A, Garcia MA, Lyu SC, Bucayu R, Kohli A, Ishida S, Berglund JP, Tsai M, Maecker H, O'Riordan G, Galli SJ, and Nadeau KC. Peanut oral immunotherapy results in increased antigen-induced regulatory t-cell function and hypomethylation of forkhead box protein 3 (foxp3). J Allergy Clin Immunol 2014. 133(2): p. 500-10.
23. van Wijk F, Nierkens S, Hassing I, Feijen M, Koppelman SJ, de Jong GA, Pieters R, and Knippels LM. The effect of the food matrix on in vivo immune responses to purified peanut allergens. Toxicol Sci 2005. 86(2): p. 333-41.
24. Schouten B, van Esch BC, Hofman GA, van den Elsen LW, Willemsen LE, and Garssen J. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. Int Arch Allergy Immunol 2008. 147(2): p. 125-34.
25. Li XM, Schofield BH, Huang CK, Kleiner GI, and Sampson HA. A murine model of ige-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 1999. 103(2 Pt 1): p. 206-14.
26. Thyagarajan A, Jones SM, Calatroni A, Pons L, Kulis M, Woo CS, Kamalakannan M, Vickery BP, Scurlock

AM, Wesley Burks A, and Shreffler WG. Evidence of pathway-specific basophil anergy induced by peanut oral immunotherapy in peanut-allergic children. Clin Exp Allergy 2012. 42(8): p. 1197-205.

27. Jutel M, Muller UR, Fricker M, Rihs S, Pichler WJ, and Dahinden C. Influence of bee venom immunotherapy on degranulation and leukotriene generation in human blood basophils. Clin Exp Allergy 1996. 26(10): p. 1112-8.
28. Xiang Z, Moller C, and Nilsson G. Readministration of ige is required for repeated passive cutaneous anaphylaxis in mice. Int Arch Allergy Immunol 2006. 141(2): p. 168-71.
29. Xiang Z, Block M, Lofman C, and Nilsson G. Ige-mediated mast cell degranulation and recovery monitored by time-lapse photography. J Allergy Clin Immunol 2001. 108(1): p. 116-21.
30. Van Ree R, Van Leeuwen WA, Dieges PH, Van Wijk RG, De Jong N, Brewczyski PZ, Kroon AM, Schilte PP, Tan KY, Simon-Licht IF, Roberts AM, Stapel SO, and Aalberse RC. Measurement of ige antibodies against purified grass pollen allergens (lol p 1, 2, 3 and 5) during immunotherapy. Clin Exp Allergy 1997. 27(1): p. 68-74.
31. Varshney P, Jones SM, Scurlock AM, Perry TT, Kemper A, Steele P, Hiegel A, Kamilaris J, Carlisle S, Yue X, Kulis M, Pons L, Vickery B, and Burks AW. A randomized controlled study of peanut oral immunotherapy: Clinical desensitization and modulation of the allergic response. J Allergy Clin Immunol 2011. 127(3): p. 654-60.
32. Burks AW, Jones SM, Wood RA, Fleischer DM, Sicherer SH, Lindblad RW, Stablein D, Henning AK, Vickery BP, Liu AH, Scurlock AM, Shreffler WG, Plaut M, Sampson HA, and Consortium of Food Allergy R. Oral immunotherapy for treatment of egg allergy in children. N Engl J Med 2012. 367(3): p. 233-43.
33. Rachid R and Umetsu DT. Immunological mechanisms for desensitization and tolerance in food allergy. Semin Immunopathol 2012. 34(5): p. 689-702.
34. Suarez-Fueyo A, Ramos T, Galan A, Jimeno L, Wurtzen PA, Marin A, de Frutos C, Blanco C, Carrera AC, Barber D, and Varona R. Grass tablet sublingual immunotherapy downregulates the th2 cytokine response followed by regulatory t-cell generation. J Allergy Clin Immunol 2014. 133(1): p. 130-8 e1-2.
35. Chehade M and Mayer L. Oral tolerance and its relation to food hypersensitivities. J Allergy Clin Immunol 2005. 115(1): p. 3-12; quiz 13.
36. Smit JJ, Willemsen K, Hassing I, Fiechter D, Storm G, van Bloois L, Leusen JH, Pennings M, Zaiss D, and Pieters RH. Contribution of classic and alternative effector pathways in peanut-induced anaphylactic responses. PLoS One 2011. 6(12): p. e28917.
37. Shamji MH and Durham SR. Mechanisms of immunotherapy to aeroallergens. Clin Exp Allergy 2011. 41(9): p. 1235-46.

38. Berin MC. Pathogenesis of ige-mediated food allergy. Clin Exp Allergy 2015.
39. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, and Akdis CA. Il-10 and tgf-beta cooperate in the regulatory t cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003. 33(5): p. 1205-14.
40. Trinchieri G. Interleukin-10 production by effector t cells: Th1 cells show self control. J Exp Med 2007. 204(2): p. 239-43.
41. Polukort SH, Rovatti J, Carlson L, Thompson C, Ser-Dolansky J, Kinney SR, Schneider SS, and Mathias CB. Il-10 enhances ige-mediated mast cell responses and is essential for the development of experimental food allergy in il-10-deficient mice. J Immunol 2016. 196(12): p. 4865-76.
42. Finkelman FD. Anaphylaxis: Lessons from mouse models. J Allergy Clin Immunol 2007. 120(3): p. 506-15;quiz516-7.



CHAPTER 4

IMPROVED EFFICACY OF ORAL IMMUNOTHERAPY USING NON-DIGESTIBLE OLIGOSACCHARIDES IN A MURINE COW'S MILK ALLERGY MODEL: A POTENTIAL ROLE FOR FOXP3+ REGULATORY T CELLS

Marlotte M. Vonk[1,2]
Mara A.P. Diks[1]
Laura Wagenaar[3]
Joost J. Smit[3]
Raymond H.H. Pieters[3]
Johan Garssen[1,2]
Betty C.A.M. van Esch[1,2]
Léon M.J. Knippels[1,2*]

1. Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
2. Immunology Platform, Danone Nutricia Research, Utrecht, the Netherlands
3. Department of Immunotoxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

* All authors are part of the NUTRALL research consortium

Published in Frontiers in Immunology (2017)

ABSTRACT

Background: Oral immunotherapy (OIT) is a promising therapeutic approach to treat food allergic patients. However, there are some concerns regarding its safety and long-term efficacy. The use of non-digestible oligosaccharides might improve OIT efficacy since they are known to directly modulate intestinal epithelial and immune cells in addition to acting as prebiotics.

Aim: To investigate whether a diet supplemented with plant-derived fructo-oligosaccharides (FOS) supports the efficacy of OIT in a murine cow's milk allergy model and to elucidate the potential mechanisms involved.

Methods: After oral sensitization to the cow's milk protein whey, female C3H/HeOuJ mice were fed either a control diet or a diet supplemented with FOS (1% w/w) and received OIT (10 mg whey) 5 days a week for 3 weeks by gavage. Intradermal (i.d.) and intragastric (i.g.) challenges were performed to measure acute allergic symptoms and mast cell degranulation. Blood and organs were collected to measure antibody levels and T cell and dendritic cell populations. Spleen-derived T cell fractions (whole spleen- and CD25-depleted) were transferred to naïve recipient mice to confirm the involvement of regulatory T cells (Tregs) in allergy protection induced by OIT+FOS.

Results: OIT+FOS decreased acute allergic symptoms and mast cell degranulation upon challenge and prevented the challenge-induced increase in whey-specific IgE as observed in sensitized mice. Early induction of Tregs in the mesenteric lymph nodes (MLN) of OIT+FOS mice coincided with reduced T cell responsiveness in splenocyte cultures. CD25 depletion in OIT+FOS-derived splenocyte suspensions prior to transfer abolished protection against signs of anaphylaxis in recipients. OIT+FOS increased serum galectin-9 levels. No differences in short-chain fatty acid (SCFA) levels in the cecum were observed between the treatment groups. Concisely, FOS supplementation significantly improved OIT in the acute allergic skin response, %Foxp3+ Tregs and %LAP+ Th3 cells in MLN, and serum galectin-9 levels.

Conclusion: FOS supplementation improved the efficacy of OIT in cow's milk allergic mice. Increased levels of Tregs in the MLN and abolished protection against signs of anaphylaxis upon transfer of CD25-depleted cell fractions, suggest a role for Foxp3+ Tregs in the protective effect of OIT+FOS.

INTRODUCTION

The prevalence of food allergies has been increasing in recent decades, in particular in Western countries. Persistence of food allergies instead of natural outgrowth is observed in patients and is likely to contribute to this increase in the future [1]. To date, strict avoidance of the culprit foods and symptomatic treatments are the only options in the management of food allergies. The significant impact of food allergies on health-related quality of life for patients and their families emphasizes the need for safe and efficacious curative treatments [2].

The strategy to induce desensitization and/or oral tolerance to food allergens via antigen-specific immunotherapy (AIT) has been studied extensively. Several routes of administration are possible, with the majority of the studies focusing on oral administration. Oral Immunotherapy (OIT) with milk, peanut and hen's egg effectively desensitized food allergic patients in randomized controlled clinical trials, measured as the absence of clinical symptoms upon food challenge [3]. However, discontinuation of OIT for a period of weeks to months leads to 'sustained unresponsiveness' in only a minority of the formerly desensitized patients [3]. In addition, safety concerns are relevant, since adverse events ranging from mild to near-fatal reactions have been reported [4]. 95% of cow's milk allergic children subjected to OIT experienced adverse events during treatment, including 25% suffering from severe, frequent and unpredictable reactions [5]. A systematic review and meta-analysis focused on AIT for IgE-mediated food allergies concluded that AIT may be effective in increasing the threshold of reactivity toward allergens, but simultaneously increases the risk of local and systemic adverse events [6]. Current limitations regarding safety and long-term protection restrict the use of OIT to treat food allergies in routine clinical practice.

Understanding the mechanism of OIT-induced desensitization and tolerance will contribute to optimizing the therapeutic strategy. A key role has been identified for naturally occurring CD4+CD25+Foxp3+ regulatory T cells (Tregs) and inducible type 1 Tregs (Tr1) in securing tolerance toward (food) antigens [7]. During immunotherapy, new antigen-specific Tregs are formed under the influence of IL-10 and TGFβ, and they suppress allergen-specific T helper 2 (Th2) and Th1 cells [8]. In addition, Tregs control the allergic response by suppressing the antigen-presenting cells responsible for effector T cell induction, shifting the production of antigen-specific IgE to antigen-specific IgG4 and suppressing mast cell and basophil activity [7]. Hence, improved Treg responses might be key in successful tolerance induction by OIT.

Nutritional interventions may provide a new window of opportunity to improve the efficacy of OIT for food allergic patients. Dietary non-digestible oligosaccharides

(i.e., carbohydrates) mimic the immunomodulatory effects exerted by human milk oligosaccharides (HMOS) in breast-fed infants and have been shown to reduce the risk of developing allergic diseases [9]. Non-digestible oligosaccharides show prebiotic activities by stimulating the growth of protective commensal microbes in the gut [10] and are fermented into short-chain fatty acids (SCFA), e.g., butyric acid, by the intestinal bacteria [11]. SCFA directly stimulate both immune cells and intestinal epithelial cells (IEC) via G-protein coupled receptors and thereby enhance gut integrity [12] and promote oral tolerance [13]. In addition to the prebiotic effect, non-digestible oligosaccharides can cross the intestinal epithelial barrier and directly affect immune cells involved in the process of oral tolerance induction [14, 15]. The capacity of non-digestible oligosaccharides to induce generic modulation of the immune response [16] and dampen allergic reactions in murine food allergy models [17-19] suggests they may provide a potential benefit in combination with OIT strategies.

With this research we aimed to assess whether dietary supplementation with non-digestible oligosaccharides supports the efficacy of OIT in a murine cow's milk allergy (CMA) model, and we aimed to elucidate the potential mechanisms involved. To that end, sensitized female C3H/HeOuJ mice were fed either a control diet or a diet supplemented with plant-derived fructo-oligosaccharides (FOS) and were subjected to OIT for 3 weeks. Subsequently, acute allergic symptoms and mast cell degranulation were measured upon intradermal (i.d.) and intragastric (i.g.) challenges. Blood and organs were collected to measure antigen-specific antibody levels and T and dendritic cell (DC) populations. Donor spleens derived from sensitized control mice and OIT+FOS mice were used to transfer whole spleen- and CD25-depleted cell fractions to naïve recipient mice to confirm the involvement of Tregs in allergy protection induced by OIT+FOS.

MATERIALS AND METHODS

Diets

A specific mixture of FOS derived from chicory inulin consisted of short-chain FOS [scFOS: oligofructose, Raftilose P95, degree of polymerization (DP) <6] and long-chain FOS (lcFOS: long-chain inulin, Raftiline HP, average DP 23 or higher, <1% DP <5) and was provided by Orafti (Wijchen, the Netherlands). FOS were added to the base recipe of the semi-purified cow's milk protein-free pelleted AIN-93G diet (scFOS/lcFOS ratio 9:1, 1% w/w) at Ssniff Spezialdiäten GmbH (Soest, Germany) (**Table 1**). The AIN-93G diet without FOS supplementation was used as control diet. Both diets were similar in color and were kept in sealed packages at 4°C prior to use.

Table 1. Dietary composition of control diet and FOS supplemented diet.

	Control diet AIN-93G (g/kg)	**FOS diet** scFOS:lcFOS (9:1, 1%) (g/kg)
Carbohydrates		
Cornstarch	397.5	397.5
Dextrinized cornstarch	132.0	132.0
Sucrose	100.0	100.0
Fibers		
Arbocel B800	50.0	39.9
Inulin HP (lcFOS) (97%)	0.0	1.03
Raftilose P95 (scFOS) (95%)	0.0	9.47
Protein		
Soy protein	200.0	200.0
L-cystine*	3.0	3.0
Fat		
Soybean oil	70.0	70.0
Others		
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.014	0.014

*0.2% DL-Met & 0.1% L-Cys
FOS, fructo-oligosaccharides; lc, long-chain; sc, short-chain.

Mice

Specific-pathogen-free 6-week-old C3H/HeOuJ female mice were purchased from Charles River Laboratories (Erkrath, Germany). Upon arrival, all mice were randomly allocated to the control and experimental groups: sham-sensitized control group (n=5/subgroup), sham no IT; sensitized control group (n=6/subgroup), sens no IT; FOS supplemented group (n=6/subgroup), FOS no IT; OIT group (n=6/subgroup), OIT; and the OIT with FOS supplementation group, OIT+FOS (n=6/subgroup) (**Figure 1**). Mice were housed in filter-topped macrolon cages (n=5-6/cage) at the animal facility of Utrecht University, Utrecht, the Netherlands on a 12 h light/dark cycle with

unlimited access to food and water. All mice were fed the AIN-93G control diet and were acclimatized for 6 days. Experimental procedures were approved by the Ethical Committee of Animal Research of Utrecht University and complied with the principles of good laboratory animal care following the European Directive for the protection of animals used for scientific purposes.

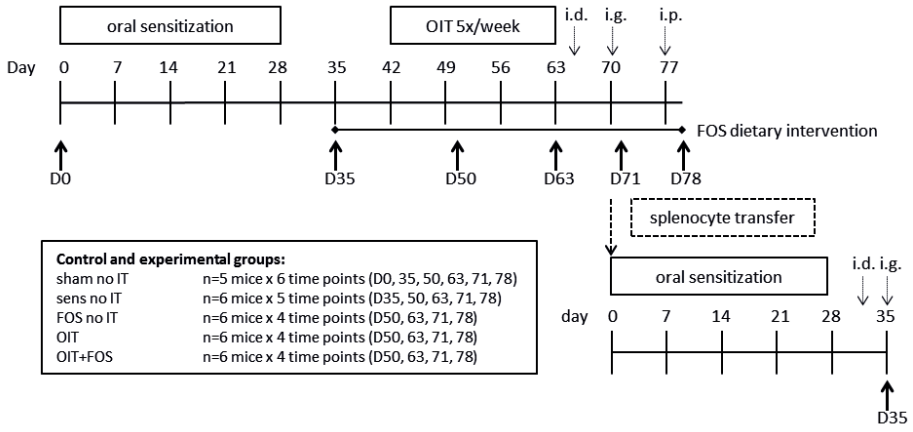


Figure 1. Experimental timeline of the animal experiment. Female C3H/HeOuJ mice were randomly allocated to the control and experimental groups: sham-sensitized control group (n=5/subgroup), sham no IT; sensitized control group (n=6/subgroup), sens no IT; FOS supplemented group (n=6/subgroup), FOS no IT; OIT group (n=6/subgroup), OIT; and the OIT with FOS supplementation group, OIT+FOS (n=6/subgroup). Mice were i.g. sensitized to the cow's milk protein whey (20 mg in 0.5 ml PBS) with cholera toxin as an adjuvant (15 µg in 0.5 ml PBS). The FOS supplemented diet was provided from D35 to the end of the protocol and OIT with 10 mg whey in 0.5 ml PBS was given from D42-D59 (five oral gavages/week for 3 weeks). Acute allergic symptoms were measured upon i.d. challenge at D64 (10 µg whey in 20 µl PBS/ear), mast cell degranulation was measured upon i.g. challenge at D70 (50 mg whey in 0.5 ml PBS) and an i.p. challenge (50 µg whey in 200 µl PBS) was conducted at D77 to stimulate T cell responses prior to organ collection. At 6 time points throughout the animal experiment (D0, D35, D50, D63, D71 and D78), subgroups of mice (n=5-6/group) from each control and experimental group were killed by cervical dislocation, and blood and organs were collected. Additional groups of donor mice were used in a follow-up experiment to perform the adoptive splenocyte transfer experiment: whole splenocyte suspensions and CD25-depleted fractions collected at D71 from sensitized control mice and OIT+FOS mice (n=8/group) were adoptively transferred to naïve recipient mice (n=6/group). Recipients were sensitized and challenged as described above prior to section at D35. OIT, oral immunotherapy; FOS, fructo-oligosaccharides; i.d., intradermal; i.g., intragastric; i.p., intraperitoneal.

Intragastric sensitization, OIT, and challenges

The timeline of the animal experiment is depicted in **Figure 1**. At experimental day (D) 0, 7, 14, 21 and 28, mice were sensitized i.g. to the cow's milk protein whey (DMV International, Veghel, the Netherlands) dissolved in PBS (20 mg whey in 0.5 ml PBS, Lonza, Verviers, Belgium) using cholera toxin (CT) as an adjuvant (15 µg CT in 0.5 ml PBS, List Biological Laboratories Inc., Campbell, CA, USA). After sensitization (D35), the FOS and OIT+FOS groups received the FOS diet until the end of the protocol. The sham-sensitized control groups, whey-sensitized control groups and OIT groups were fed the control diet throughout the experiment. Starting at D42, OIT was administered five times per week for three consecutive weeks (i.g. 10 mg whey in 0.5 ml PBS) (D42-D59). On D64, all mice were challenged i.d. in both ear pinnae (10 µg whey in 20 µl PBS/ear) to induce an anaphylactic response. The acute allergic skin response (i.e., ear swelling after i.d. injection), drop in body temperature, and severity of clinical symptoms were measured. Subsequently, mice received an i.g. challenge at D70 (50 mg whey in 0.5 ml PBS) and mast cell-derived mucosal mast cell protease-1 (mMCP-1) was measured in serum samples collected via cheek puncture 30 min after challenge. Finally, all mice were challenged intraperitoneally (i.p.) on D77 to stimulate T cell responses prior to organ collection (50 µg whey in 200 µl PBS). At 6 time points throughout the animal experiment (D0, D35, D50, D63, D71 and D78), subgroups of mice (n=5-6/group) from each control and experimental group were killed by cervical dislocation, and blood and organs were collected (**Figure 1**). Additional groups of donor mice were used in a follow-up experiment to perform the adoptive splenocyte transfer experiment and histological analyses in colon tissue.

Acute allergic skin response, body temperature and anaphylaxis symptom scores upon i.d. challenge

The magnitude of the acute allergic skin response after i.d. injection of the allergen was measured as Δ ear swelling by subtracting the mean basal ear thickness from the mean ear thickness 1 h post-challenge (in duplicate in both ears). Ear thickness in micrometers was measured using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). After blinding the cages, all mice were anesthetized using inhalation of isoflurane to perform the i.d. injection and both ear measurements [n=10-12/group, data represent the mean of two subgroups: mice scheduled for section at D71 and D78 (**Figure 1**)]. Body temperature was measured 45 min after i.d. challenge to monitor the anaphylaxis-associated drop in temperature using a rectal thermometer and severity of anaphylaxis symptoms was scored according to the method described by Li *et al.* [20].

Adoptive splenocyte transfer

At D71, pooled spleen suspensions derived from sensitized control and OIT+FOS mice (n=8/group) from a follow-up experiment were adoptively transferred to naïve specific-pathogen-free 6-week-old C3H/HeOuJ female mice (Charles River Laboratories, n=6/group), which were fed the control diet and were housed under similar conditions as described earlier. After homogenization and red blood cell lysis, whole spleen suspensions and CD25-depleted fractions were intravenously (i.v.) injected into the tail vein of recipient mice ($1x10^6$ cells in 100 µl PBS) prior to sensitization and i.d. and i.g. challenges as described in previous sections. CD25-depleted CD4+ fractions were obtained using a CD4+CD25+ purification kit according to the manufacturer's instructions (Miltenyi Biotec, Leiden, the Netherlands). Flow cytometric analysis of CD25-depleted fractions showed that <1% of the cells were positive for Foxp3. In addition, whole spleen suspensions showed on average 14% Foxp3 positivity. At D35 after transfer, recipient mice were killed by cervical dislocation 30 min after i.g. challenge, and blood and spleens were collected for further analysis.

Serum levels of whey-specific antibodies, mMCP-1 and galectin-9

Blood was collected via cheek puncture at D0, D35, D50, D63, D71 and D78 prior to sectioning of the mice and from one corresponding subgroup (n=10-12 samples/group) and at D35 in case of the recipient mice (n=6 samples/group). Blood samples were centrifuged (10,000 rpm for 10 min) and serum was stored at -20°C until analysis of mMCP-1, whey-specific antibodies and galectin-9 by means of ELISA. Determination of whey-specific antibodies was performed as described previously [21]. Concentrations of mMCP-1 in serum collected 30 min after i.g. challenge [D70; n=10-12 samples/group, data represent the mean of two subgroups: mice scheduled for section at D71 and D78 (**Figure 1**) and D35 in recipients; n=6/group)] were measured by using a mMCP-1 Sandwich ELISA Kit (Mouse MCPT-1 ELISA Ready-SET-Go kit, eBioscience, Breda, the Netherlands) according to the manufacturer's instructions. Serum collected from mice killed at D50, D63 and D71 was used to measure galectin-9 concentrations. Overnight incubation (4°C) with 100 µl of coating antibody in coating buffer (0.75 µg/ml, mouse galectin-9 affinity purified polyclonal goat IgG antibody, R&D Systems, Oxon, UK) in 96-wells high-binding plates (Corning Incorporated, Corning, NY, USA) was followed by a washing step. The wells were blocked for 1 h (RT) with 200 µl blocking buffer (PBS with 1% BSA) and washed prior to 2 h (RT) incubation of serum samples diluted (100x) in dilution buffer (PBS with 1% BSA and 0.05% Tween20) and a standard curve with recombinant mouse galectin-9 (serial 1:1 dilution starting with 500,000 pg/ml, R&D Systems). After washing, the

plates were incubated with 100 µl capture antibody (0.75 µg/ml, mouse galectin-9 biotinylated affinity purified goat IgG antibody, R&D Systems) for 1 h at RT. Afterward, the plates were washed and incubated with 100 µl Strep-HRP (Sanquin, Amsterdam, the Netherlands) for 1 h at RT in the dark. The color reaction was initiated by adding 100 µl 3,3',5,5'-tetramethylbenzidine (1-Step Ultra TMB, Thermo Fisher Scientific, Waltham, MA, USA) and the reaction was stopped with 4 M H_2SO_4 (50 µl). Optical density was measured with a Benchmark microplate reader (Biorad, Hercules, CA, USA) at a wavelength of 450 nm.

Spleen, mesenteric lymph nodes (MLN), and lamina propria (LP) cell isolation

Spleen and MLN were collected (n=5-6/group) at D0, D35, D50, D63, D71 and D78 and homogenized using a syringe and a 70-µm cell strainer. Red blood cells were lysed in the splenocyte suspensions using lysis buffer (8.3 g NH_4Cl, 1 g KHC_3O and 37.2 mg EDTA dissolved in 1 L demi water, filter sterilized). Cell suspensions were either dissolved in RPMI 1640 medium (Lonza) supplemented with 10% fetal bovine serum (FBS) and penicillin (pen, 100 U/ml)/streptomycin (strep, 100 µg/ml, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) and β-mercaptoethanol (20 µM) prior to *ex vivo* antigen-specific stimulation assays or dissolved in FACS buffer (PBS with 1% BSA) prior to flow cytometry stainings. At D63 and D71, small intestine LP tissue (n=4/group) was collected to isolate lymphocytes as follows: fat and Peyer's patches were removed from small intestine tissue and after washing in Hank's Balanced Salt Solution (HBSS, Invitrogen, Life Technologies, Carlsbad, CA, USA) with 15 mM Hepes (Gibco, Life Technologies) at pH 7.2, longitudinally opened tissue was cut into small fragments (0.5 cm). After washing in HBSS/Hepes, tissue samples were incubated in HBSS/Hepes buffer supplemented with 10% FBS, pen/strep, 5 mM EDTA and 1 mM dithiothreitol (DTT) for 20 min at 37°C (2x). Subsequently, tissue samples were washed in RPMI/FBS/DTT to remove EDTA followed by incubation in RPMI/FBS/DTT with collagenase type D (1 mg/ml, Roche Diagnostics Inc., Almere, the Netherlands) and DNAse (20 µg/ml, Sigma) for 45 min on a plate shaker at 37°C (2x). After digestion, remaining tissue fragments were re-suspended using a syringe (10 ml) and a needle (18G) and suspensions were filtered with a 100-µm cell strainer afterward. LP-derived cell suspensions were washed in HBSS/Hepes and purified using a percoll (GE Healthcare, Uppsala, Sweden) 40-80% mediated separation after centrifugation. Purified cell fractions were washed to remove traces of percoll and cells were taken up in FACS buffer prior to flow cytometry stainings.

Ex vivo antigen-specific stimulation of splenocytes for cytokine measurements

$8x10^5$ cells/well in 200 µl culture medium (RPMI 1640, 10% FBS, pen/strep, β-mercaptoethanol) in 96-wells U-bottom plates (Greiner, Frickenhausen, Germany) were stimulated with either culture medium, anti-CD3 (1 µg/ml, eBioscience) or anti-CD3/CD28 (10 µg/ml anti-CD3 and 1 µg/ml anti-CD28, eBioscience, transfer experiment) or whey (50 µg/ml). Polyclonal stimulation (48 h) and whey stimulation (96 h) were conducted at 37°C and 5% CO_2. Culture supernatant was collected and stored at -20°C until measurements of IL-5, IL-10, IL-13 and IFNγ production by means of ELISA according to the protocol described earlier for galectin-9. Purified rat anti-mouse coating antibodies (1 µg/ml for IL-5 and IFNγ and 2 µg/ml for IL-10 and IL-13), recombinant mouse cytokines for the standard curve and biotinylated detection antibodies (1 µg/ml for IL-5, IL-10 and IFNγ and 400 ng/ml for IL-13) were purchased at BD Biosciences.

Flow cytometry

To increase the expression of latency-associated peptide (LAP) on the surface of MLN-derived lymphocytes, cells were incubated in culture medium (RPMI 1640, 10% FBS, pen/strep) and received polyclonal stimulation with anti-CD3/CD28 (10 µg/ml anti-CD3 and 1 µg/ml anti-CD28, eBioscience) for 24 h at 37°C and 5% CO_2 prior to staining. Otherwise spleen, MLN and LP-derived cell suspensions in FACS buffer were plated in 96-wells U-bottom Falcon plates (BD Biosciences, 1-0.5 x 10^6 cells/well). The cells were incubated with anti-mouse CD16/CD32 (mouse Fc Block, BD Biosciences) in FACS buffer to block non-specific binding sites (15 min, on ice). Subsequently, cells were stained extracellularly with the following fluorescent antibodies (all purchased at eBioscience unless stated otherwise) in FACS buffer for 30 min on ice in the dark: anti-CD4-PerCpCy5.5 (1:100, clone RM4-5), anti-CD69-APC (1:100, clone H1.2F3), anti-CXCR3-PE (1:50, clone CXCR3-173), anti-T1St2-FITC (1:50, clone DJ8, mdbioproducts, St. Paul, MN, USA), anti-CD25-AlexaFluor 488 (1:100, clone PC61.5), anti-F4/80-APC-eFluor 780 (1:100, clone BM8), anti-CD103-APC (1:100, clone 2E7), anti-CD11b-PE (1:50, clone M1/70), anti-CD11c-PerCpCy5.5 (1:50, clone N418), anti-MHCII-FITC (1:100, clone NIMR-4), anti-CD45-PE-Cy7 (1:100, clone 30-F11), anti-CD4-FITC (1:100, clone GK1.5) and anti-LAP-PerCP-eFluor 710 (1:50, clone TW7-16B4). Cells stained for extracellular markers were fixed using 1% IC fixation buffer (eBioscience), and cells receiving additional intracellular staining for the transcription factor Foxp3 were fixed and permeabilized using the Foxp3 staining buffer set purchased at eBioscience according to the manufacturer's instructions. Afterward, cells were incubated for 30 min on ice in the dark with anti-Foxp3-APC (1:50, clone FJK-16s) in permeabilization

buffer. Live cells were distinguished from dead cells using Fixable Viability Dye eFluor 780 (FVD, 1:2000, eBioscience) and single cells were separated from aggregated cells based on forward/sideward scatter properties. Isotype controls were used for each antibody and cutoff gates for positivity were established using the fluorescence-minus-one (FMO) technique. Fluorescence was measured on the FACS Canto II (BD Biosciences) and analyzed with Flowlogic software (Inivai Technologies, Mentone, Australia).

Histological staining for mast cells and Foxp3+ Tregs in colon tissue

The colon was dissected (n=3-6/group), opened longitudinally, washed in PBS and Swiss roles were prepared by rolling the tissue from distal to proximal end with the mucosal side down. Tissue roles were fixed in formalin (10% v/v) and embedded in paraffin (Leica IG1150c, Leica Microsystems, Rijswijk, the Netherlands). Tissue sections (5 µm) were cut with a microtome (Leica Microsystems) and mounted on slides prior to deparaffinization and hydration. To stain mast cells in the tissue sections, May-Grunwald and Giemsa solutions were used according to the manufacturer's instructions (Giemsa Stain, Abcam, Cambridge, UK). Intracellular Foxp3 expression was stained as described previously [22]. After dewaxing, the sections were boiled in sodium citrate buffer (0.01 M) for 15 min. Then the sections were incubated with 0.2% Tween20 in PBS for 20 min. After blocking with 5% rabbit serum (Dako, Heverlee, Belgium) in PBS with 1% BSA (PBS/BSA) for 30 min, the sections were incubated overnight (4°C) with rat anti-mouse Foxp3 purified antibody (10 µg/ml, eBioscience). Afterward, sections were incubated with 3% H_2O_2 in PBS for 30 min. Detection of the primary antibody was conducted with a biotinylated rabbit anti-rat IgG (2.5 µg/ml in PBS/BSA, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h (RT). Then sections were incubated with avidin biotin complex (ABC HRP kit, Vector Laboratories, Peterborough, UK) in PBS/BSA for 1 h. The color reaction was developed with 3,3'-diaminobenzidine (Sigma) and counterstaining was performed with hematoxylin. After dehydration, the sections were covered with Pertex mounting medium (Histolab, Göteborg, Sweden) and cover glass. Stained cells were counted per 100 intact cripts in each colon section.

Foxp3 and IL-10 mRNA expression in colon tissue

One centimetre of the proximal colon was dissected and stored in RNAlater (Sigma) at -80°C until further processing (n=5-6/group). After homogenization, RNA extraction was conducted using the Qiagen RNeasy isolation kit and the RNAse-free DNAse Set (Qiagen GmbH, Hilden, Germany) and cDNA was synthesized using the iScript™ cDNA synthesis kit (Biorad) according to the manufacturer's instructions. RT^2 qPCR Primer Assays to measure pPIP5k1 (housekeeping gene), Foxp3 and IL-10 were purchased at SA Biosciences (Qiagen, German Town, MD, USA) and quantitative real-time PCR was performed on a CFX96 real-time PCR detection system (Biorad) using iQ SYBR green supermix as described previously [19]. Foxp3 and IL-10 mRNA expression data were normalized to pPIPk1 and depicted as the fold change in expression compared to the sham-sensitized control group.

SCFA analysis in cecum content

Short-chain fatty acid (SCFA) analysis was conducted as described elsewhere [23]. Briefly, cecum content was collected (n=5-6/group) and frozen at -80°C until further analysis. Samples were defrosted, homogenized by vortexing and diluted in ice cold PBS (1:10). After centrifugation (13,000 rpm for 10 min), the supernatant was analyzed using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) and acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acid concentrations were quantitated (based on 2-ethylbutyric acid internal standard).

Data analysis and statistics

Data are depicted as mean ± SEM and were statistically analyzed with GraphPad Prism software version 6.00 (GraphPad software, La Jolla, CA, USA) using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. Calculated p-values were adjusted for the total number of comparisons made and were considered statistically significant when $p<0.05$. In addition, whey-specific antibody data were log transformed prior to testing. The anaphylaxis symptom scores were analyzed using Kruskal-Wallis test for non-parametric data with Dunn's post-hoc test to compare pre-selected combinations.

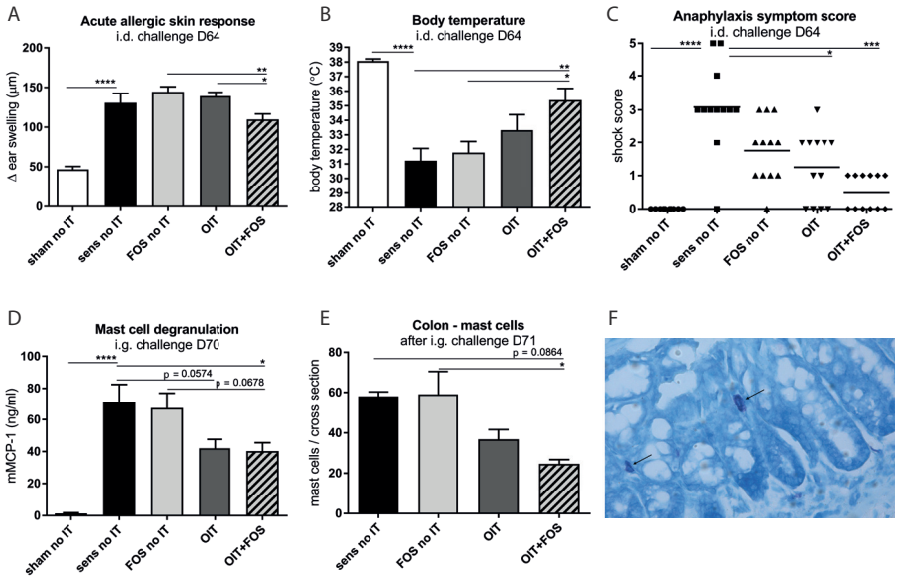


Figure 2. Acute allergic symptoms and mast cell degranulation upon challenge. (A) Acute allergic skin response measured as Δ ear swelling 1 h after i.d. injection at D64. **(B)** Body temperature levels and **(C)** anaphylaxis symptom scores during anaphylaxis measured 45 min after i.d. challenge at D64. **(D)** Serum mMCP-1 concentrations 30 min after i.g. challenge at D70. **(E)** Mast cell numbers in colon Swiss role cross sections after May-Grunwald/Giemsa staining (total number in one cross section). **(F)** Representative slide with stained mast cell (OIT+FOS). Data are represented as mean ± SEM n=10-12/group in **(A-D)** and n=3-6/group in **(E)**. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test and anaphylaxis symptom scores were analyzed using Kruskal-Wallis test for non-parametric data with Dunn's post hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Sens, sensitization; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; no IT, no immunotherapy; i.d., intradermal; i.g., intragastric; mMCP-1, mucosal mast cell protease-1.

RESULTS
Allergic symptoms

Sensitization to the cow's milk protein whey increased the acute allergic skin response measured as ear swelling (p<0.0001) and caused severe anaphylaxis (p<0.0001) with a characteristic drop in body temperature (p<0.0001) upon i.d. challenge (D64) compared to sham-sensitization (**Figures 2A-C**). An increased serum mMCP-1 concentration, indicative for mast cell degranulation, was observed upon i.g. challenge (D70) in the sensitized control animals compared to sham-sensitized control animals (p<0.0001) (**Figure 2D**). OIT+FOS induced protection against these allergic symptoms: the acute allergic skin response was reduced after OIT+FOS compared to FOS (p=0.0048) or OIT alone (p=0.023) (**Figure 2A**), indicating the improved efficacy

of the combination strategy. OIT+FOS protected against the anaphylaxis-associated drop in body temperature compared to sensitized control mice (p=0.0045) and FOS no IT (p=0.0123) (**Figure 2B**) and both OIT and OIT+FOS reduced the severity of anaphylaxis symptoms (**Figure 2C**) compared to the sensitized control (p=0.0322 and p=0.0002, respectively). After i.g. challenge (D70), OIT+FOS reduced mast cell degranulation (mMCP-1) compared to the sensitized control (p=0.0321) (**Figure 2D**). The reduction in mMCP-1 coincided with reduced local mast cell numbers in colon tissue of OIT+FOS mice, but only compared to FOS supplementation alone (FOS no IT, p=0.0227) (**Figures 2E,F**).

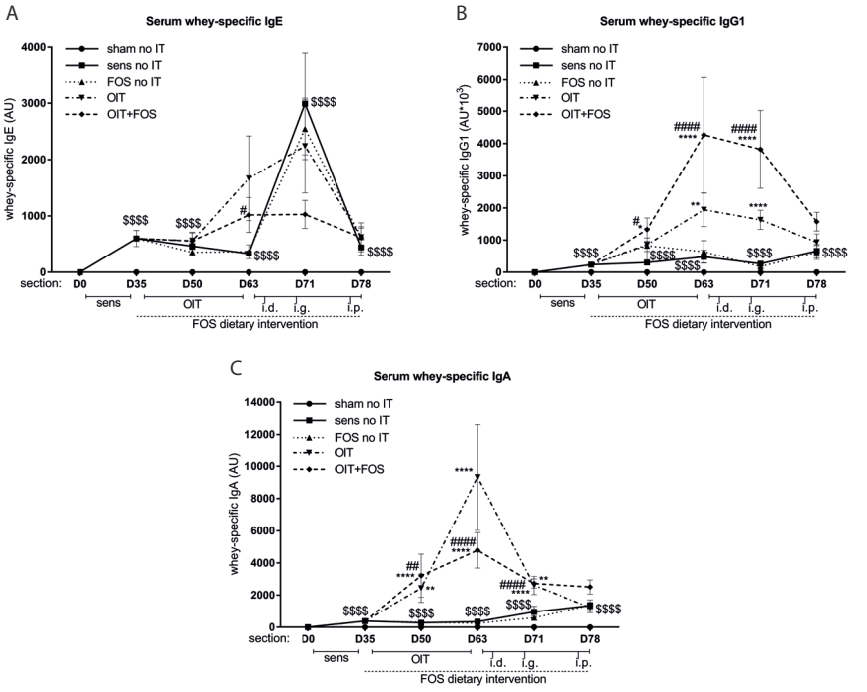


Figure 3. Whey-specific IgE, IgG1 and IgA levels in serum. (A) Whey-specific IgE levels, **(B)** Whey-specific IgG1 levels and **(C)** Whey-specific IgA levels in serum measured by means of ELISA. Data are represented as mean ± SEM n=10-12/group/time point and n=5-6/group at D78 in **(A-C)**. Data were log transformed and statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test per individual time point. $^{\$\$\$\$}$p<0.0001 for whey-sensitized control group compared to sham-sensitized control group. *p<0.05, **p<0.01, ****p<0.0001 compared to sensitized control group. $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#\#}$p<0.0001 compared to FOS no IT group. Sens, sensitization; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; no IT, no immunotherapy; i.d., intradermal; i.g., intragastric; i.p., intraperitoneal; AU, arbitrary units.

Whey-specific antibody levels in serum

Oral sensitization to whey increased the level of whey-specific IgE, IgG1 and IgA in serum compared to sham-sensitized mice ($p<0.0001$) (D35, D50, D63, D71 and D78) (**Figures 3A-C**). As observed after immunotherapy (D63), OIT increased whey-specific IgG1 and IgA levels compared to sensitized controls ($p=0.0026$ for IgG1 and $p<0.0001$ for IgA) and a similar pattern was observed for IgE (**Figures 3A-C**). FOS supplementation did not affect the OIT-induced rise in whey-specific antibodies, since no differences were observed between OIT and OIT+FOS. Whey-specific IgG1 and IgA levels decreased despite a series of challenges (D63-D78) in the OIT and OIT+FOS groups (**Figures 3B,C**). Interestingly, OIT+FOS protected against the rise in whey-specific IgE (D71) observed after i.d. and i.g. challenge, since levels were comparable to the time point preceding the challenges (D63) (**Figure 3A**).

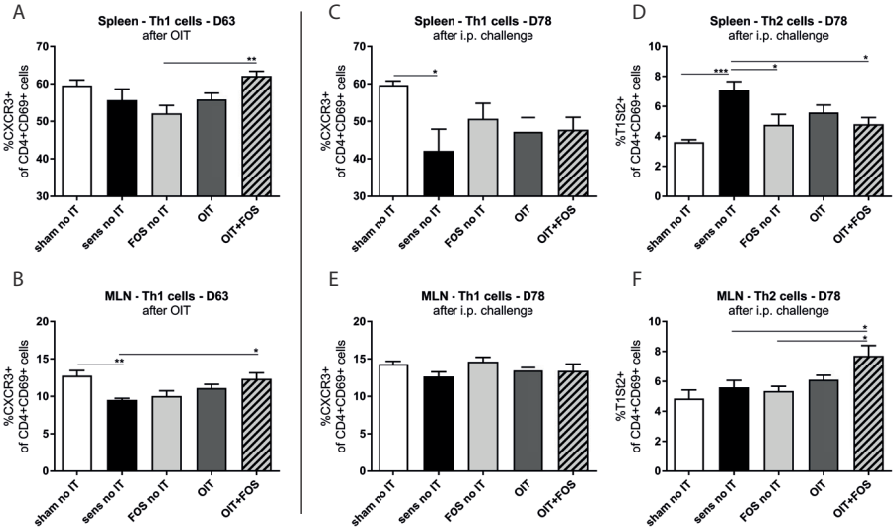
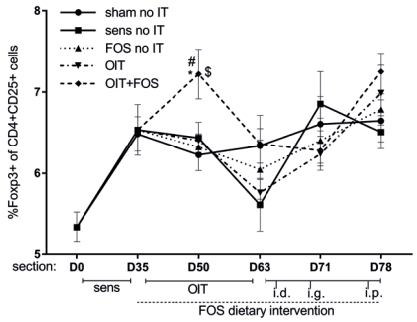
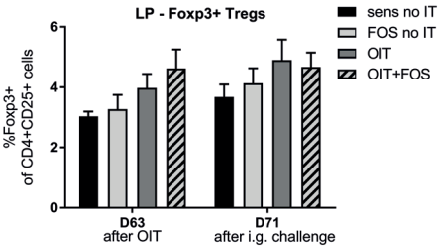


Figure 4. Flow cytometric analysis of T helper cell populations in spleen and MLN. (A) Percentage of activated Th1 cells (CXCR3+ of CD4+CD69+ cells) in spleen and **(B)** in MLN after immunotherapy (D63). **(C)** Percentage of activated Th1 cells and **(D)** Th2 cells (T1St2+ of CD4+CD69+ cells) in spleen after i.p. challenge (D78). **(E)** Percentage of activated Th1 cells and **(F)** Th2 cells in MLN after i.p. challenge (D78). Data are represented as mean ± SEM n=5-6/group in **(A-F)**. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001. Sens, sensitization; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; no IT, no immunotherapy; i.p., intraperitoneal; Th, T helper; MLN, mesenteric lymph nodes.

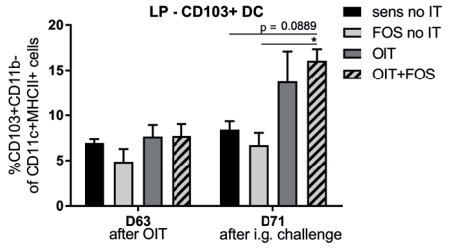
A


MLN - Foxp3+ Tregs
sham no IT
sens no IT
FOS no IT
OIT
OIT+FOS
%Foxp3+ of CD4+CD25+ cells
section:
D0
D35
D50
D63
D71
D78
sens
OIT
i.d.
i.g.
i.p.
FOS dietary intervention

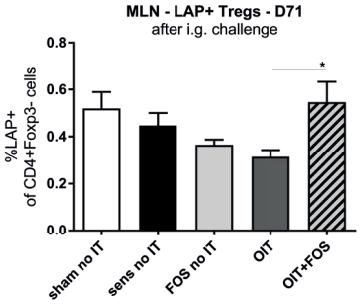
B


LP - Foxp3+ Tregs
sens no IT
FOS no IT
OIT
OIT+FOS
%Foxp3+ of CD4+CD25+ cells
D63 after OIT
D71 after i.g. challenge

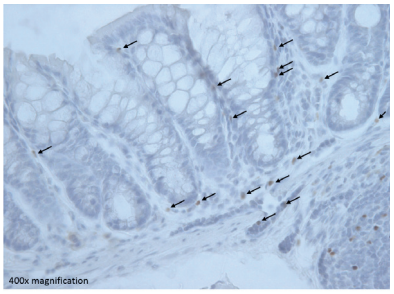
C


LP - CD103+ DC
p = 0.0889
sens no IT
FOS no IT
OIT
OIT+FOS
%CD103+CD11b- of CD11c+MHCII+ cells
D63 after OIT
D71 after i.g. challenge

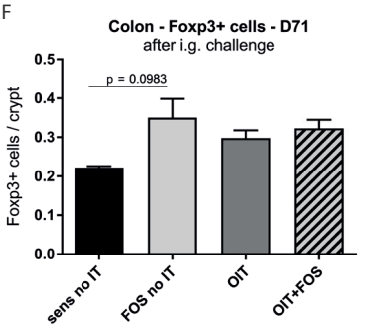
D


MLN - LAP+ Tregs - D71
after i.g. challenge
%LAP+ of CD4+Foxp3- cells
sham no IT
sens no IT
FOS no IT
OIT
OIT+FOS

E


400x magnification

F


Colon - Foxp3+ cells - D71
after i.g. challenge
p = 0.0983
Foxp3+ cells / crypt
sens no IT
FOS no IT
OIT
OIT+FOS

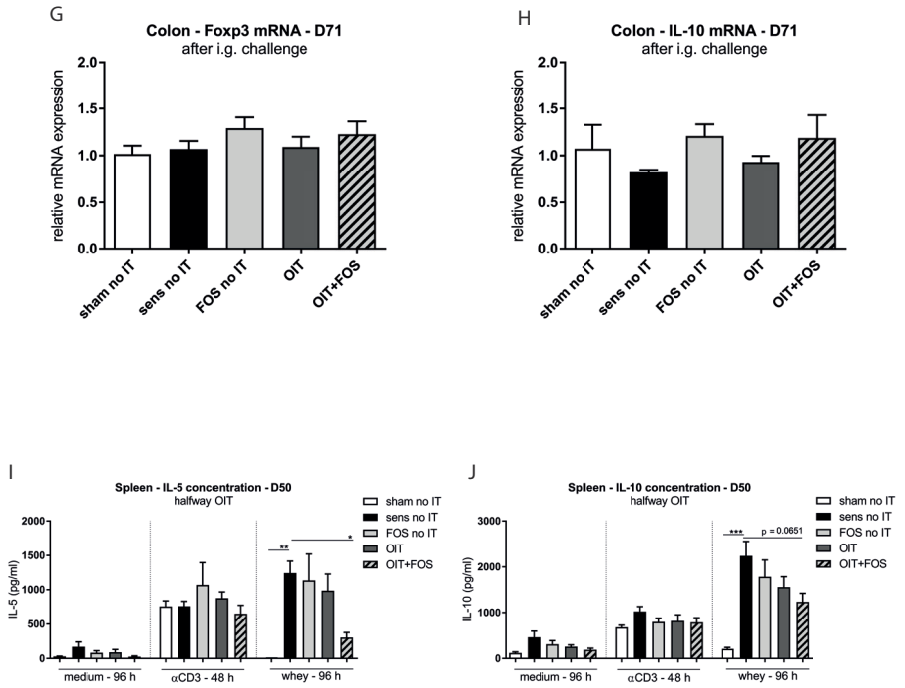


Figure 5. Treg analysis with flow cytometry, immunohistochemistry and qPCR. (A) Percentage of CD4+CD25+Foxp3+ cells in MLN measured with flow cytometry. **(B)** Percentage of CD4+CD25+Foxp3+ cells in LP measured with flow cytometry after immunotherapy (D63) and after i.g. challenge (D71). **(C)** Percentage of CD103+ DC (CD103+CD11b- of CD11c+MHCII+ cells) in LP measured with flow cytometry after immunotherapy (D63) and after i.g. challenge (D71). **(D)** Percentage of LAP+ Th3 cells (LAP+ of CD4+Foxp3- cells) in MLN measured with flow cytometry after i.g. challenge (D71). **(E)** Representative slide with Foxp3+ cells (OIT). **(F)** Foxp3+ cells in colon Swiss role cross sections stained with immunohistochemistry and counted in at least 100 intact crypts. **(G)** Relative mRNA expression of Foxp3 and **(H)** IL-10 in colon tissue measured with qPCR. **(I)** IL-5 concentrations and **(J)** IL-10 concentrations in supernatant after *ex vivo* stimulation of splenocytes with medium, anti-CD3 and whey halfway immunotherapy (D50). Data are represented as mean ± SEM n=5-6/group in **(A,D,G-J)**, n=4/group in **(B,C)** and n=3-6/group in **(F)**. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 [**(A)**: *compared to sens no IT, $^{\#}$compared to FOS no IT, $^{\$}$compared to OIT]. Sens, sensitization; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; no IT, no immunotherapy; i.d., intradermal; i.g., intragastric; i.p., intraperitoneal; Tregs, regulatory T cells; Th, T helper; DC, dendritic cell; MLN, mesenteric lymph nodes; LP, lamina propria; LAP, latency-associated peptide.

T helper cell subsets in lymphoid organs

No differences in Th1 and Th2 cell percentages were shown between OIT and OIT+FOS animals; however, differences were observed at D63 (after OIT) and D78 (after i.p. challenge) between OIT+FOS mice and sensitized controls (**Figure 4**). At D63, an increase in activated Th1 cells (CXCR3+ of CD4+CD69+) was observed in the OIT+FOS group compared to the sensitized control group in the MLN (p=0.0159) (**Figure 4B**) and compared to the FOS no IT group in the spleen (p=0.0096) (**Figure 4A**). At D78, activated Th2 cells (T1St2+ of CD4+CD69+) were increased in the sensitized control animals compared to sham-sensitized control animals (p=0.0009) and both FOS (p=0.0259) and OIT+FOS (p=0.0419) reduced the percentage of Th2 cells in spleen (**Figure 4D**). No differences were observed in the MLN at D78, except the elevated percentage of activated Th2 cells in the OIT+FOS group compared to the sensitized control (p=0.0297) and FOS no IT (p=0.0121) groups (**Figures 4E,F**).

Induction of tolerance-associated cell types

As shown in **Figure 5A**, an early increase in the percentage of CD4+CD25+Foxp3+ Tregs was observed halfway immunotherapy (D50) in the MLN of the OIT+FOS mice compared to OIT (p=0.0457). The higher percentage of Foxp3+ Tregs in MLN coincided with reduced IL-5 concentrations upon antigen-specific stimulation of splenocytes derived from OIT+FOS mice (D50) compared to the sensitized control (p=0.0365) (**Figure 5I**). A similar pattern was found for IL-10 (p=0.0651; not significant) (**Figure 5J**). No differences in cytokine concentrations were observed after medium and anti-CD3 stimulation of splenocytes (**Figures 5I,J**). At D63, the percentage of Foxp3+ Tregs in LP-derived lymphocyte fractions suggests an increase in the OIT+FOS mice compared to sensitized control mice; however, these results are not statistically significant (**Figure 5B**). A tendency toward increased percentages of CD103+CD11b- DC in LP was observed in the OIT+FOS group compared to the sensitized control (p=0.0889; not significant) after challenge (D71), but not after immunotherapy (D63) (**Figure 5C**). The percentage of LAP+ Tregs was increased in the OIT+FOS group compared to OIT at D71 in MLN (p=0.0327) (**Figure 5D**). Levels of Foxp3+ cells and Foxp3 and IL-10 mRNA expression in colon tissue were not statistically different between the groups at D71 (**Figures 5E-H**). A trend (p=0.0983) toward an increase in the number of Foxp3+ cells was observed in colon samples of FOS-treated animals compared to sensitized control animals (**Figure 5F**).

Transfer of protection against allergic symptoms

To confirm the involvement of the induced Foxp3+ Tregs in protection against allergic symptoms, pooled whole spleen suspensions and CD25-depleted fractions derived from sensitized control mice and OIT+FOS mice were adoptively transferred to naïve recipients. In contrast to body temperature (**Figure 6B**), challenge-induced acute allergic skin responses were significantly increased in the whey-sensitized recipient control mice compared to the sham-sensitized recipient control mice ($p<0.0001$), indicating that cell transfer was not responsible for the allergen-induced ear swelling response (**Figure 6A**). OIT+FOS splenocyte transfer did not protect against the increased ear swelling response (**Figure 6A**). CD25 depletion induced a more severe drop in body temperature in the OIT+FOS recipients compared to the recipients of the whole spleen suspension ($p=0.0426$) (**Figure 6B**). Similarly, challenge-induced mast cell activation was increased in mice which received the CD25-depleted fraction compared to the recipients of the OIT+FOS whole spleen suspension ($p=0.0003$) (**Figure 6C**). In addition, CD25 depletion increased the IL-10 concentration in whey-stimulated splenocyte cultures derived from OIT+FOS recipients ($p=0.0125$) (**Figure 6E**). A similar pattern was observed for IL-5, IL-13 and IFNγ; however, differences were not significant (**Figures 6D,F,G**). No differences in cytokine concentrations were observed after medium and anti-CD3/CD28 stimulation (**Figures 6D-G**).

Direct and indirect modulation of the intestinal environment by OIT+FOS

Bacterial fermentation of FOS leads to production of SCFA. After sensitization at D35, the mean total SCFA level was higher in the cecum of sensitized control mice than in sham-sensitized control mice ($p=0.017$) (**Figure 7A**). Considering individual SCFA, a similar pattern was observed for butyric acid; however, differences between the groups were not significant (**Figure 7B**). The intestinal epithelium-derived factor galectin-9 was measured in serum halfway- (D50) and after immunotherapy (D63) and after challenge (D71) (**Figures 7C-E**). OIT+FOS increased serum galectin-9 levels at D63 ($p=0.0014$ compared to sensitized control, $p=0.001$ compared to FOS no IT and $p=0.0161$ compared to OIT) (**Figure 7D**) and at D71 (compared to sensitized control, FOS no IT and OIT, all $p<0.0001$) (**Figure 7E**).

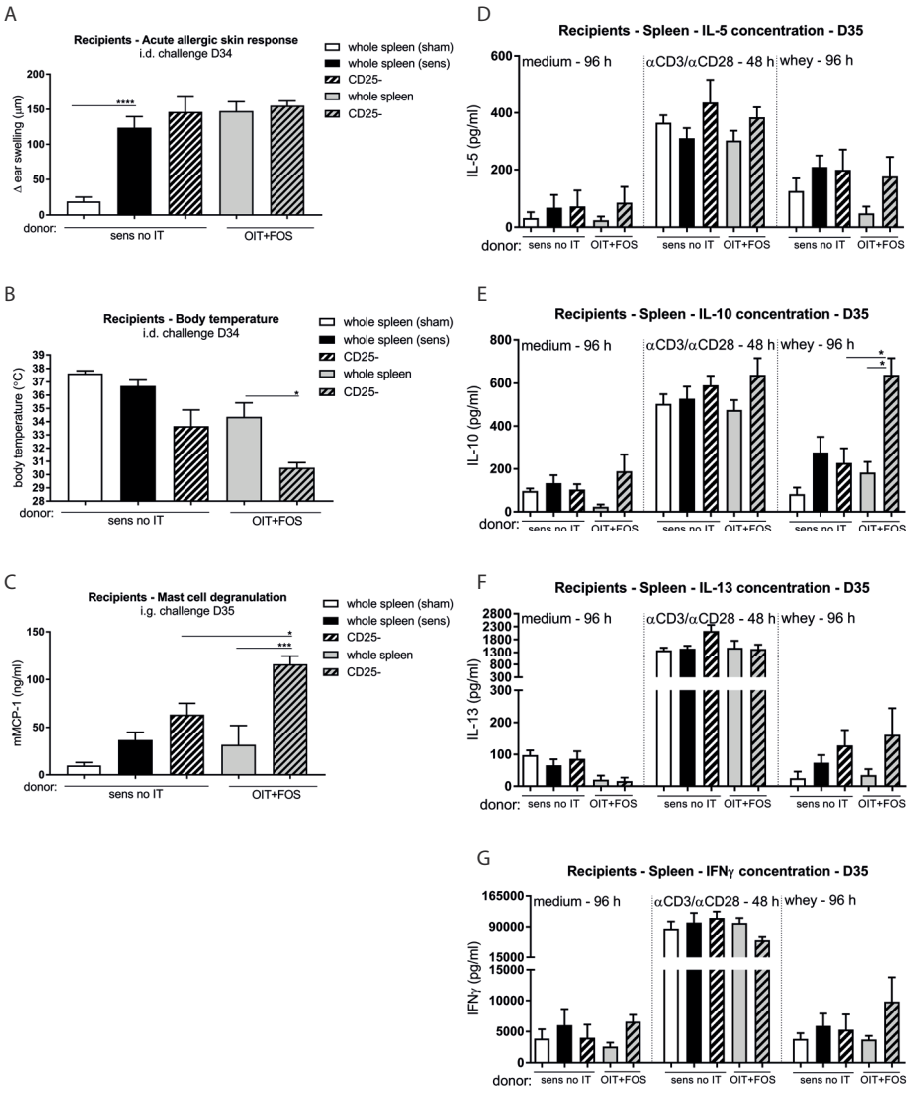


Figure 6. Adoptive cell transfer of whole spleen- and CD25-depleted pooled splenocyte suspensions derived from sensitized control mice and OIT+FOS mice. (A) Acute allergic skin response measured as Δ ear swelling 1 h after i.d. injection at D34 in the recipients. **(B)** Body temperature levels during anaphylaxis measured 45 min after i.d. challenge at D34 in the recipients. **(C)** Serum mMCP-1 concentrations 30 min after i.g. challenge at D35 in the recipients. **(D)** IL-5 concentrations, **(E)** IL-10 concentrations, **(F)** IL-13 concentrations and **(G)** IFNγ concentrations in supernatant after *ex vivo* stimulation of recipients-derived splenocytes with medium, anti-CD3/CD28 and whey. Data are represented as mean ± SEM n=6/group in **(A-G)**. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test. *p<0.05, ***p<0.001, ****p<0.0001. Sens, sensitization; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; no IT, no immunotherapy; mMCP-1, mucosal mast cell protease-1; i.d., intradermal; i.g., intragastric.

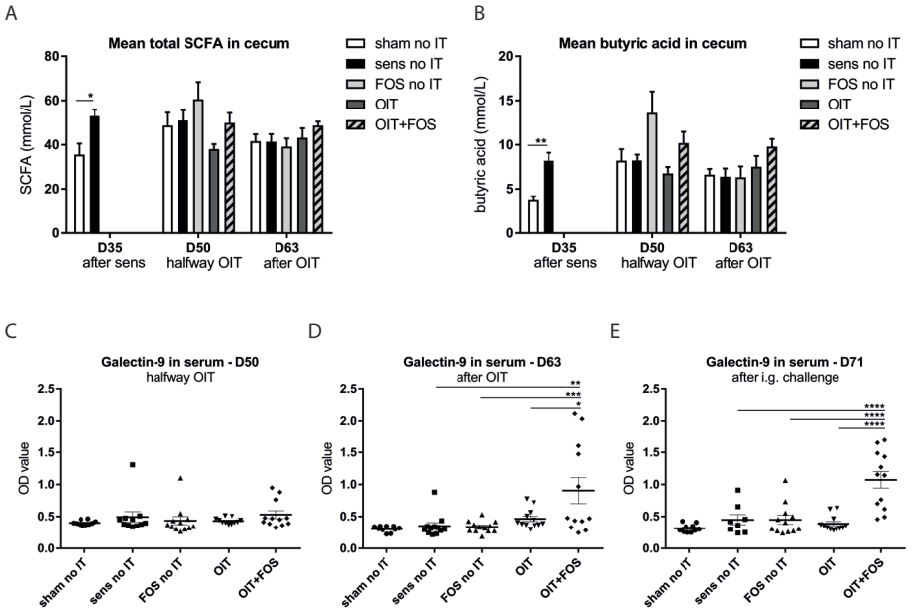


Figure 7. SCFA analysis in cecum content and serum galectin-9 levels. (A) Mean total SCFA concentrations and **(B)** mean butyric acid concentrations in homogenized cecum content supernatant collected after sensitization (D35), halfway immunotherapy (D50) and after immunotherapy (D63). **(C)** Serum galectin-9 levels halfway immunotherapy (D50), **(D)** after immunotherapy (D63) and **(E)** after i.g. challenge (D71). Data are represented as mean ± SEM n=5-6/group in **(A,B)** and n=10-12/group in **(C-E)**. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Sens, sensitization; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; no IT, no immunotherapy; i.g., intragastric; SCFA, short-chain fatty acids.

DISCUSSION

Overall, our study shows that the combination of OIT and a FOS supplemented diet effectively reduces allergic symptoms upon challenge. We observed increased percentages of Foxp3+ Tregs and a reduction in T cell responsiveness and mast cell number and activation. The presence of both FOS and whey protein in the gut induces release of IEC-derived galectin-9, a factor that directly supports the process of tolerance induction via Treg differentiation [24]. Although the exact mechanism of action has to be determined, to our knowledge, this is the first demonstration of the improved efficacy of oral immunotherapy in combination with immunomodulatory food components in a murine CMA model.

In the current study, OIT with 10 mg whey induced desensitization as shown by decreased anaphylaxis symptom scores after i.d. challenge. However, the combination of OIT and FOS was the most effective approach in terms of clinical protection against challenge-induced acute symptoms of anaphylaxis and reduced activation and number of mast cells in the gastrointestinal tract. The supporting effect of FOS during OIT was evident from the reduction of the challenge-induced ear swelling in OIT+FOS mice compared to OIT mice. In line with previous results, OIT effectively increased whey-specific IgG1 and IgA in serum [25]. As demonstrated in human studies, increased allergen-specific IgG and IgA levels observed during OIT are associated with allergy protection [8, 26]. The most striking difference in the humoral response in our study is the prevention of the challenge-induced increase in whey-specific IgE only observed in OIT+FOS mice.

Induction of Foxp3+ Tregs occurs in the MLN via CD103+ DC and involvement of TGFβ and retinoic acid (RA) [27]. Under the influence of CD103+ DC and MLN stromal cells, gut-homing receptors are expressed on the surface of Tregs [28] which facilitate migration to the intestine [29]. In the current study, a significant induction of Foxp3+ Tregs was only observed during immunotherapy in the MLN of OIT+FOS mice. Since clinical protection was observed upon challenge at a later stage of the experiment in the OIT+FOS mice, we hypothesize that the Foxp3+ Tregs traveled to other sites of action after immunotherapy. It has been described that trafficking of Tregs between multiple compartments contributes to their suppressive effect [30]. In accordance, we confirmed the presence of Foxp3+ Tregs in the LP of the small intestine after immunotherapy. A trend toward an increased percentage of CD103+ DC was observed in the LP of OIT+FOS mice after i.g. challenge. Functionality of spleen-derived Foxp3+ Tregs was shown by the loss of protection against allergic symptoms after transfer of depleted cell fractions. In addition, murine models of peanut AIT have shown that several routes of administration modulate the allergic response via IL-10-secreting Type 1 regulatory cells (Tr1) and TGFβ-secreting LAP+ Th3 Tregs [31]. In the current study, we were not able to measure IL-10 producing CD4+CD25+ Tr1 cells, but the percentage of LAP+CD4+Foxp3- cells was increased in the MLN of OIT+FOS mice compared to OIT alone at the time point of clinical protection. Together, the data of MLN-, LP- and spleen-derived Tregs indicate an important role for Tregs in the protective mechanism of OIT+FOS.

The induction of Foxp3+ Tregs in the MLN of OIT+FOS mice halfway immunotherapy was accompanied by reduced antigen-specific T cell responsiveness in splenocyte cultures. Only OIT+FOS reduced IL-5 production upon whey stimulation. Reduced cytokine production may either be due to the presence of suppressive Tregs or to

anergy of specific T cells during immunotherapy. With the current data it is not possible to distinguish between the two; however, the results of the whey stimulation assay in the adoptive transfer experiment show that depletion of CD25+ cells in OIT+FOS-derived cell fractions increases responsiveness of specific T cells toward the allergen. Mast cell suppression by Tregs via the OX40-OX40 ligand interaction is an important control mechanism of the allergic response [32]. In the current study, *ex vivo* Treg depletion in OIT+FOS- derived cell fractions prior to transfer increased mucosal mast cell activation *in vivo*. FOS supplementation might enhance the ability of Tregs to suppress mast cell activation directly. It has been reported that CD4+CD25+ T cells derived from HMOS-treated OVA allergic mice efficiently suppressed *in vitro* IgE-mediated mast cell degranulation [33].

The immunomodulatory activities of non-digestible oligosaccharides have been described in both human and animal studies of gastrointestinal disorders and allergies including asthma [34-36]. Carbohydrate structures are recognized by specific glycan receptors (e.g., C-type lectins) present on IEC and immune cells and contribute to the orchestration of the mucosal immune response [36]. Moreover, epithelial transport of non-digestible oligosaccharides was shown *in vitro* [14], indicating the possible direct interaction with lymphocytes residing in the LP. Direct modulation of human monocyte-derived DC and induction of Foxp3+ Tregs by a mixture of short-chain galacto-oligosaccharides and long-chain FOS (scGOS/lcFOS) was shown *in vitro* [37]. Galectins are soluble type lectins expressed by IEC that were identified to be involved in the immunomodulatory effects of the scGOS/lcFOS mixture in an *in vitro* setting using IEC and human peripheral blood mononuclear cells [24]. In particular IEC-derived galectin-9 played a key role in Th1 and Treg polarization possibly via conditioning of tolerogenic DC [24]. Elevated galectin-9 levels were observed in serum of cow's milk allergic mice after scGOS/lcFOS supplementation [38]. In the current study, OIT+FOS increased serum galectin-9 levels, suggesting direct modulation of intestinal epithelial cells and a potential role in allergy protection by OIT+FOS. FOS supplementation without exposure to whey protein (OIT) could not induce galectin-9 secretion.

The influence of whey protein on the gut environment was further supported by the SCFA analysis in the cecum content. Whey administration during sensitization increased bacterial butyric acid production in the allergic mice compared to the sham-sensitized mice, suggesting altered microbiota composition and/or abundance. In the colon, the microbiota are involved in the fermentation of both (non-digestible) carbohydrates and proteins that were not digested in the upper part of the digestive tract into e.g., acetate, propionate and butyrate [39]. The type and quantity of the

produced SCFA can be a reflection of both the bacterial and dietary composition in the colon [40]. Our findings could not confirm that the combination of OIT and FOS supplementation favors the growth of butyrate-producing bacteria in the gut. Butyrate is a major energy source for colonocytes [41] and can promote the production of RA by epithelial cells via inhibition of histone deacetylase [42]. RA is converted from dietary vitamin A by retinaldehyde-dehydrogenase-2, an enzyme expressed by epithelial cells and CD103+ DC. Increased RA levels were associated with improved tolerogenic activity of CD103+ DC *in vitro* [34] and stimulate the differentiation of naïve T cells into Tregs [43]. Oral butyrate administration via the drinking water of antibiotic-treated mice induced extrathymic differentiation of Foxp3+ Tregs, showing direct modulation of the immune response independent of the microbiota [44]. In addition, preventive butyrate administration mimicked the high fiber diet-induced protection against allergic symptoms and reduced total IgE levels in the serum of peanut allergic mice [34]. The involvement of butyrate in the tolerance-associated immune response suggests a role in the protective mechanism induced by OIT+FOS; however, since no significant differences were found, butyric acid levels cannot explain the changes in allergic- and immunologic parameters observed in the current study.

The use of immunomodulatory food components in combination with AIT is a promising strategy to combine specific- and generic modulation of the immune response. A first attempt was made by co-administration of the probiotic *Lactobacillus rhamnosus* CGMCC during OIT in peanut allergic children. The authors report sustained unresponsiveness to a food challenge in 82.1% of the treated children after 2-5 weeks off-therapy [45]. However, human data concerning the additive effect of pre- and/or probiotic supplementation on the efficacy of OIT is limited.
In conclusion, we show the potency of a specific mixture of FOS to support the efficacy of OIT in cow's milk allergic mice. Foxp3+ Tregs play a potential role in the protective effect induced by OIT+FOS; however, the exact contribution of galectin-9 and butyric acid should be further studied. In addition, future research is necessary to investigate the potential of the current strategy to improve the safety of OIT and induce long-term tolerance toward food proteins after discontinuation of therapy. Understanding the complex interplay between the gut epithelium, the microbiota, and the immune system during OIT will contribute to knowledge-based application of nutritional interventions in human food allergy trials in the future.

ACKNOWLEDGEMENTS

We would like to thank D. Veening, G.M. Dingjan and G.A. Hofman for their technical assistance and S. Abbring for the useful discussions.

REFERENCES

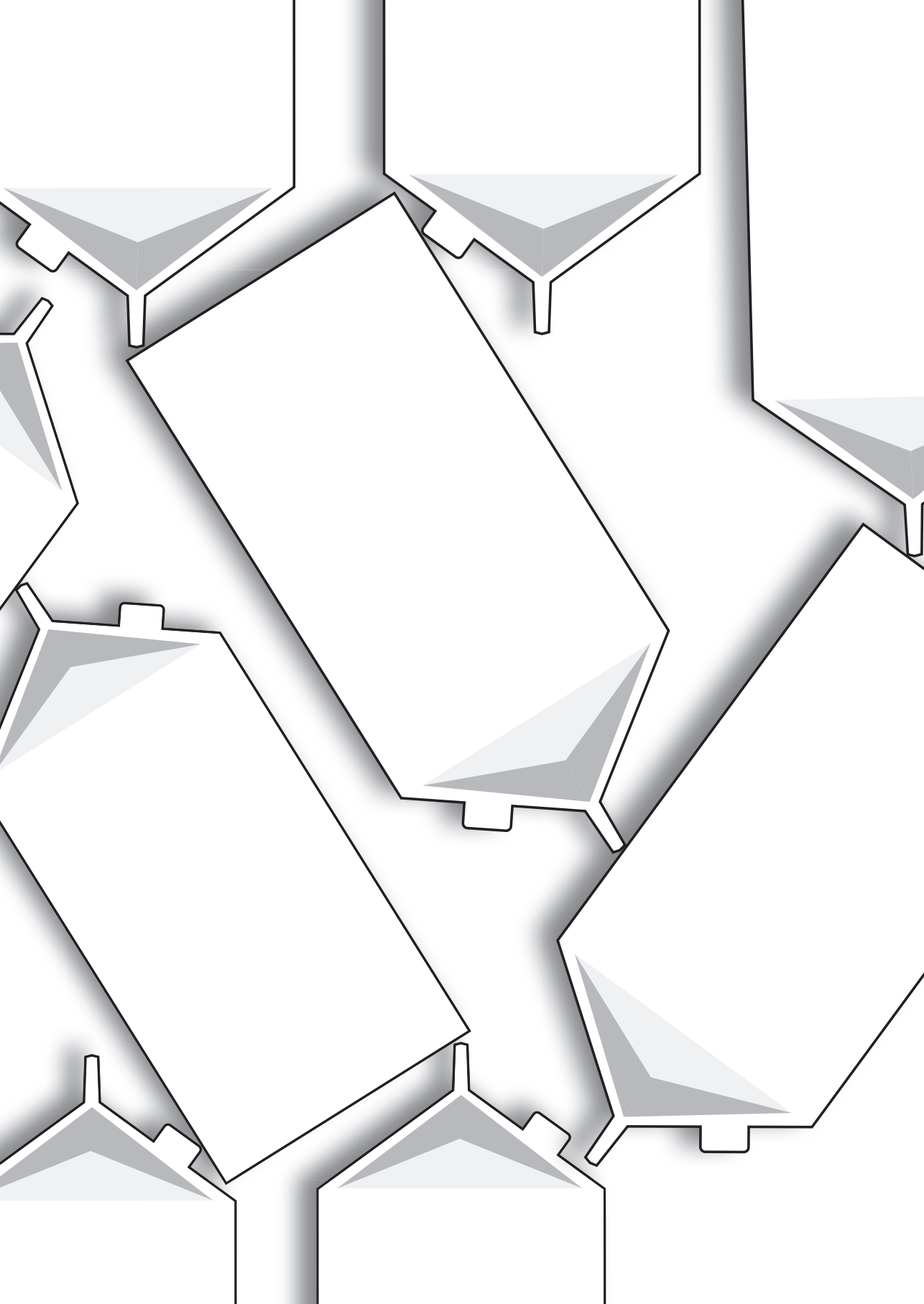
1. Tang ML and Mullins RJ. Food allergy: is prevalence increasing? Intern Med J 2017. 47(3): p. 256-261.
2. Vazquez-Ortiz M, Alvaro M, Piquer M, Dominguez O, Giner MT, Lozano J, Jimenez-Feijoo R, and Plaza AM. Impact of oral immunotherapy on quality of life in egg-allergic children. Pediatr Allergy Immunol 2015. 26(3): p. 291-4.
3. Chiang D and Berin MC. An Examination of Clinical and Immunologic Outcomes in Food Allergen Immunotherapy by Route of Administration. Curr Allergy Asthma Rep 2015. 15(6): p. 35.
4. Vazquez-Ortiz M and Turner PJ. Improving the safety of oral immunotherapy for food allergy. Pediatr Allergy Immunol 2016. 27(2): p. 117-25.
5. Vazquez-Ortiz M, Alvaro-Lozano M, Alsina L, Garcia-Paba MB, Piquer-Gibert M, Giner-Munoz MT, Lozano J, Dominguez-Sanchez O, Jimenez R, Dias M, Martin-Mateos MA, and Plaza-Martin AM. Safety and predictors of adverse events during oral immunotherapy for milk allergy: severity of reaction at oral challenge, specific IgE and prick test. Clin Exp Allergy 2013. 43(1): p. 92-102.
6. Nurmatov U, Dhami S, Arasi S, Pajno GB, Fernandez-Rivas M, Muraro A, Roberts G, Akdis C, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, du Toit G, Ebisawa M, Eigenmann P, Knol E, Makela M, Nadeau KC, O'Mahony L, Papadopoulos N, Poulsen LK, Sackesen C, Sampson H, Santos A, van Ree R, Timmermans F, and Sheikh A. Allergen immunotherapy for IgE-mediated food allergy: a systematic review and meta-analysis. Allergy 2017: p. 72(8):1133-1147.
7. Akdis M, Blaser K, and Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. J Allergy Clin Immunol 2005. 116(5): p. 961-8; quiz 969.
8. Akdis CA and Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. World Allergy Organ J 2015. 8(1): p. 17.
9. Jeurink PV, van Esch BC, Rijnierse A, Garssen J, and Knippels LM. Mechanisms underlying immune effects of dietary oligosaccharides. Am J Clin Nutr 2013. 98(2): p. 572S-7S.
10. Boehm G, Stahl B, Jelinek J, Knol J, Miniello V, and Moro GE. Prebiotic carbohydrates in human milk and formulas. Acta Paediatr Suppl 2005. 94(449): p. 18-21.
11. Rijnierse A, Jeurink PV, van Esch BC, Garssen J, and Knippels LM. Food-derived oligosaccharides exhibit pharmaceutical properties. Eur J Pharmacol 2011. 668 Suppl 1: p. S117-23.
12. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA, Fagarasan S,

and Mackay CR. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun 2015. 6: p. 6734.
13. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, and Macia L. The role of short-chain fatty acids in health and disease. Adv Immunol 2014. 121: p. 91-119.
14. Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, Urbanek R, and Szepfalusi Z. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatr Allergy Immunol 2010. 21(8): p. 1179-88.
15. Nauta AJ and Garssen J. Evidence-based benefits of specific mixtures of non-digestible oligosaccharides on the immune system. Carbohydr Polym 2013. 93(1): p. 263-5.
16. Gourbeyre P, Denery S, and Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 2011. 89(5): p. 685-95.
17. Kerperien J, Jeurink PV, Wehkamp T, van der Veer A, van de Kant HJ, Hofman GA, van Esch EC, Garssen J, Willemsen LE, and Knippels LM. Non-digestible oligosaccharides modulate intestinal immune activation and suppress cow's milk allergic symptoms. Pediatr Allergy Immunol 2014. 25(8): p. 747-54.
18. Schouten B, van Esch BC, Hofman GA, Boon L, Knippels LM, Willemsen LE, and Garssen J. Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. J Nutr 2010. 140(4): p. 835-41.
19. van Esch BC, Abbring S, Diks MA, Dingjan GM, Harthoorn LF, Vos AP, and Garssen J. Post-sensitization administration of non-digestible oligosaccharides and Bifidobacterium breve M-16V reduces allergic symptoms in mice. Immun Inflamm Dis 2016. 4(2): p. 155-165.
20. Li XM, Schofield BH, Huang CK, Kleiner GI, and Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 1999. 103(2 Pt 1): p. 206-14.
21. Schouten B, van Esch BC, Hofman GA, van den Elsen LW, Willemsen LE, and Garssen J. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. Int Arch Allergy Immunol 2008. 147(2): p. 125-34.
22. van den Elsen LW, van Esch BC, Hofman GA, Kant J, van de Heijning BJ, Garssen J, and Willemsen LE. Dietary long chain n-3 polyunsaturated fatty acids prevent allergic sensitization to cow's milk protein in mice. Clin Exp Allergy 2013. 43(7): p. 798-810.
23. de Theije CG, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J, Garssen J, Kraneveld AD, and Oozeer R. Altered gut microbiota and activity in a murine model of autism spectrum disorders. Brain Behav Immun 2014. 37: p. 197-206.

24. de Kivit S, Kraneveld AD, Knippels LM, van Kooyk Y, Garssen J, and Willemsen LE. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. J Innate Immun 2013. 5(6): p. 625-38.
25. Vonk MM, Wagenaar L, Pieters RHH, Knippels LMJ, Willemsen LEM, Smit JJ, van Esch B, and Garssen J. The efficacy of oral and subcutaneous antigen-specific immunotherapy in murine cow's milk- and peanut allergy models. Clin Transl Allergy 2017. 7: p. 35.
26. Wright BL, Kulis M, Orgel KA, Burks AW, Dawson P, Henning AK, Jones SM, Wood RA, Sicherer SH, Lindblad RW, Stablein D, Leung DY, Vickery BP, Sampson HA, and Consortium of Food Allergy R. Component-resolved analysis of IgA, IgE, and IgG4 during egg OIT identifies markers associated with sustained unresponsiveness. Allergy 2016. 71(11): p. 1552-1560.
27. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, and Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007. 204(8): p. 1757-64.
28. Molenaar R, Greuter M, van der Marel AP, Roozendaal R, Martin SF, Edele F, Huehn J, Forster R, O'Toole T, Jansen W, Eestermans IL, Kraal G, and Mebius RE. Lymph node stromal cells support dendritic cell-induced gut-homing of T cells. J Immunol 2009. 183(10): p. 6395-402.
29. Chinthrajah RS, Hernandez JD, Boyd SD, Galli SJ, and Nadeau KC. Molecular and cellular mechanisms of food allergy and food tolerance. J Allergy Clin Immunol 2016. 137(4): p. 984-97.
30. Siegmund K, Feuerer M, Siewert C, Ghani S, Haubold U, Dankof A, Krenn V, Schon MP, Scheffold A, Lowe JB, Hamann A, Syrbe U, and Huehn J. Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo. Blood 2005. 106(9): p. 3097-104.
31. Dioszeghy V, Mondoulet L, Puteaux E, Dhelft V, Ligouis M, Plaquet C, Dupont C, and Benhamou PH. Differences in phenotype, homing properties and suppressive activities of regulatory T cells induced by epicutaneous, oral or sublingual immunotherapy in mice sensitized to peanut. Cell Mol Immunol 2016: p. 14(9):770-782.
32. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, Viola A, Odom S, Rivera J, Colombo MP, and Pucillo CE. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. Immunity 2008. 29(5): p. 771-81.
33. Castillo-Courtade L, Han S, Lee S, Mian FM, Buck R, and Forsythe P. Attenuation of food allergy symptoms following treatment with human milk oligosaccharides in a mouse model. Allergy 2015. 70(9): p. 1091-102.
34. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE,

Macia L, and Mackay CR. Dietary Fiber and Bacterial SCFA Enhance Oral Tolerance and Protect against Food Allergy through Diverse Cellular Pathways. Cell Rep 2016. 15(12): p. 2809-24.

35. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, and Marsland BJ. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 2014. 20(2): p. 159-66.
36. Wilson B and Whelan K. Prebiotic inulin-type fructans and galacto-oligosaccharides: definition, specificity, function, and application in gastrointestinal disorders. J Gastroenterol Hepatol 2017. 32 Suppl 1: p. 64-68.
37. Lehmann S, Hiller J, van Bergenhenegouwen J, Knippels LM, Garssen J, and Traidl-Hoffmann C. In Vitro Evidence for Immune-Modulatory Properties of Non-Digestible Oligosaccharides: Direct Effect on Human Monocyte Derived Dendritic Cells. PLoS One 2015. 10(7): p. e0132304.
38. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BC, Knol J, Sprikkelman AB, van der Aa LB, Knippels LM, Garssen J, van Kooyk Y, and Willemsen LE. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012. 67(3): p. 343-52.
39. Macfarlane S and Macfarlane GT. Regulation of short-chain fatty acid production. Proc Nutr Soc 2003. 62(1): p. 67-72.
40. Krajmalnik-Brown R, Ilhan ZE, Kang DW, and DiBaise JK. Effects of gut microbes on nutrient absorption and energy regulation. Nutr Clin Pract 2012. 27(2): p. 201-14.
41. Wong JM, de Souza R, Kendall CW, Emam A, and Jenkins DJ. Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol 2006. 40(3): p. 235-43.
42. Schilderink R, Verseijden C, Seppen J, Muncan V, van den Brink GR, Lambers TT, van Tol EA, and de Jonge WJ. The SCFA butyrate stimulates the epithelial production of retinoic acid via inhibition of epithelial HDAC. Am J Physiol Gastrointest Liver Physiol 2016. 310(11): p. G1138-46.
43. Coombes JL and Powrie F. Dendritic cells in intestinal immune regulation. Nat Rev Immunol 2008. 8(6): p. 435-46.
44. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, and Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 2013. 504(7480): p. 451-5.
45. Tang ML, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, and Donath S. Administration of a probiotic with peanut oral immunotherapy: A randomized trial. J Allergy Clin Immunol 2015. 135(3): p. 737-44 e8.



CHAPTER 5

A NETWORK-BASED APPROACH FOR IDENTIFYING SUITABLE BIOMARKERS FOR ORAL IMMUNOTHERAPY OF FOOD ALLERGY

Jolanda H.M. van Bilsen[1]
Lars Verschuren[1]
Laura Wagenaar[2]
Marlotte M. Vonk[3,4]
Betty C.A.M. van Esch[3,4]
Léon M.J. Knippels[3,4]
Johan Garssen[3,4]
Joost J. Smit[2]
Raymond H.H. Pieters[2]
Tim J. van den Broek[1*]

1. TNO, Zeist, the Netherlands
2. Department of Immunotoxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
3. Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
4. Immunology Platform, Danone Nutricia Research, Utrecht, the Netherlands

* All authors are part of the NUTRALL research consortium

To be submitted

ABSTRACT

Background: Oral immunotherapy (OIT) is a promising therapeutic approach to treat food allergic patients. Recently, we have shown that the use of a mixture of short-chain and long-chain fructo-oligosaccharides (scFOS/lcFOS) improves the efficacy of OIT in cow's milk and peanut allergic mice. However, concerns with regards to safety and long-term efficacy of OIT remain. There is a need to identify novel biomarkers that predict, monitor and/or evaluate the effects of OIT. Here we present a method to select candidate biomarkers for efficacy and safety assessment of OIT using the computational approaches Bayesian networks (BN) and topological data analysis (TDA).

Methods: Data were used from scFOS/lcFOS diet-supported OIT experiments performed in two independent cow's milk allergy (CMA) and two independent peanut allergy (PNA) experiments in mice. First, a subset of the data was analyzed to understand the data structure and its interactions in terms of a BN. This BN was used to compare the key parameters in CMA and PNA. Finally, the relations within the dataset in combination with the BN were explored to identify and rank candidate biomarkers that show the effect of OIT by applying TDA.

Results: The BN predicted the efficacy of OIT in the CMA and the PNA model with 82% and 80% accuracy respectively, thereby identifying a set of 5 parameters (allergen-specific IgE and IgG1, body temperature, mMCP-1, ear swelling) which are key players in the mechanisms involved in both scFOS/lcFOS-aided OIT food allergy models. The TDA zoomed in on the full set of 67 previously analyzed parameters and identified clusters of biomarkers closely linked to biologically relevant clinical symptoms and also unrelated and redundant parameters within the network. Taken together, this enabled the prioritization of candidate biomarkers. Moreover, the TDA indicated differences between PNA and CMA models in how the data are related to each other.

Conclusion: Here we provide promising computational approaches to a) compare mechanistic features during OIT between two different food allergies b) determine the biological relevance of candidate biomarkers c) generate new hypotheses to explain why CMA has a different disease pattern than PNA and d) select relevant biomarkers for future studies.

INTRODUCTION

Food allergy is an important socio-economic and health problem estimated to occur in 6-8% of children and in 1-2% of adults [1-3]. Unfortunately, to date there is no effective and safe therapy available and only symptomatic treatment and elimination diets are currently available.

Human studies have shown that both subcutaneous immunotherapy (SCIT) and oral immunotherapy (OIT) which are based on the regular administration of the culprit food in increasing doses, have promising therapeutic potential in allergy. Even though SCIT may have some clinical efficacy for food allergy (increased food allergen thresholds), treatment has been shown to be associated with a high incidence of allergic side effects, which currently limits its application in clinical practice [4-6]. Few clinical trials have shown encouraging results of specific OIT in CM and PN allergic children [7-10]. OIT increases food allergen thresholds, diminishes skin prick test responses, enhances allergen-specific IgG4, decreases allergen-specific IgE, increases the activation threshold of basophils and temporarily increases regulatory T cells (Tregs) and relevant cytokine levels [8-11]. OIT is considered safer than SCIT, and hence more suitable for human treatment [5, 9]. However, although OIT has some efficacy, it is hampered by the high incidence of allergic side effects [10, 12-14]. Moreover, to date OIT has not yet resulted in long-lasting protection against food allergy: children subjected to OIT appear desensitized (i.e., protection against clinical effects), but not tolerized to peanut (i.e., induction of complete non-responsiveness or selective modulation of B and T cell responses) [8, 15], so the continuous ingestion of the allergen is still required to protect against clinical symptoms.

Data suggest that in addition to the food itself, an immune modulating agent (adjuvant) may be helpful to induce tolerance rather than desensitization [16-20]. In addition, an immune modulating agent may enhance the safety of the IT procedure by reducing the optimal allergen dose required to induce tolerance or by direct suppression of the allergic effector response over shorter treatment periods. Recent *in vitro* studies, as well as studies in animals and in allergic children, suggest that non-digestible carbohydrates, such as fructo-oligosaccharides (FOS) may improve both the efficacy and the safety of subcutaneous and oral therapeutic approaches. FOS has been shown to directly interact with the epithelium and to modulate the intestinal mucosal and systemic immune system from an allergic tuning toward a Treg and Th1 setting [21], thereby suppressing allergic inflammation [22-24].

One of the major challenges in immunotherapy of food allergy is the lack of food allergy-specific biomarkers for disease diagnosis, illness monitoring, therapy evaluation, and prognosis prediction. To improve our understanding and ability to intervene in complex multifactorial food allergy, it is important to investigate the molecular networks underlying the biological system and elucidate which interactions contribute to pathology and how this occurs. Biomarkers involved in these processes should be measurable indicators of normal biologic processes, pathogenic processes, or therapeutic responses, for the risk assessment, early diagnosis, and predicting and monitoring responses to therapies and toxicities.

This article focuses on applying data mining to search for hidden trends within large data sets. Here, Bayesian modeling in combination with technologies from topological data analysis and network science were used to analyze complex data from experimental OIT studies in mice by unraveling the complex relationships between analyzed parameters and prioritizing candidate biomarkers.

A Bayesian network (BN) is a type of probabilistic graphical model that lies at the intersection between statistics and machine learning. A BN is a compact representation of a probability distribution over a set of discrete variables. It can help to create a simplified overview of a complicated experiment, depicting an intuitive representation of relationships between variables, where it combines prior knowledge (such as the known relationships between variables) with data from observations. It captures the relationships between variables, and may be used to make inferences about unobserved variables. BNs are particularly suitable to deal with multiple cause-effect relationships within a complex system. Furthermore, a trained BN describing general relationships among variables can be used to make inferences about what-if scenarios and can as such be used to test hypotheses. Application of BNs has progressed enormously over the last decades leading to its use spanning all fields.

We use techniques that borrow extensively from topological data analysis (TDA) and network science to extract information from high-dimensional data sets. The 'shape' of data, as it can be elucidated by topological analysis, can provide information about the observed system. The geometric shape of our application of these techniques corresponds to the way in which different features within the system interact. The use of Bayesian networks in combination with topological analysis enables the discovery of therapeutic mechanisms that trigger a specific cascade of processes underlying OIT and subsequently identify a wide range of relevant disease parameters. In this way the study design of future studies may be optimized *in silico*, saving time and resources.

The aim of this study was i) to compare the key drivers of the mechanisms of scFOS/lcFOS diet-supported OIT in peanut allergy and cow's milk allergy and ii) to identify the biological relevance of biomarker (panels) of immunotherapy of food allergy thereby enabling the prioritization of candidate biomarkers.

MATERIALS AND METHODS

Data sources

Data were obtained from previously published studies describing experimental peanut allergy (PNA) and cow's milk allergy (CMA) models, in which female C3H/HeOuJ mice were sensitized to the allergens and treated with/without OIT and fed a diet supplemented with/without scFOS/lcFOS [24] (Wagenaar *et al.*, manuscript submitted). The treatment efficacy was assessed with an intradermal (i.d.), intragastric (i.g.) and intraperitoneal (i.p.) food provocation. The outline of the studies is depicted in **Figure 1**. The results of these murine studies indicated that scFOS/lcFOS supplementation improved the efficacy of OIT in cow's milk allergic mice.

Bayesian network analyses

For Bayesian data analysis, a selection of variables was made from both the CMA and PNA model datasets to ensure that the selected variables were present in all data and that these were measured under equivalent circumstances. The model used here was trained using data from two CMA model datasets. In order to integrate the data to train a single model, some considerations had to be made. Because several variables, which were to be included, were measured in assays that use relative values, pooling the data for use in the model required normalization and discretization. The two datasets were normalized by rescaling the variables with relative values to a new mutual scale, while keeping the variables with an absolute scale (such as temperature) unchanged.

Because of the relatively small amount of data available for model training, it was decided to train a discrete Bayesian network. In a discrete Bayesian network, each node represents a variable. Each node contains a conditional probability table that represents the joint probabilities of the states of this node and the states of the parent nodes. In order to express the variables and their dependencies as conditional probabilities, the variables have to be discrete. Discretization was therefore performed, using 'Hartemink's algorithm'. This is a method of discretization that automatically finds quantiles that preserve and maximize mutual information among variables within the network. It was applied as implemented by package 'bnlearn' [25, 26].

The structure of the network was not taken from the data, but provided by expert knowledge. After the model structure was defined, conditional probabilities were estimated using maximum likelihood estimation as implemented in package 'bnlearn' [26]. Model performance was assessed using a multiclass area under the ROC curve algorithm [27].

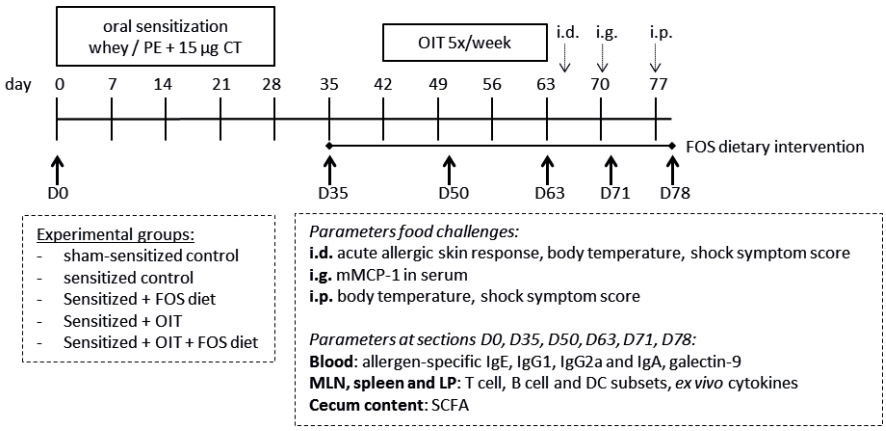


Figure 1. Experimental timelines of PNA and CMA models. Six-week-old female C3H/HeOuJ mice were randomly allocated to the control and experimental groups: sham-sensitized control group, sensitized control group, FOS supplemented group, OIT group, and the OIT with FOS supplementation group. Mice were i.g. sensitized to the cow's milk protein whey or PE (20 mg whey in 0.5 ml or 6 mg PE in 0.5 ml PBS) with cholera toxin as an adjuvant (15 µg in 0.5 ml PBS). The FOS supplemented diet was provided from D35 to the end of the protocol and OIT with 10 mg whey or 1.5 or 15 mg PE in 0.5 ml PBS was given from D42-D59 (5 oral gavages/week for 3 weeks). Acute allergic symptoms were measured upon i.d. challenge at D64 (10 µg whey or 1 µg PE in 20 µl PBS/ear), mast cell degranulation was measured upon i.g. challenge at D70 (50 mg whey or 15 mg PE in 0.5 ml PBS) and an i.p. challenge (50 µg whey or 100 µg PE in 200 µl PBS) was conducted at D77 to stimulate T cell responses prior to organ collection. At six time points throughout the animal experiment (D0, D35, D50, D63, D71 and D78), subgroups of mice from each control and experimental group were killed by cervical dislocation and blood and organs were collected. PE, peanut extract; CT, cholera toxin; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; i.d., intradermal; i.g., intragastric; i.p., intraperitoneal; LP, lamina propria of small intestine; SCFA, short-chain fatty acids.

Topological data analyses

Data were taken from the CMA and PNA model datasets for topological visualization. For the CMA model, two experiments were merged into one dataset while for the PNA model data from one experiment was used. During this merge, features were discarded when only one of the two datasets contained said feature. This was done to prevent situations where the similarity of two features cannot be determined because of mutually exclusive sample sets. The processing procedure for both datasets was identical.

Among the included variables were mucosal mast cell protease-1 (mMCP-1) upon i.g. food challenge and allergen-specific IgE, IgG1, IgG2a in serum, acute allergic skin response (ear swelling) upon i.d. food challenge, anaphylaxis symptom score and body temperature after challenge, leukocyte phenotypes by flow cytometry, cytokine release of MLN, LP and spleen-derived lymphocytes after *ex vivo* stimulation with anti-CD3, whey protein or peanut extract (PE) and short-chain fatty acids (SCFA). To construct the graph, an adjacency matrix was calculated using Spearman's rank correlation similarity. Using the resulting adjacency matrix, a mutual k-nearest neighbors graph was constructed as described [28]. The same publication shows that the graph, given large enough n, will be connected if we choose k on the order of log(n) where n is the number of samples in the data. Therefore, for each of the datasets, k equaled log(n).

To aid visualization and interpretation of the mutual nearest neighbors network, the nodes of the network were assigned to clusters using the multilevel modularity optimization algorithm [29].

RESULTS

Bayesian network illustrates beneficial effects of scFOS/lcFOS diet and OIT on CMA

Internal validation of the performance of the BN-model trained on the CMA model data was performed to assess how well the model can infer the clinical severity of allergy in light of the diet and treatment effects. mMCP-1 was chosen as a meaningful objective representation of allergic severity. The predictive performance of the model on mMCP-1 quantiles from CMA model data was good, as assessed from a multiclass area under the ROC curve of 0.86.

Next we used the BN to make inferences to test the influence of sensitization with or without OIT on the probability distribution of BN variables. In **Figure 2A** the probability distribution of the BN parameters is shown irrespective of the animal treatments (sensitization, scFOS/lcFOS dietary supplementation, or OIT), showing 84.6% probability of being sensitized, 54.2% probability of having received the scFOS/lcFOS supplemented diet and 42.6% probability of having received OIT treatment and the other probability distributions of the parameters analyzed in the animals.

The probability distributions of the analyzed parameters changed upon the assumption of the model that all animals were sensitized (**Figure 2B**) resulting in only a small shift in probability distributions of the variables. This can be explained by the fact that the chance of sensitization irrespective of the animal treatments was already 84.6% (**Figure 2A**), so the increase to 100% sensitization does not have a major effect. The effect on the probability distributions change significantly upon the assumption of the model that all animals were sensitized and received OIT treatment (**Figures 2B,C**). This results in a clear decrease in probability of high specific IgE (from 21.51% to 17.82%), specific IgG1 (from 81.72% to 60.40%), ear swelling upon i.d. challenge(from 64.58% to 60.79%) and mMCP-1 (from 84.96% to 51.99%) levels, indicating the clear effect of OIT on these allergy parameters.

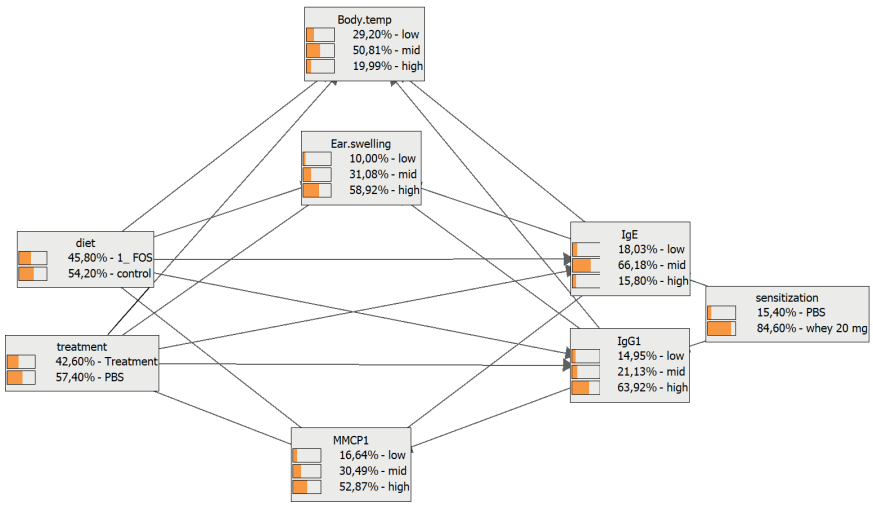


Figure 2A. Bayesian network.

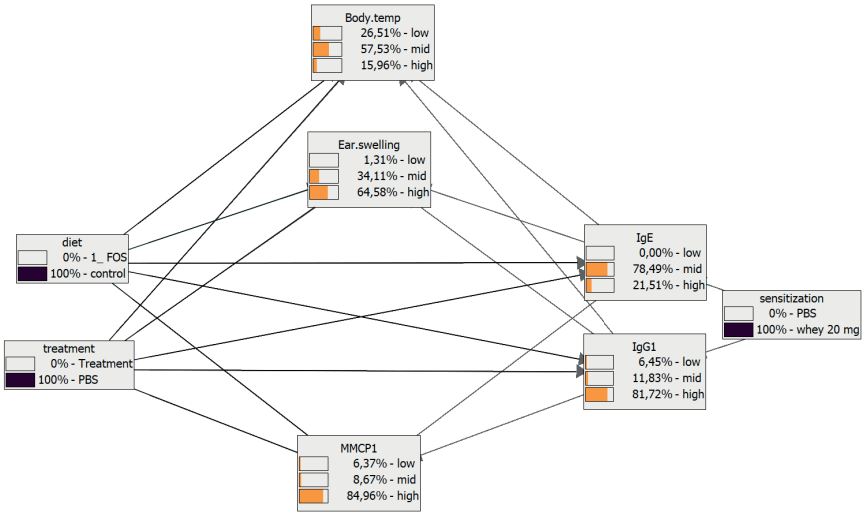


Figure 2B. Effect of sensitization.

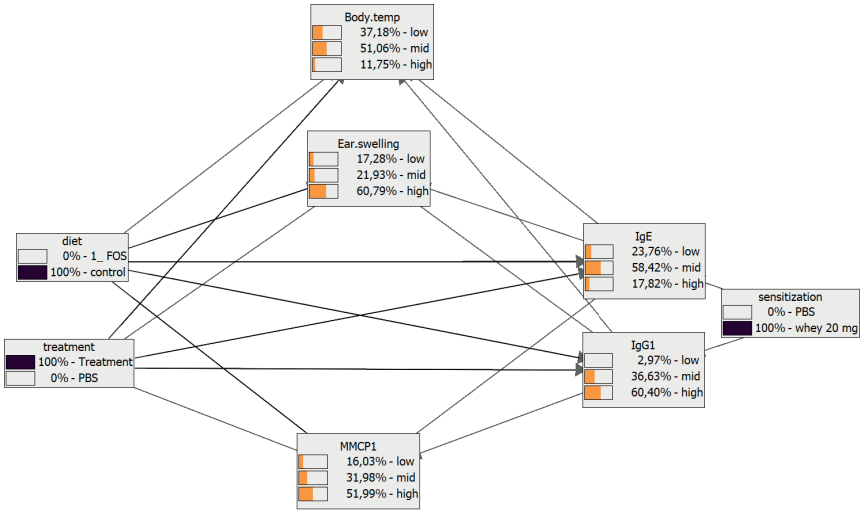


Figure 2C. Effect of sensitization + OIT.

Figure 2. Bayesian network trained on CMA data, effects of sensitization and/or OIT. BN depicting the relationships between the analyzed parameters and the animal treatments (sensitization, scFOS/lcFOS diet, or OIT treatment) using CMA data. Moreover, the probability distributions of all BN variables are depicted **(A)** irrespective of animal treatments, **(B)** assuming that all animals were sensitized and **(C)** assuming that all animals were sensitized and received OIT.

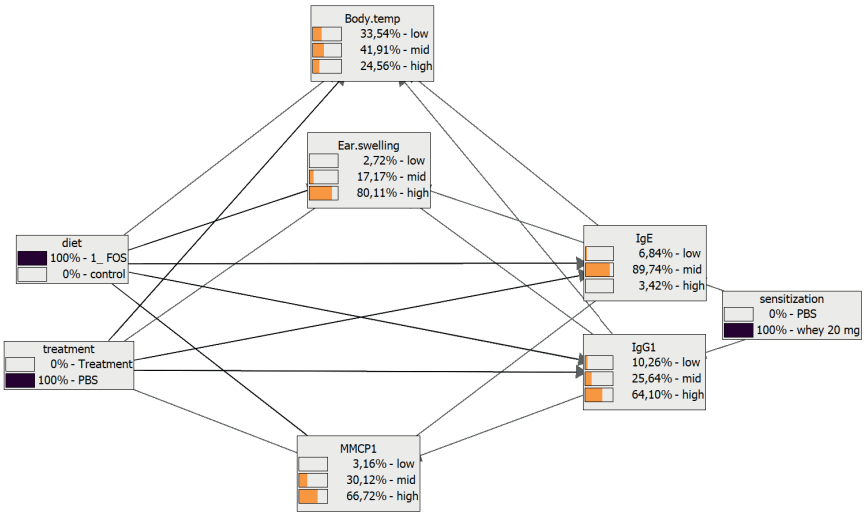


Figure 3A. Effect of sensitization + FOS diet.

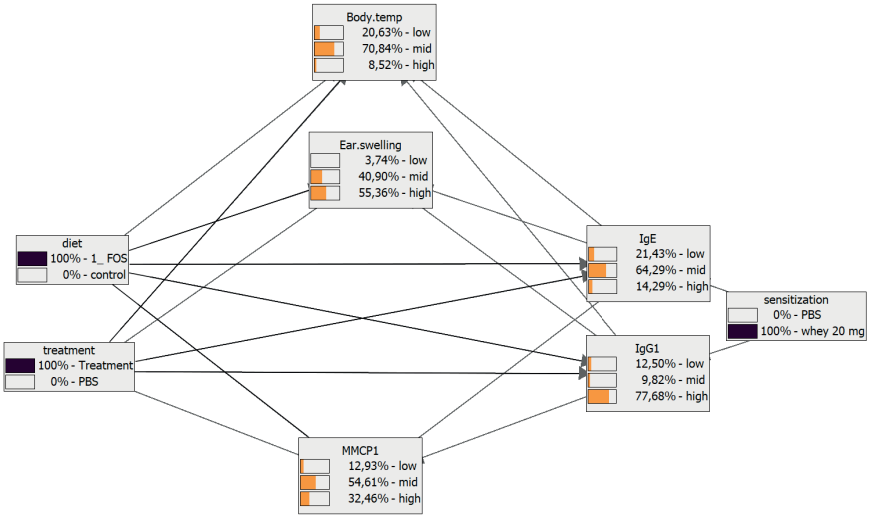


Figure 3B. Effect of sensitization + FOS diet + OIT.

Figure 3. Bayesian network trained on CMA data, effects of scFOS/lcFOS diet with or without OIT. BN depicting the relationships between the analyzed parameters and the animal treatments (sensitization, scFOS/lcFOS diet, OIT treatment) using the CMA data. The probability distributions of all BN variables are depicted assuming that all animals were sensitized and **(A)** received scFOS/lcFOS diet or **(B)** received scFOS/lcFOS diet in combination with OIT.

Next we investigated the effect of the scFOS/lcFOS supplemented diet on the probability distributions in sensitized animals (**Figures 2B and 3A**). This modeling showed a clear decrease in probability of high specific IgE (from 21.51% to 3.42%), high specific IgG1 (from 81.72% to 64.10%), high mMCP-1 (from 84.96% to 66.72%) and an unexpected increase in high ear swelling (from 64.58% to 80.11%). Together, these calculations indicate a clear effect of scFOS/lcFOS diet on these allergy parameters. This effect of the scFOS/lcFOS diet on the probability distributions in sensitized animals is further increased by the extra addition of OIT in the model (**Figures 2B and 3B**). This results in a major decrease in probability of high mMCP-1 (from 84.96% to 32.46%) and high ear swelling (from 64.58% to 55.36%).

Together these data indicate clearly the added effect of supplementing the diet with scFOS/lcFOS on the efficacy of OIT in CMA, which confirms previous work [24]. These examples illustrate how the BN is suitable to easily test and generate hypotheses by visualizing the consequences of what-if scenarios. Instead of analyzing the effects of interventions on the entire population, the modeling also enables to focus on the effects of interventions on subpopulations of subjects, which clearly makes this approach even more valuable for future applications (e.g., mechanism elucidation, patient stratification), although this goes beyond the scope of this manuscript.

Bayesian network indicates similar key drivers in PNA and CMA

Although both CMA and PNA seem clinically similar diseases, they differ in the fact that CMA is most prevalent during early childhood, but is often outgrown [30] while PNA is more persistent and is the most frequent cause of life-threatening allergic reactions in adults [31].

To assess whether key features of the CMA and PNA models have similar properties, we used the Bayesian network that was trained on data from the CMA model to make inferences on the PNA model data. Inferences on mMCP-1 quantiles in the BN from a PNA model experiment, using the network trained on the CMA model data gave a multiclass area under the ROC curve of 0.76; which is considered a fair performance. From these analyses, it is reasonable to deduce that the effects of the allergen, treatment and diet are similar within our Bayesian network abstraction of CMA across peanut and cow's milk models. Therefore, we can regard the model as a reasonable representation of the core mechanisms and characteristics of allergy, treatment and diet.

Topological data analyses prioritize candidate biomarkers

The BN indicated that the core characteristics of allergy, treatment and diet in both the PNA and CMA models are similar. Because many more parameters were analyzed throughout the course of each study in each individual mouse, the next question was whether it would be possible to indicate whether the relevance of the analyzed parameters differs between PNA and CMA by determining how the parameters are related to each other and to the clinical endpoints of food allergy.

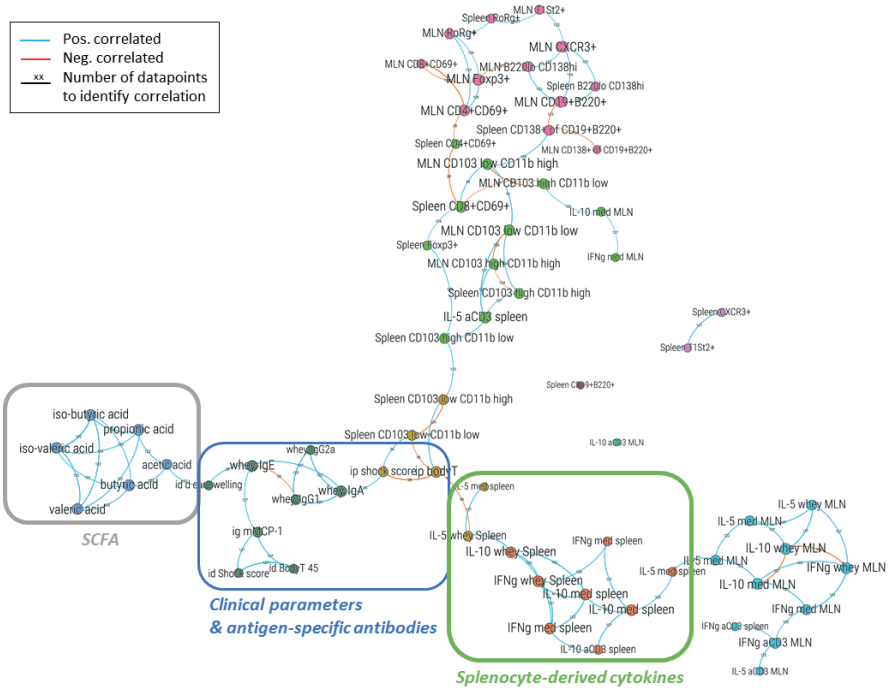


Figure 4. The mutual nearest neighbors network of the CMA model. Topological network showing the clustering of parameters (dots with same color). The clusters were used to identify the cluster relationships in CMA. Moreover, the encircled clusters were used to compare the cluster relationships between CMA and PNA (**Figure 5**).

Figure 4 shows the overview of the mutual nearest neighbors network of parameters that were measured in the CMA model. From this experiment, 66 parameters were available for analysis. These were organized in 7 large (6-12 parameters) and 4 small clusters (1-2 parameters). The parameters most closely related to clinical outcomes (ear swelling, shock scores, body temperature and mMCP-1) were located in the same cluster as antigen-specific antibodies (dark green dots), indicating how closely they are connected. The remaining parameters closely linked to clinical outcomes (shock score and body temperature after i.p. challenge) were clustered together with splenocyte-derived parameters, both located in a cluster (yellow dots) which was quite closely linked to this antibody/clinical parameter cluster.

The antibody/clinical parameter cluster (dark green dots) was most closely related to the SCFA cluster (purple dots) and to the cluster with spleen-derived/clinical parameters (yellow dots). The remaining harboring MLN and remaining splenocyte-derived parameters showed quite indirect relationships (light blue, orange, magenta and green dots) with the antibody/clinical parameter cluster or had no relationship (purple, brown and seagreen dots) at all (spleen CXCR3+ cells, T1St2+ cells, CD19+B220+ cells and IL-10 production of anti-CD3 stimulated MLN-derived lymphocytes). The indirect or absent relationship with clusters harboring clinical parameters indicates that they are of lesser importance to the process of OIT of food allergy.

Figure 5 shows the TDA of the PNA model. In the PNA model, 33 parameters were analyzed which were organized in 6 clusters containing 4-7 parameters. The parameters most closely related to clinical outcomes (ear swelling, body temperature, shock scores and mMCP-1) were located in the same cluster as spleen Th1 (CD183+CD69+) and Th2 (T1St2+CD69+) cells. In contrast to the CMA model, all clinical parameters were located in the same cluster (purple dots). Moreover, the antigen-specific antibodies were not linked in the same cluster as the clinical parameters, although they were closely linked (light blue dots). Another cluster (sea green dots) closely linked to the clinical parameter cluster, consisted of the Tregs of spleen and MLN, Th2 cells in MLN, IFNγ production of anti-CD3/CD28 stimulated splenocytes and the %CD11b-CD103+ cells of CD11c+MHCII+ DC in MLN. The cluster (orange dots) harboring parameters of cytokine production by stimulated splenocytes was indirectly linked to the clinical symptoms via the antibody cluster (light blue dots). The remaining clusters showed quite an indirect relationship (dusky pink dots) with the clinical parameter cluster or had no relationship at all (light green dots, SCFA cluster). The latter finding is quite remarkable, since in the CMA model the SCFA cluster was quite closely linked to the clinical parameter clusters.

In summary, even though not all analyzed parameters were identical in both models, there is a substantial similarity in topology and clustering of features from both PNA and CMA models (clinical parameters, antibodies, SCFA, cytokines from stimulated splenocytes) and the connections between the clusters, indicating that both models have largely similar mechanistic relationships. Nevertheless, topological data analyses also indicate differences between parameters and the clinical outcome (e.g., importance of SCFA). These differences identified by in the mutual nearest neighbors networks may be useful to generate new hypotheses for observed clinical differences and prognoses of CMA and PNA.

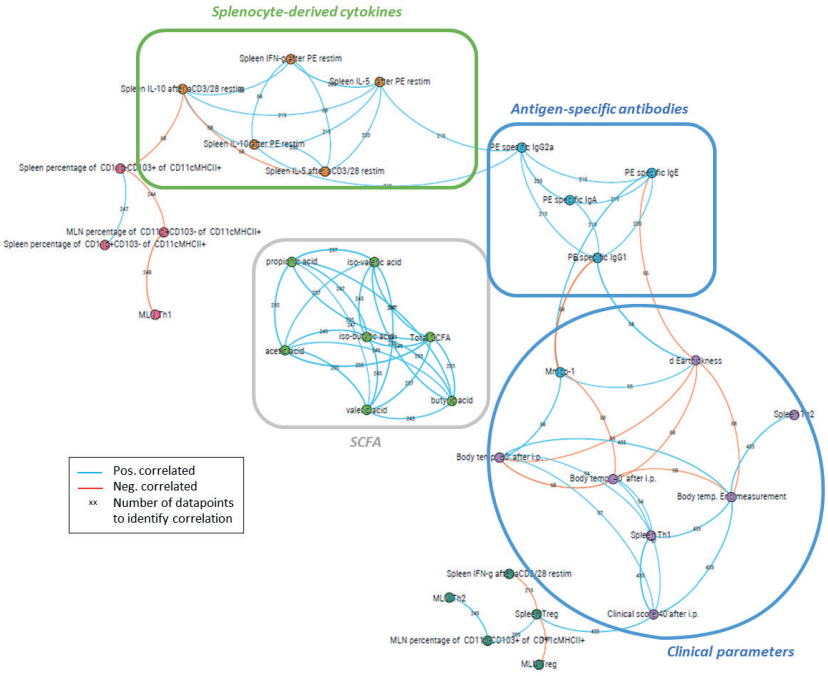


Figure 5. The mutual nearest neighbors network of the PNA model. Depicted is the topological network showing the clustering of parameters (dots with same color). The clusters were used to identify the cluster relationships in PNA. Moreover, the encircled clusters were used to compare the cluster relationships between PNA and CMA (**Figure 4**).

DISCUSSION

Immunotherapy is currently the most promising therapy for patients with food allergy, who now rely on avoidance and carrying adrenaline auto injectors in case of accidental exposure. Unfortunately, current immunotherapy treatments of food allergy are too often accompanied by allergic side effects and do not appear to give long-term protection [17]. Recent *in vitro* studies, studies in animal models and studies in children with atopic dermatitis indicate that the addition of non-digestible sugars may improve the efficacy and safety of therapeutic approaches [22, 23, 32, 33]. However, the mechanism of action of these approaches is still largely unclear and as a result possibilities, limitations and safety /risks of these types of interventions are not known. The lack of insight into the mechanism also results in a large number of parameters being measured in studies, while the usefulness of results for a large part of these parameters is unclear and the studies become extremely elaborate.

Here we use ways of data mining to search for hidden trends within existing sets of data, by using computational solutions (including algorithms, models and tools) which can be used to optimize experimental designs, data analyses and interpretation and hypotheses generation. We show that network analysis methods can be applied to investigate the underlying molecular mechanisms involved in immunotherapy of food allergy and the prioritization of biomarkers. By applying Bayesian networks and topological data analyses, 'hidden' information was discovered in the available data by visualizing the complex relationships between measured parameters and symptoms. In this study, we analyzed data from animal experiments with the major allergenic foods, peanut and cow's milk, which show different disease patterns. CMA is most prevalent during early childhood, but is often outgrown [30] while PNA is more persistent and is the most frequent cause of life-threatening allergic reactions in adults [31]. Our analyses suggest that the mechanisms involved in immunotherapy of CMA and PNA are very similar but not completely identical on the basis of the measured parameters. Possibly, slight differences can help to explain differences between patients.

One of the most striking differences was that our data clearly indicates the role of SCFA in CMA, but not in PNA. Previously, we have shown that in PNA and CMA, increased levels of SCFA, specifically butyric acid, coincided with allergy reduction [24] (Wagenaar *et al.*, manuscript submitted). These findings are confirmed in literature, where accumulating evidence indicates that SCFA have several anti-allergic properties by amongst others Treg induction and enhancement of the gut barrier function [34]. Previous findings also show that dietary fibers which are metabolized by the gut microbiota into SCFA are able to down-regulate PNA [35]

and inflammatory airway responses in asthma [36]. Moreover, in CMA, levels of faecal butyrate were increased in tolerant infants [37]. So even though we have observed that increased levels of SCFA coincided with an allergy reduction in both PNA and CMA using the dataset used in current analyses [24] (Wagenaar *et al.*, manuscript submitted), we here show that the structure of how the experimental data are correlated with each other are different between the allergy models. This means that the relationship between the SCFA and the clinical outcomes in PNA is a) more indirect and/or b) occurs via different mechanisms or parameters which were not analyzed in the studies and/or c) is not essential for the outcome of the allergy, so the level of SCFA could be an epiphenomenon which is in contrast to the current opinion in literature as mentioned before. This example nicely illustrates how these types of network-based analyses, enable the generation of new hypotheses, in this case the role of the different biomarkers in (treatment of) food allergy and to explain the differences between the disease patterns of CMA and PNA.

Another important feature of these types of network analyses is that they create a new view of the dataset which can be used to determine the biological relevance of the measured parameters. Using the mutual nearest neighbors networks from the topological data analyses, several criteria can be applied to prioritize the measured parameters: i) it became clear that several more or less 'standard' study parameters seem to have little relevance because they had no clear link to the clinical outcomes of immunotherapy, while others had a very direct link; ii) clusters of parameters were identified that individually were linked in a comparable manner to the biologically relevant parameters, so one could argue that analyses of only a few cluster-members would be sufficient instead of analyzing the entire panel; iii) mutual nearest neighbors networks enabled the prioritization of parameters based on the invasiveness of the measurements of the parameter in case of 'equally' relevant linked parameters to the clinical parameters. For instance, SCFA analyses in faecal samples or IgE in serum are far less invasive for the subject than determining the skin response upon challenge, both in experimental animal models and in humans.

The BN approach demonstrated here allows investigators to more productively mine the currently available and/or future data sets of phenotypes for food allergy-related traits to discover testable hypotheses for physiological mechanisms that lead to a food allergic phenotype. Here we show that in this approach, the addition of oligosaccharides with or without immunotherapy reduced the food allergy in both CMA and PNA. Moreover, even though the analyzed parameters in CMA and PNA were not identical, we showed that the key mechanisms between CMA and PNA are comparable. The BN shown here is quite simple in this experimental setting

containing a limited set of parameters. For future clinical applications, it would be very interesting to expand this BN with patient characteristics (e.g., epigenetic factors, genetic factors, age, sex, medication), analyzing multiple parameters on multiple time points. This would result in a so called dynamic BN which would enable a stratification strategy to predict before the start of treatment whether a patient will benefit from undergoing immunotherapy. These new insights provide good starting points for selecting relevant biomarkers to monitor and predict safety and efficacy in later clinical studies, but also eventually in clinical applications.

CONCLUSION

Here we provide a promising bioinformatics method to compare mechanistic features between different food allergies and to identify the biological relevance of biomarker (panels) of immunotherapy of food allergy. We have shown that the key drivers that influence PNA and CMA are similar but that these phenotypically similar diseases show mechanistic differences in their subnetworks. This method may be useful to generate new hypotheses to explain why CMA has a different disease pattern than PNA and to select biomarkers that are useful in for future clinical studies.

AVAILABILITY OF DATA

Vonk MM, Diks MAP, Wagenaar L, Smit JJ, Pieters RHH, Garssen J, van Esch B, and Knippels LMJ. Improved efficacy of oral immunotherapy using non-digestible oligosaccharides in a murine cow's milk allergy model: A potential role for foxp3+ regulatory t cells. Front Immunol 2017. 8: p. 1230.

ACKNOWLEDGEMENTS

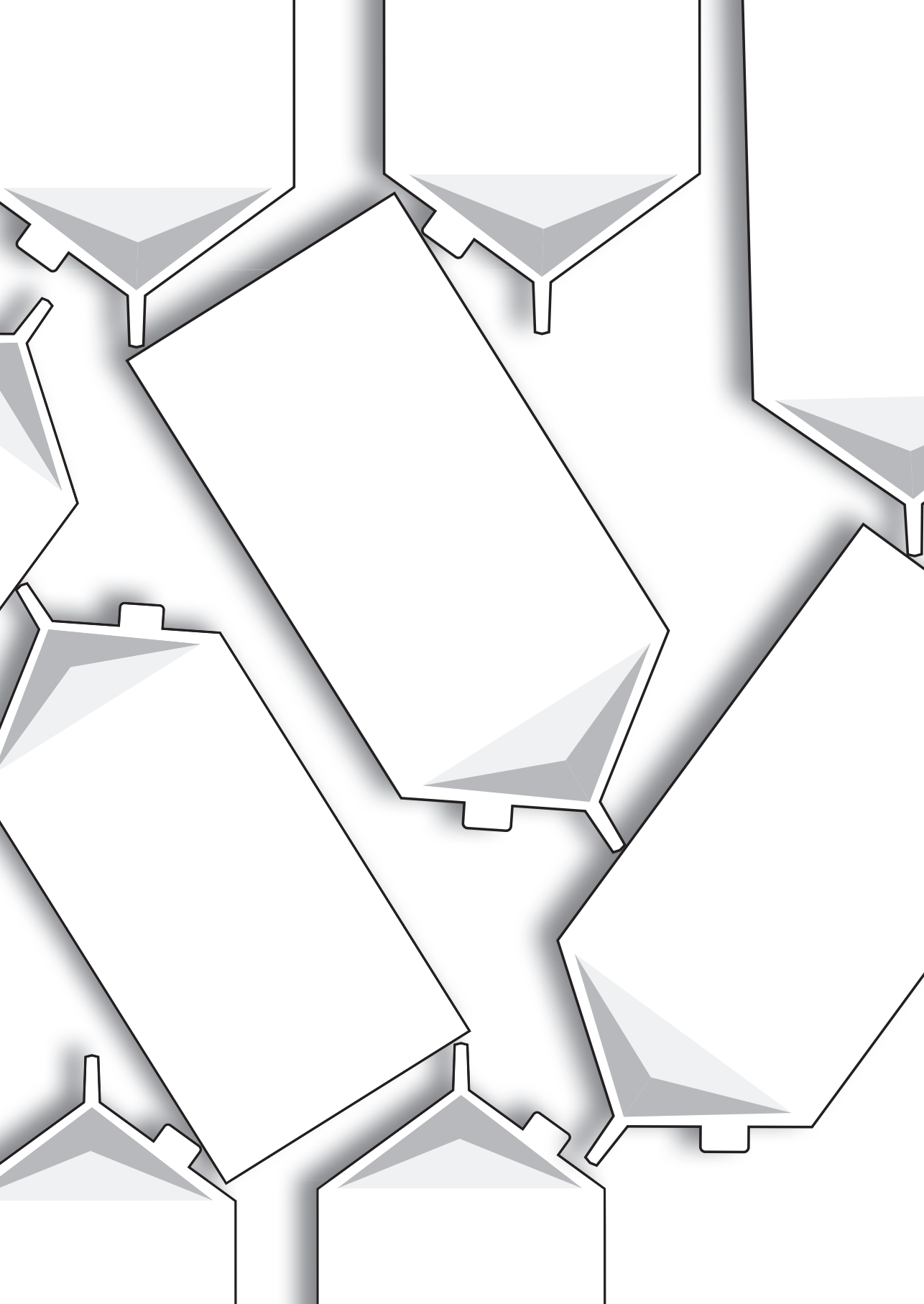
The authors are all part of the Utrecht Center for Food Allergy (UCFA; www.ucfa.nl) and the NUTRALL research consortium. This research was financially supported by the NWO 'Open Technology Program' grant 12652 and by Dutch Governmental TNO Research Cooperation Funds.

REFERENCES

1. Carrard A, Rizzuti D, and Sokollik C. Update on food allergy. Allergy 2015. 70(12): p. 1511-1520.
2. Prescott SL, Pawankar R, Allen KJ, Campbell DE, Sinn J, Fiocchi A, Ebisawa M, Sampson HA, Beyer K, and Lee BW. A global survey of changing patterns of food allergy burden in children. World Allergy Organ J 2013. 6(1): p. 21.
3. Sampson HA. Update on food allergy. J Allergy Clin Immunol 2004. 113(5): p. 805-19; quiz 820.
4. Chiang D and Berin MC. An examination of clinical and immunologic outcomes in food allergen immunotherapy by route of administration. Current Allergy and Asthma Reports 2015. 15(6).
5. Nelson HS, Lahr J, Rule R, Bock A, and Leung D. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. Journal of Allergy and Clinical Immunology 1997. 99(6): p. 744-751.
6. Oppenheimer JJ, Nelson HS, Bock SA, Christensen F, and Leung DYM. Treatment of peanut allergy with rush immunotherapy. Journal of Allergy and Clinical Immunology 1992. 90(2): p. 256-262.
7. Blumchen K, Ulbricht H, Staden U, Dobberstein K, Beschorner J, de Oliveira LCL, Shreffler WG, Sampson HA, Niggemann B, Wahn U, and Beyer K. Oral peanut immunotherapy in children with peanut anaphylaxis. Journal of Allergy and Clinical Immunology 2010. 126(1): p. 83-91.
8. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, Shreffler WG, Steele P, Henry KA, Adair M, Francis JM, Durham S, Vickery BP, Zhong XP, and Burks AW. Clinical efficacy and immune regulation with peanut oral immunotherapy. Journal of Allergy and Clinical Immunology 2009. 124(2): p. 292-300.
9. MacDonald TT and Di Sabatino A. The immunologic basis for gastrointestinal food allergy. Current Opinion in Gastroenterology 2009. 25(6): p. 521-526.
10. Nowak-Wegrzyn A and Fiocchi A. Is oral immunotherapy the cure for food allergies? Current Opinion in Allergy and Clinical Immunology 2010. 10(3): p. 214-219.
11. Larenas-Linnemann D. Certainties and doubts about sublingual and oral immunotherapy in children. Current Opinion in Allergy and Clinical Immunology 2009. 9(6): p. 558-567.
12. Thyagarajan A, Varshney P, Jones SM, Sicherer S, Wood R, Vickery BP, Sampson H, and Burks AW. Peanut oral immunotherapy is not ready for clinical use. J Allergy Clin Immunol 2010. 126(1): p. 31-2.
13. Vazquez-Ortiz M, Alvaro-Lozano M, Alsina L, Garcia-Paba MB, Piquer-Gibert M, Giner-Munoz MT, Lozano J, Dominguez-Sanchez O, Jimenez R, Dias M, Martin-Mateos MA, and Plaza-Martin AM. Safety and predictors of adverse events during

oral immunotherapy for milk allergy: Severity of reaction at oral challenge, specific ige and prick test. Clin Exp Allergy 2013. 43(1): p. 92-102.
14. Virkud YV, Burks AW, Steele PH, Edwards LJ, Berglund JP, Jones SM, Scurlock AM, Perry TT, Pesek RD, and Vickery BP. Novel baseline predictors of adverse events during oral immunotherapy in children with peanut allergy. J Allergy Clin Immunol 2017. 139(3): p. 882-888 e5.
15. Rolinck-Werninghaus C, Staden U, Mehl A, Hamelmann E, Beyer K, and Niggemann B. Specific oral tolerance induction with food in children: Transient or persistent effect on food allergy? Allergy 2005. 60(10): p. 1320-2.
16. Keet CA and Wood RA. Emerging therapies for food allergy. Journal of Clinical Investigation 2014. 124(5): p. 1880-1886.
17. Kobernick AK and Burks AW. Active treatment for food allergy. Allergology International 2016. 65(4): p. 388-395.
18. Loh WY and Tang MM. Adjuvant therapies in food immunotherapy. Immunology and Allergy Clinics of North America 2018. 38(1): p. 89-+.
19. Prescott SL, Smith P, Tang M, Palmer DJ, Sinn J, Huntley SJ, Cormack B, Heine RG, Gibson RA, and Makrides M. The importance of early complementary feeding in the development of oral tolerance: Concerns and controversies. Pediatr Allergy Immunol 2008. 19(5): p. 375-80.
20. Tang MLK, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, and Donath S. Administration of a probiotic with peanut oral immunotherapy: A randomized trial. Journal of Allergy and Clinical Immunology 2015. 135(3): p. 737-U248.
21. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJG, Schouten B, van Esch BCAM, Knol J, Sprikkelman AB, van der Aa LB, Knippels LMJ, Garssen J, van Kooyk Y, and Willemsen LEM. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012. 67(3): p. 343-352.
22. de Kivit S, Kostadinova AI, Kerperien J, Morgan ME, Muruzabal VA, Hofman GA, Knippels LMJ, Kraneveld AD, Garssen J, and Willemsen LEM. Dietary, nondigestible oligosaccharides and bifidobacterium breve m-16v suppress allergic inflammation in intestine via targeting dendritic cell maturation. Journal of Leukocyte Biology 2017. 102(1): p. 105-115.
23. Hayen SM, Kostadinova AI, Garssen J, Otten HG, and Willemsen LEM. Novel immunotherapy approaches to food allergy. Current Opinion in Allergy and Clinical Immunology 2014. 14(6): p. 549-556.
24. Vonk MM, Diks MAP, Wagenaar L, Smit JJ, Pieters RHH, Garssen J, van Esch B, and Knippels LMJ. Improved efficacy of oral immunotherapy using non-digestible oligosaccharides in a murine cow's milk allergy model: A potential role for foxp3+ regulatory t

cells. Front Immunol 2017. 8: p. 1230.
25. Hartemink AJ. Principled computational methods for the validation discovery of genetic regulatory networks. Massachusetts Institute of Technology. Dept. of Electrical Engineering and Computer Science, PhD Thesis 2001.
26. Scutari M. Learning bayesian networks with the bnlearn r package. Journal of Statistical Software 2010. 35(3): p. 1-22.
27. Hand DJ and Till RJ. A simple generalisation of the area under the roc curve for multiple class classification problems. Machine Learning 2001. 45(2): p. 171-186.
28. Brito MR, Chavez EL, Quiroz AJ, and Yukich JE. Connectivity of the mutual k-nearest-neighbor graph in clustering and outlier detection. Statistics & Probability Letters 1997. 35(1): p. 33-42.
29. Blondel VD, Guillaume JL, Lambiotte R, and Lefebvre E. Fast unfolding of communities in large networks. Journal of Statistical Mechanics-Theory and Experiment 2008. p. 10008
30. Ludman S, Shah N, and Fox AT. Managing cows' milk allergy in children. Bmj-British Medical Journal 2013. 347.
31. Al-Ahmed N, Alsowaidi S, and Vadas P. Peanut allergy: An overview. Allergy Asthma Clin Immunol 2008. 4(4): p. 139-43.
32. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, and Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Archives of Disease in Childhood 2006. 91(10): p. 814-819.
33. Vonk MM, Wagenaar L, Pieters RHH, Knippels LMJ, Willemsen LEM, Smit JJ, van Esch B, and Garssen J. The efficacy of oral and subcutaneous antigen-specific immunotherapy in murine cow's milk- and peanut allergy models. Clin Transl Allergy 2017. 7: p. 35.
34. Hirata S and Kunisawa J. Gut microbiome, metabolome, and allergic diseases. Allergology International 2017. 66(4): p. 523-528.
35. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, and Mackay CR. Dietary fiber and bacterial scfa enhance oral tolerance and protect against food allergy through diverse cellular pathways. Cell Reports 2016. 15(12): p. 2809-2824.
36. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, and Marsland BJ. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 2014. 20(2): p. 159-66.
37. Canani RB, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, Calignano A, Khan AA, Gilbert JA, and Nagler CR. Lactobacillus rhamnosus gg-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. Isme Journal 2016. 10(3): p. 742-750.



CHAPTER 6

BUTYRATE ENHANCES DESENSITIZATION INDUCED BY ORAL IMMUNOTHERAPY IN COW'S MILK ALLERGIC MICE

Marlotte M. Vonk[1,2]
Bart R.J. Blokhuis[1]
Mara A.P. Diks[1]
Laura Wagenaar[3]
Joost J. Smit[3]
Raymond H.H. Pieters[3]
Johan Garssen[1,2]
Léon M.J. Knippels[1,2]
Betty C.A.M. van Esch[1,2*]

1. Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
2. Immunology Platform, Danone Nutricia Research, Utrecht, the Netherlands
3. Department of Immunotoxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

* All authors are part of the NUTRALL research consortium

Submitted for publication

ABSTRACT

Background: In previous studies, we showed that a fructo-oligosaccharide (FOS) supplemented diet enhanced oral immunotherapy (OIT) efficacy in a mouse model for cow's milk allergy. Fermentation of FOS by intestinal bacteria leads to production of short-chain fatty acids (SCFA) including butyrate.

Aim: To investigate the contribution of butyrate in the enhanced efficacy of OIT+FOS.

Methods: C3H/HeOuJ mice were sensitized and received OIT with or without FOS or butyrate supplementation. After treatment, whole blood was collected to conduct a basophil activation test (BAT) and allergen challenges were performed to measure acute allergic symptoms. CD4+CD25+ regulatory T cells (Tregs) were isolated from treated mice or differentiated *in vitro* and used in a bone marrow-derived mast cell (BMMC) suppression assay. Cecum content was collected to analyze SCFA concentrations.

Results: Allergen-induced basophil activation was reduced in OIT+butyrate samples compared to OIT. In accordance, the acute allergic skin response and mast cell degranulation were reduced in OIT+butyrate and OIT+FOS mice compared to sensitized controls. Butyric acid was increased in the cecum content of OIT+FOS mice compared to OIT mice and sensitized controls. Treg-mediated BMMC suppression was enhanced after *in vivo* butyrate and FOS exposure in combination with OIT.

Conclusion: Butyrate supplementation enhanced OIT-induced desensitization of basophils and mast cells and Treg functionality. Both butyrate and FOS are promising candidates to improve OIT efficacy in human studies to treat food allergies.

INTRODUCTION

Population-based sampling of Australian one-year-old infants showed oral challenge-proven IgE-mediated food allergy in over 10% of the infants [1]. This high prevalence of food allergies among infants, in combination with an associated reduced growth and increased risk of asthma development later in life [2, 3], stress the need for effective interventions. To date, food allergy management largely consists of allergen avoidance and administration of epinephrine in case of systemic anaphylaxis. Human trials with antigen-specific immunotherapy (AIT) to treat food allergies have shown promising results. However, safety and efficacy concerns have obstructed wide-spread clinical application [4, 5]. A recent meta-analysis confirmed an AIT-induced increase in the tolerated dose in food allergic patients, but also reported an increased risk of mild to severe adverse (systemic) reactions [6].

Oral immunotherapy (OIT) to treat cow's milk, peanut and hen's egg allergies has been shown to reduce clinical symptoms upon food challenge, but unsuccessfully maintained the protective state upon discontinuation of the therapy [7]. Effective desensitization of effector cells like mast cells and basophils in combination with active modulation of the adaptive immune response via antigen-presenting cells and T and B lymphocytes are key mechanisms in OIT [8]. The use of dietary adjuvants with immunomodulatory properties might open a new window of opportunities to improve the efficacy of OIT for food allergies.

Pre- and probiotics have been shown to promote oral tolerance and attenuate the allergic phenotype via the growth of beneficial microbes in the gut and the increased production of short-chain fatty acids (SCFA) [9, 10]. Co-administration of a probiotic during OIT in peanut allergic children induced sustained unresponsiveness to a food challenge in 82.1% of the participants, even after 2-5 weeks without therapy [11]. Previous studies from our group have shown that dietary supplementation with fructo-oligosaccharides (FOS, prebiotics) during OIT improved the efficacy of the therapy in a murine cow's milk allergy model [12]. We observed a reduction in clinical symptoms upon food challenge and showed the involvement of Foxp3+ regulatory T cells (Tregs) in the protective effect induced by OIT+FOS [12]. In addition, the interaction of proteins and non-digestible oligosaccharides with intestinal epithelial cells (IEC) can induce release of soluble galectin-9, a glycan-recognizing protein involved in tolerance induction and direct suppression of IgE-mediated mast cell degranulation [13]. A significant increase in serum galectin-9 levels was observed after OIT+FOS treatment in cow's milk allergic mice [12].

Fermentation of non-digestible oligosaccharides and proteins by commensal microbes, present in the colon and cecum, leads to formation of SCFA. Specific bacterial groups are responsible for the production of butyrate from acetyl-CoA and butyryl-CoA, propionate from propionyl-CoA and acetate from acetyl-CoA [14]. After absorption into colonic or cecal epithelial cells via diverse mechanisms, SCFA enter the circulation and modulate metabolic and immune processes in peripheral tissues [15]. Via the inhibition of histone deacetylases (HDAC) and activation of G-protein coupled receptors (GPCR), e.g., GPR41, GPR43 and GPR109a, on epithelial and immune cells, SCFA can alter gene expression and inflammatory responses [16]. To gain more insight into the role of butyrate in the allergy protective effect induced by OIT and FOS supplementation, we administered butyrate directly to cow's milk allergic mice during OIT and evaluated the allergic response to food challenges.

MATERIALS AND METHODS

Mice

Six-week-old female specific-pathogen free C3H/HeOuJ mice were purchased (Charles River laboratories, Erkrath, Germany) and randomly allocated to the control and experimental groups (**Figure 1**). All mice were housed in filter-topped macrolon cages (one cage/group) on a 12 h light/dark cycle with unlimited access to food and water at the animal facility of Utrecht University and were acclimatized for 6 days. All experimental procedures were approved by the Ethical Committee of Animal Research of Utrecht University and complied with the principles of good laboratory animal care as stated by the European Directive for the protection of animals used for scientific purposes.

Control diet and FOS supplemented diet

All animals were fed the AIN-93G control diet during acclimatization and oral sensitization (D0 to D35). The FOS supplemented diet was provided from D35 to the end of the protocol in the FOS and OIT+FOS groups (**Figure 1**). Shortly, a specific mixture of plant-derived short-chain FOS (scFOS: oligofructose, Raftilose P95, degree of polymerization (DP) <6) and long-chain FOS (lcFOS: long-chain inulin, Raftiline HP, average DP of 23 or higher with <1% DP of 5 or lower) was provided by Orafti (Wijchen, the Netherlands) and added to the base recipe of the semi-purified cow's milk protein-free AIN-93G diet (scFOS/lcFOS, 9:1, 1%, w/w, Ssniff Spezialdiäten GmbH, Soest, Germany) [12].

Experimental animal procedures

All mice were intragastrically (i.g.) sensitized to the cow's milk protein whey (DMV International, Veghel, the Netherlands) dissolved in PBS (20 mg whey in 0.5 ml PBS, Lonza, Verviers, Belgium) with cholera toxin (CT, 15 µg CT in 0.5 ml, List Biological Laboratories Inc., Campbell, CA, USA) to induce food allergy or were sham-sensitized with PBS and CT alone on D0, 7, 14, 21 and 28 (**Figure 1**). OIT consisted of 10 mg whey in 0.5 ml PBS and was provided per oral gavage from D42-D59 (5x/week, for 3 weeks). The butyrate and OIT+butyrate mice were weighted prior to D42, D49 and D56 and received 0.6 g/kg bodyweight/day sodium butyrate (Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in PBS with or without whey based on mean bodyweight of the group. All mice received an intradermal (i.d.) challenge in both ear pinnae (10 µg whey in 20 µl PBS/ear) to measure the acute allergic skin response and symptoms of anaphylaxis (D64). Mean basal ear thickness in µm (using a digital micrometer, Mitutoyo, Veenendaal, the Netherlands) was subtracted from mean ear thickness 1 h post-challenge (in duplicate, in both ears, blinded measurement) to calculate Δ ear swelling (i.e., the acute allergic skin response) per mouse. To perform the i.d. injection and both ear measurements, all mice were anesthetized twice using inhalation of isoflurane. On D69, all mice were i.g. challenged with 50 mg whey in 0.5 ml PBS. Serum samples were collected via cheek puncture 30 min after i.g. provocation to measure mucosal mast cell protease-1 (mMCP-1) concentrations. The mice were sectioned at D70 to collect blood and organs.

Table 1. Experimental groups and treatments.

group	n	sens	treatment	challenge	diet
sham	5	PBS + CT	PBS	whey	control
sens	9	whey + CT	PBS	whey	control
FOS	8	whey + CT	PBS	whey	FOS
butyrate	8	whey + CT	PBS + butyrate	whey	control
OIT	8	whey + CT	10 mg whey	whey	control
OIT+FOS	8	whey + CT	10 mg whey	whey	FOS
OIT+butyrate	8	whey + CT	10 mg whey + butyrate	whey	control

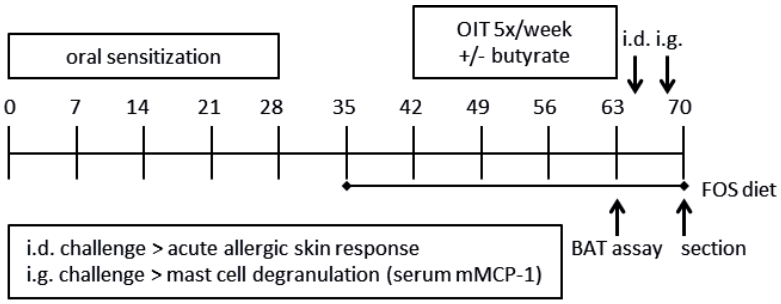


Figure 1. Experimental timeline. Female C3H/HeOuJ mice were grouped as depicted in **Table 1**: sham, sham-sensitized control; sens, whey-sensitized control; FOS, FOS supplemented group; butyrate, butyrate supplemented group; OIT, OIT group; OIT+FOS, OIT with FOS supplementation group; OIT+butyrate, OIT with butyrate supplementation group. All mice were fed the AIN-93G control diet upon arrival and during i.g. sensitization with whey (20 mg in 0.5 ml PBS) and cholera toxin (15 µg in 0.5 ml PBS) on day 0, 7, 14, 21 and 28. The FOS supplemented diet was provided from D35 to the end of the protocol in the FOS and OIT+FOS groups. OIT with 10 mg whey in 0.5 ml PBS was given 5x/week for 3 weeks (D42-59). Sodium-butyrate was co-administered during OIT (0.6 g/kg bodyweight/day) based on mean bodyweight per group. At D63, whole blood samples were collected via cheek puncture to perform a BAT and an i.d. challenge (D64, 10 µg whey in 20 µl PBS/ear) and i.g. challenge (D69, 50 mg whey in 0.5 ml PBS) were conducted to measure the acute allergic skin response and mucosal mast cell degranulation (mMCP-1), respectively. At D70, all mice were sectioned and blood and organs were collected. OIT, oral immunotherapy; FOS, fructo-oligosaccharides; CT, cholera toxin; i.d., intradermal; i.g., intragastric; BAT, basophil activation test; mMCP-1, mucosal mast cell protease-1.

Basophil activation test

Whole blood samples from all control and experimental groups were collected via cheek puncture at D63 to conduct a basophil activation test (BAT) according to the method described by Torrero et al [17]. Briefly, whole blood was incubated (1.5 h at 37°C) with RPMI-1640 medium (Lonza), αIgE (125 ng/ml, eBioscience, Breda, the Netherlands) or whey (20 µg/ml, DMV International) to activate basophils. After red blood cell lysis (Whole Blood Lysing Reagents, Beckman Coulter, Fullerton, CA, USA), cells were stained with anti-IgE-FITC, anti-CD49b-APC, anti-CD4-PE and anti-B220-PE (eBioscience) to select the basophil population while excluding T and B cells. Median fluorescence intensity (MFI) of activation marker CD200R-PerCp-eFluor 710 was determined with flow cytometry using a FACS Canto II (BD Biosciences, Alphen a/d Rijn, the Netherlands).

ELISA

Serum samples collected prior to sectioning were stored at -20°C until analysis of whey-specific antibodies (IgE, IgA, IgG1 and IgG2a), mMCP-1 and galectin-9. Culture

supernatants from bone marrow-derived mast cell (BMMC) assays were collected and stored at -20°C to measure IL-6 and IL-13 concentrations. All procedures were conducted as described elsewhere [12].

SCFA analysis in cecum supernatant

To determine SCFA concentrations, cecum content was collected and stored at -80°C until further processing according to the method described previously [18]. Cecum supernatant was analyzed using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan).

Tregs and BMMC suppression assays

BMMC were cultured from naïve female C3H/HeOuJ mice in RPMI-1640 medium (Lonza) supplemented with 10% FCS, 26 mM Hepes, 0.12 mM MEM non-essential amino acids, 2.4 mM Glutamax, 1.2 mM sodium pyruvate (all from Gibco Thermo Fisher Scientific, Waltham, MA, USA), penicillin-streptomycin (100 U/ml-100 µg/ml, Sigma-Aldrich) and IL-3 and stem cell factor (SCF, both 10 ng/ml, Prospec, Ness-Ziona, Israel) at 37°C with 5% CO_2.

CD4+CD25+ Tregs were purified from pooled whole spleen suspensions derived from all control and experimental groups of mice in a follow-up experiment according to the manufacturer's instructions (Miltenyi Biotec, Leiden, the Netherlands) and were co-cultured in a 1:1 ratio with BMMC sensitized with anti-DNP-IgE (dinitrophenol) according to the method described elsewhere [19]. Subsequently, BMMC were activated with 25 ng/ml DNP-HSA (DNP hapten conjugated to human serum albumin) and release of β-hexosaminidase (β-hex) was measured. Activated BMMC were incubated in fresh medium for 24 h to collect culture supernatant for cytokine analysis.

In vitro Treg induction from naïve CD4+CD25- splenocytes was conducted according to the method described previously [20]. 125 µM sodium butyrate (Sigma-Aldrich) and 0.05% scFOS/lcFOS (9:1, Orafti) were added to the culture medium. After 6 days of incubation at 37°C with 5% CO_2 in the presence of IL-2 and TGFβ, cells were harvested and co-cultured with BMMC sensitized with anti-DNP-IgE as described earlier. Beta-hex release was measured upon BMMC activation using DNP-HSA. An aliquot of cells was stained for anti-CD4-FITC, anti-CD25-PE, anti-Foxp3-APC and anti-OX40-PerCp-eFluor 710 (eBioscience) and analysed using flow cytometry.

Data analysis and statistics

Data were presented as mean ± SEM and were analyzed using GraphPad Prism software version 7 (GraphPad software, La Jolla, CA, USA). We used one-way ANOVA and Bonferroni's post-hoc test to compare sham with sens; sens with FOS, butyrate, OIT, OIT+FOS and OIT+butyrate; FOS with OIT+FOS; butyrate with OIT+butyrate and OIT with OIT+FOS and OIT+butyrate. Whey-specific antibody data were log-transformed prior to testing and the median is depicted per group. Beta-hex data (mean ± SD) in **Figure 6** were analyzed using a two-way ANOVA for non-repeated measures. Calculated p-values were corrected for the number of comparisons and were considered statistically significant when $p<0.05$.

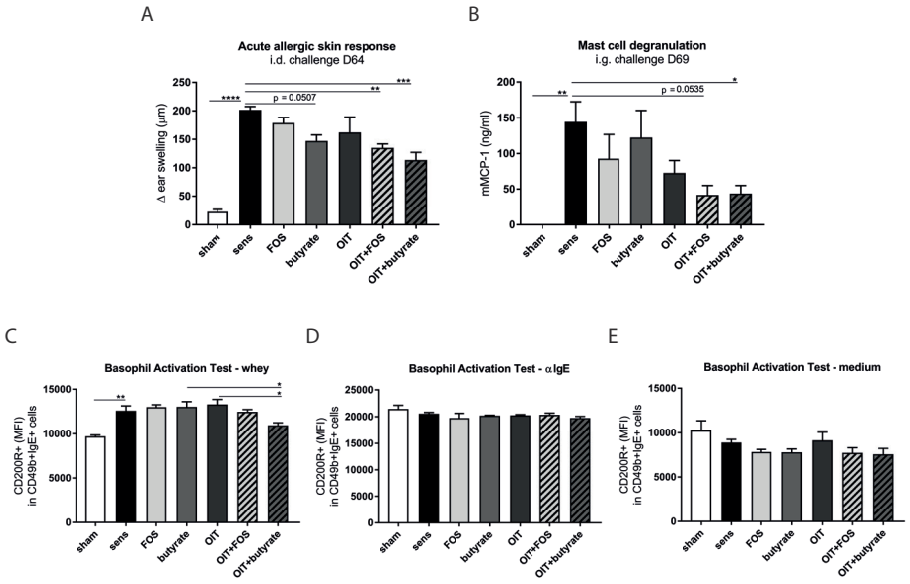


Figure 2. Reduced activation of allergic effector cells upon challenge after OIT and butyrate or FOS supplementation. (A) Reduced acute allergic skin response (Δ ear swelling 1 h after i.d. injection with whey) in OIT+FOS and OIT+butyrate mice compared to sensitized controls. **(B)** Reduced mucosal mast cell degranulation (serum mMCP-1 concentration) in OIT+butyrate mice compared to sensitized controls. Decreased MFI of CD200R in basophils activated with **(C)** whey in OIT+butyrate blood samples compared to OIT and butyrate samples. No differences in MFI observed after basophil stimulation with **(D)** αIgE and **(E)** medium. Data are represented as mean ± SEM, n=5-9/group in **(A,B)** and n=3-5/group in **(C,D)** (whole blood samples were pooled per 2 mice). Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. i.d., intradermal; i.g., intragastric; mMCP-1, mucosal mast cell protease-1; MFI, median fluorescence intensity.

RESULTS

Butyrate supplementation supported OIT efficacy shown by reduced effector cell activation upon challenge

The sensitized control group showed increased acute allergic skin responses (i.e., magnitude of the ear swelling response) and increased mucosal mast cell degranulation (i.e., serum mMCP-1) after challenge compared to the sham-sensitized control group (**Figures 2A,B**). OIT+butyrate reduced the acute allergic skin response and mucosal mast cell degranulation compared to sensitized controls (**Figures 2A,B**). A trend (p=0.0507) toward a reduction in ear swelling was observed in the animals which were only exposed to oral butyrate supplementation (**Figure 2A**). In addition, basophils derived from whole blood of OIT+butyrate mice showed reduced expression of activation-associated receptor CD200R upon antigen-specific stimulation compared to OIT- and butyrate-derived basophils (**Figure 2C**). No differences between the groups were observed after stimulation with αIgE and medium, indicating modulation of the antigen-specific basophil response (**Figures 2D,E**). In accordance with previous results [12], OIT+FOS effectively reduced the acute allergic skin response (**Figure 2A**) and a trend (p=0.0535) toward a reduction in mucosal mast cell degranulation was observed compared to sensitized controls (**Figure 2B**). Despite the observed reduction in the acute allergic skin responses in OIT+butyrate and OIT+FOS mice, no protection against symptoms of anaphylaxis, e.g., body temperature drop, induced by the i.d. challenge was observed (data not shown).

OIT influenced whey-specific IgE levels in serum

Levels of whey-specific IgE, IgA, IgG1 and IgG2a in serum collected at D70 were increased in the sensitized controls compared to the sham-sensitized controls (**Figures 3A-D**). Except the whey-specific IgE levels which were lowered in the OIT+butyrate group compared to butyrate (**Figure 3A**), no significant differences in whey-specific antibody levels were found between the control and experimental groups. However, in accordance with previous results [21], the level of whey-specific IgE was lower in OIT-exposed animals. No additional effect of the dietary intervention with either FOS or butyrate was observed in the current study with respect to whey-specific IgE (**Figure 3A**). In contrast, whey-specific IgA, IgG1 and IgG2a levels were higher in the combination group OIT+FOS (**Figures 3B-D**).

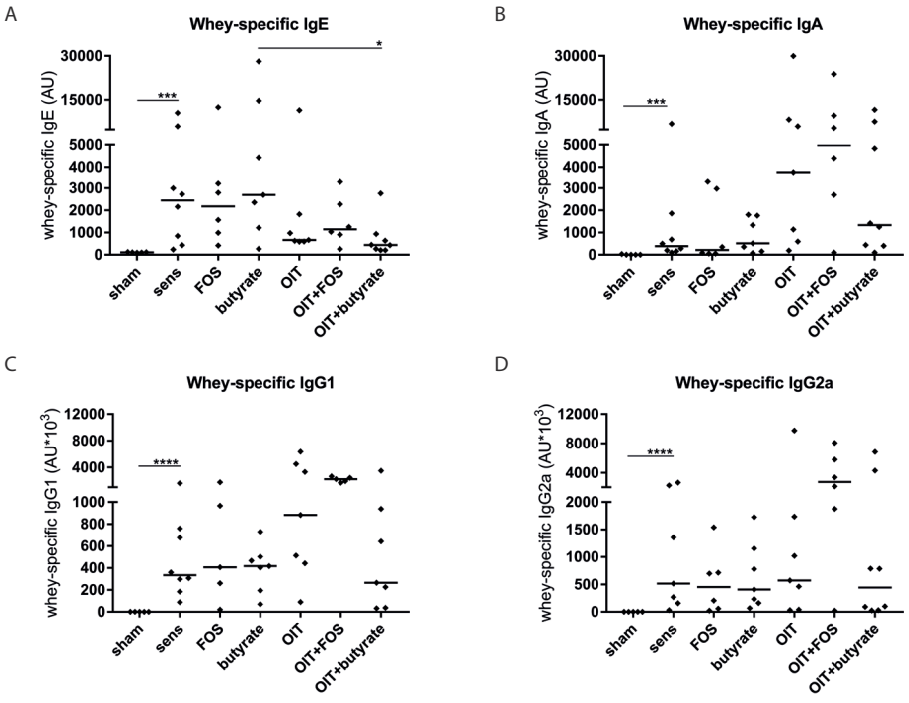


Figure 3. OIT influenced whey-specific IgE responses in mice. Whey-specific antibodies were measured by means of ELISA in serum collected at D70. **(A)** whey-specific IgE, **(B)** whey-specific IgA, **(C)** whey-specific IgG1 and **(D)** whey-specific IgG2a levels were increased in whey-sensitized control mice compared to sham-sensitized controls. OIT decreased whey-specific IgE independent of the dietary interventions. OIT+FOS had the most pronounced effect on whey-specific IgA, IgG1 and IgG2a. Data are depicted as individual data points with the median per group, n=5-8/group. Statistical analysis was performed by log-transforming the data followed by a one-way ANOVA with Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, ***p<0.001, ****p<0.0001. AU, arbitrary units.

OIT+FOS increased local butyric acid levels

OIT+FOS increased levels of butyric acid in cecum content compared to the sensitized control and the OIT groups which were fed the control diet (**Figure 4A**). As expected, no increase in butyric acid in the cecum content was observed in butyrate and OIT+butyrate mice, suggesting systemic uptake of orally administered butyrate. Oral sensitization against whey increased propionic acid and acetic acid concentrations as observed in cecum content of the whey-sensitized controls compared to the sham-sensitized controls (**Figures 4B,C**). Total SCFA levels in cecum content of sensitized control mice were increased accordingly (**Figure 4D**). In the current study, no significant differences in serum galectin-9 concentrations were observed between the groups (**Figure 4E**).

Tregs derived from OIT+butyrate mice showed enhanced suppression of BMMC responses

A schematic representation of the *ex vivo* Treg-BMMC suppression assay is shown in **Figure 5A**. Spleen-derived CD4+CD25+ cells were isolated and verified for Foxp3 expression (**Figures 5B-D**, approximately 70% positivity) and co-cultured with anti-DNP-IgE-sensitized naïve BMMC. OIT+butyrate and OIT+FOS-derived Tregs reduced β-hex release upon BMMC activation with DNP-HSA compared to the control condition without Tregs. Butyrate- and OIT-derived Tregs also reduced BMMC degranulation; however, a reduction in IL-13 release by BMMC was only observed in OIT+butyrate compared to OIT (**Figures 5E,F**). No difference in release of IL-6 by activated BMMC was observed in the Treg-BMMC co-cultures (**Figure 5G**).

FOS exposure, but not butyrate exposure, during *in vitro* Treg induction enhanced BMMC suppression

In vitro stimulation of naïve spleen-derived CD4+CD25- cells with TGFβ and IL-2 led to differentiation into Foxp3+ Tregs with functional suppressive capacities shown by reduced BMMC activation (**Figures 6A,B**). The percentage of Foxp3+ cells after 6 days of stimulation was similar in all conditions (**Figure 6C**). Although exposure to butyrate during Treg induction could not further improve the suppressive action of the Tregs toward BMMC (**Figure 6A**), direct exposure to FOS did enhance Treg-mediated BMMC suppression (**Figure 6B**). In particular, BMMC activation with 50 ng/ml antigen was significantly reduced in the presence of FOS exposed Tregs compared to control Tregs (**Figure 6B**). We hypothesized that the OX40-OX40L interaction is involved in the Treg-mediated suppression of BMMC; however, FOS exposure did not increase expression of OX40 by CD4+CD25+Foxp3+ cells compared to the control condition (**Figure 6D**).

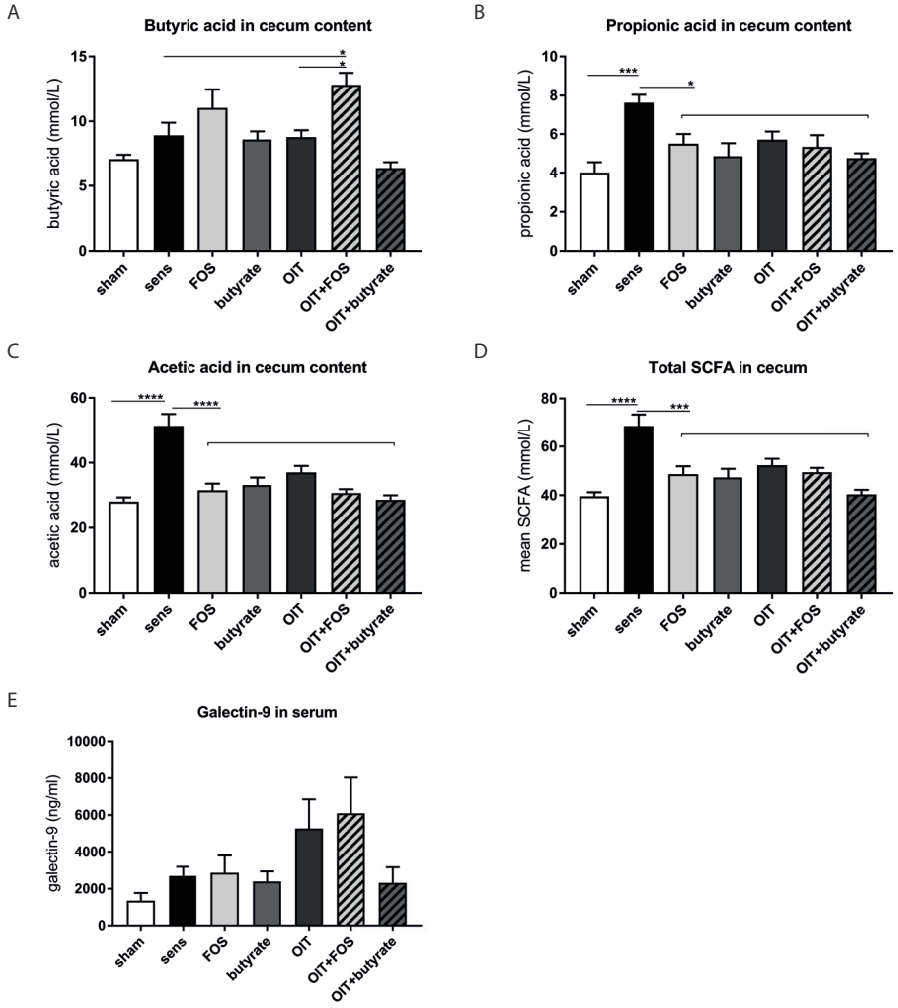


Figure 4. Increased butyric acid concentrations in cecum content of OIT+FOS mice. SCFA analysis in cecum content (upon local fermentation) indicated that **(A)** butyric acid levels were increased in OIT+FOS mice compared to OIT mice and sensitized controls. **(B)** Propionic acid and **(C)** acetic acid levels in cecum content were increased in sensitized controls compared to sham-sensitized controls. **(D)** Mean total SCFA levels were increased in the sensitized controls correspondingly. **(E)** Serum galectin-9 concentrations measured by means of ELISA. Data are represented as mean ± SEM, n=5-8/group. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, ***p<0.001, ****p<0.0001. SCFA, short-chain fatty acids.

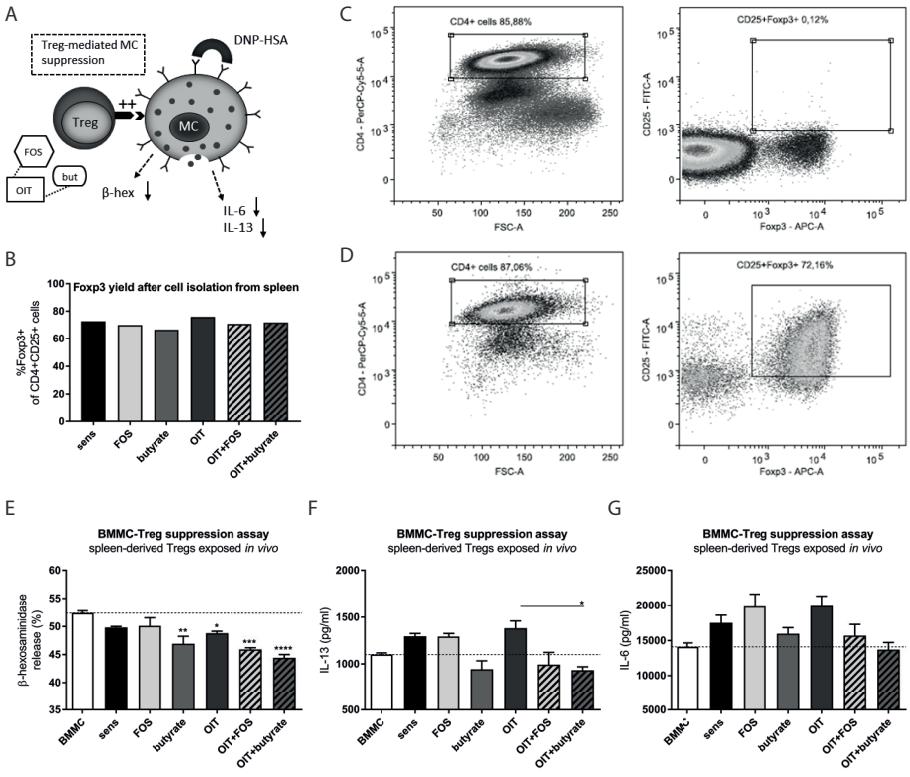


Figure 5. Enhanced Treg-mediated BMMC suppression after exposure to OIT and butyrate or FOS supplementation *in vivo*. CD4+ cells derived from pooled spleen suspensions were separated based on CD25 positivity. **(A)** Schematic representation of BMMC-Treg suppression assay. **(B)** CD4+CD25+ cells were verified for Foxp3 expression with flow cytometry and showed ± 70% positivity in all groups. **(C)** CD4+CD25- cells and **(D)** CD4+CD25+ cells after MACS separation. Subsequently, CD4+CD25+ cells were co-cultured with BMMC sensitized with anti-DNP-IgE. **(E)** Reduced release of β-hex upon BMMC activation with 25 ng/ml DNP-HSA was observed in the presence of Tregs derived from butyrate, OIT, OIT+FOS and OIT+butyrate mice compared to the control condition without Tregs. Additional 24 h incubation of BMMC in fresh culture medium indicated **(F)** reduced production of IL-13 after co-culture with OIT+butyrate-Tregs compared to OIT-Tregs and **(G)** no differences in IL-6 release by activated BMMC. Data are represented as mean ± SD in **(E-G)**, duplicate measurements. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. FSC-A, forward scatter-area; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; but, butyrate; Treg, regulatory T cell; MC, mast cell; BMMC, bone marrow-derived mast cell; β-hex, β-hexosaminidase; DNP-HSA, dinitrophenol hapten conjugated to human serum albumin.

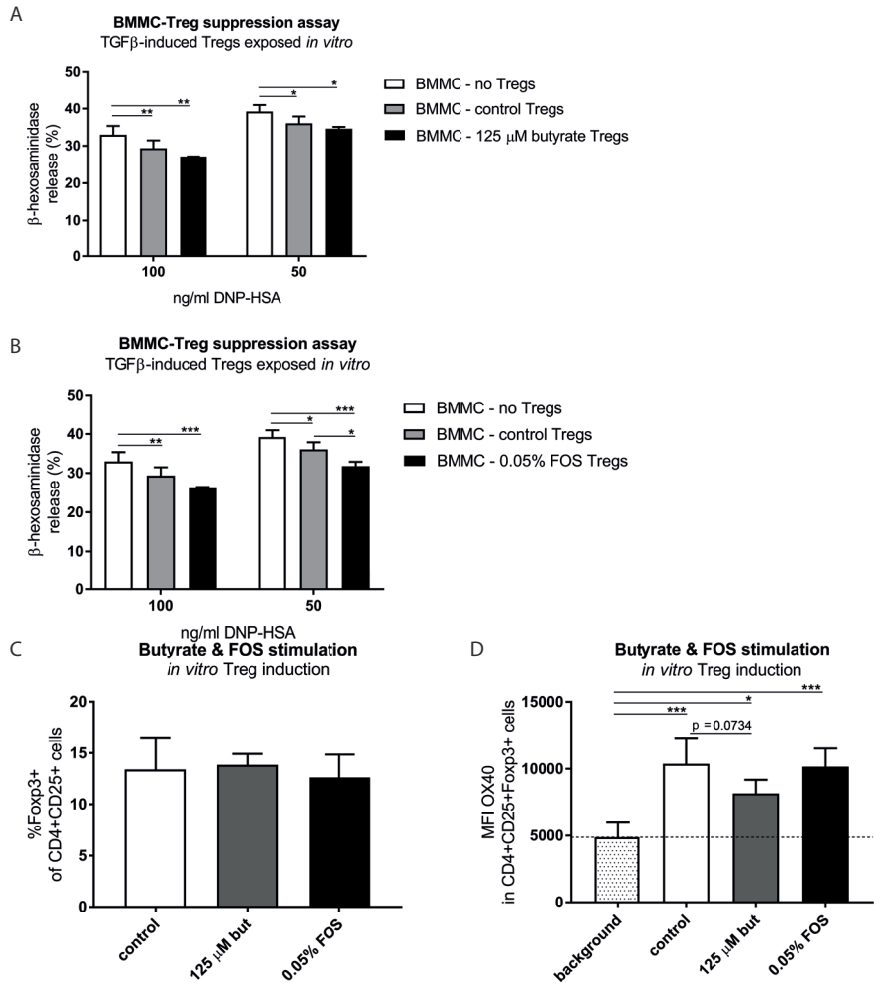


Figure 6. Enhanced Treg-mediated BMMC suppression after *in vitro* exposure to FOS, but not butyrate. TGFβ and IL-2-mediated induction of Tregs from naïve CD4+CD25- splenocytes resulted in functional cells, shown by reduced BMMC activation. **(A)** Exposure to butyrate (125 μM) did not enhance Treg-mediated suppression of BMMC. **(B)** Exposure to FOS (0.05%) significantly improved Treg-mediated BMMC suppression upon activation with 50 ng/ml DNP-HSA compared to control Tregs. No differences in Foxp3 yield **(C)** and OX40 expression **(D)** were observed in the control, butyrate or FOS condition. Data are depicted as mean ± SD, **(A,B)** representative experiment, duplicate measurements per concentration DNP-HSA. **(C,D)** mean of 3 independent experiments, duplicate measurements. Statistical analysis was performed using two-way ANOVA for non-repeated measures in **(A,B)** and one-way ANOVA in **(C,D)** with Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, **p<0.01, ***p<0.001. BMMC, bone marrow-derived mast cells; Treg, regulatory T cells; FOS, fructo-oligosaccharides; DNP-HSA, dinitrophenol hapten conjugated to human serum albumin; but, butyrate; MFI, median fluorescence intensity.

DISCUSSION

The presented data indicate that butyrate supplementation is as effective as FOS supplementation in supporting OIT-induced desensitization in a murine cow's milk allergy model. Both therapeutic strategies reduced responsiveness of effector cells to an antigen-specific challenge and enhanced the suppressive capacity of Tregs toward cultured mast cells. Interestingly, differences in local butyric acid levels suggest specific alteration of the microbiome in the gastrointestinal tract.

In the current study, mice receiving OIT without a dietary intervention showed no reduction in acute allergic symptoms upon antigen-specific challenge. It has been described that desensitization of effector cells like mast cells and basophils is one of the earliest events observed in antigen-specific immunotherapy [22]. Despite the presence of (high levels of) specific IgE, mast cells and basophils show reduced degranulation capacity followed by suppressed systemic anaphylaxis symptoms [22]. Cross-linking of surface-bound IgE by intact allergens simultaneously up-regulates expression of the inhibitory histamine receptor 2 on basophils, thereby providing a control system for cell activation [23]. The combination therapies OIT+butyrate and OIT+FOS effectively induced desensitization of mast cells as observed at two distinct sites in the mice: connective tissue-mast cells residing in the skin of the ear (upon i.d. challenge) and mucosal-mast cells residing in the gastrointestinal tract (upon i.g. challenge). Systemically available butyrate might have affected mast cell functionality at both sites, since it has been shown *in vitro* that butyrate reduced proliferation and cytokine production by mast cells via HDAC inhibition [24].

Firstly, as observed in the current study, oral butyrate supplementation alone did not lead to a significant reduction in mast cell degranulation provoked by food challenge compared to the sensitized controls. Moreover, *ex vivo* whey-specific basophil activation in whole blood samples was only reduced after providing OIT and butyrate supplementation simultaneously. Thereby, it was demonstrated that both butyrate and whey are key components in the desensitization process of basophils in cow's milk allergic mice. Secondly, a direct effect of FOS supplementation on *in vivo* mast cell degranulation was not observed; however, it has been shown *in vitro* that specific human milk oligosaccharides (HMOS) are able to directly inhibit IgE-mediated mast cell activation, but only at a high concentration of 1 mg/ml [19]. Previous studies showed epithelial transfer of non-digestible oligosaccharides *in vitro* [25] and confirmed systemic availability of HMOS in breast-fed infants [26]. It needs to be elucidated whether physiologically relevant FOS concentrations reach mast cells residing in the mucosal or connective tissues, and whether FOS directly contribute to the observed reduced degranulation response.

The involvement of CD4+CD25+Foxp3+ Tregs in controlling the allergic response has been described earlier [27]. Here, we showed enhanced suppression of BMMC responses upon FcεRI-mediated activation by Tregs derived from OIT+butyrate and OIT+FOS treated mice. Enhanced Treg functionality was also observed in an adoptive transfer experiment using OIT+FOS donor mice: *ex vivo* Treg-depleted cell fractions could not control mucosal mast cell degranulation upon allergen challenge in recipients [12]. Data derived from *in vitro* experiments indicate that FOS exposure, but not butyrate exposure, directly affects *in vitro* Treg functionality without increasing expression of the co-stimulatory OX40 molecule. Direct cell-cell contact between Tregs and mast cells via the OX40-OX40 ligand interaction was previously shown to directly suppress mast cell degranulation and the following hypersensitivity response via increased intracellular cyclic adenosine monophosphate (cAMP) concentrations and reduced Ca^{2+} influx [28, 29].

As described previously, oral butyrate supplementation exerts anti-inflammatory effects in pre-clinical models of colitis and liver disease [30, 31]. In addition, butyrate enemas were shown to ameliorate symptoms in ulcerative colitis patients [32]. In the context of food allergy prevention, protection against anaphylaxis and a reduction in total IgE were observed in peanut allergic mice after oral supplementation with butyrate or acetate during sensitization, mimicking the beneficial effects mediated by high fiber intake [33]. In the current experiment, butyrate was either directly available per oral gavage or indirectly available after FOS fermentation. However, administration of FOS or butyrate might lead to different alterations in microbial communities present in the gastrointestinal tract. A reflection of microbial activity and/or abundance can be observed in SCFA levels in cecum content: OIT+FOS mice showed increased butyric acid levels whereas OIT+butyrate mice did not. FOS supplementation in combination with *Bifidobacterium breve* M16V (synbiotics) in non-IgE mediated cow's milk allergic infants that were fed an amino acid-based infant formula, improved microbiota composition shown by *Bifidobacteria* levels approximating healthy breast-fed infants [34]. To date, the direct effect of (high) butyrate exposure on composition and activity of the microbiome is poorly defined. Butyrate treatment in mice suffering from enteritis affected the abundance of specific bacterial species in addition to reduced intestinal inflammation [35]. However, clinical trials on the application of SCFA to treat colonic inflammatory disorders reported varying success rates and do not include microbiome data [36].

In addition to the microbiota effects, direct interaction of non-digestible oligosaccharides via glycan receptors with IEC and immune cells resident in the lamina propria of the gut contributes to orchestration of the mucosal immune response [37]. *In vitro* stimulation of IEC with non-digestible oligosaccharides in combination with bacterial DNA or a TLR-9 ligand, leads to release of galectin-9. IEC-derived galectin-9 induced IFNγ secretion by activated PBMC and stimulated proliferation of Th1 cells and Tregs [38]. Galectin-9 present in serum derived from whey-sensitized mice supplemented with non-digestible oligosaccharides and *Bifidobacterium breve M16V* suppressed *in vitro* degranulation of RBL-2H3 cells [39]. However, the contribution of galectin-9 in effector cell suppression remains elusive in this experimental model, since no differences in serum galectin-9 levels were observed in the current study.

The used butyrate dose was previously shown to be safe in mice [30] and no signs of (mild) toxicity were detected. However, butyrate administration should be performed with caution, since previous studies reported cases of hypokalemia, nausea and seizure after intravenous injection [40, 41]. FOS supplementation has been considered to be safe and is used as a supplement in infant formula for cow's milk allergic infants [42].

CONCLUSION

Butyrate supplementation enhanced desensitization of effector cells induced by OIT in cow's milk allergic mice. Improvement of OIT efficacy was previously only described for FOS supplementation. We showed effective reduction of mast cell and basophil activation and alteration of the suppressive activity of Tregs. More insight into the interaction of butyrate and FOS with the intestinal epithelium, the immune system and the microbiota is needed; however, both supplements are promising candidates to improve OIT efficacy in human food allergy trials.

REFERENCES

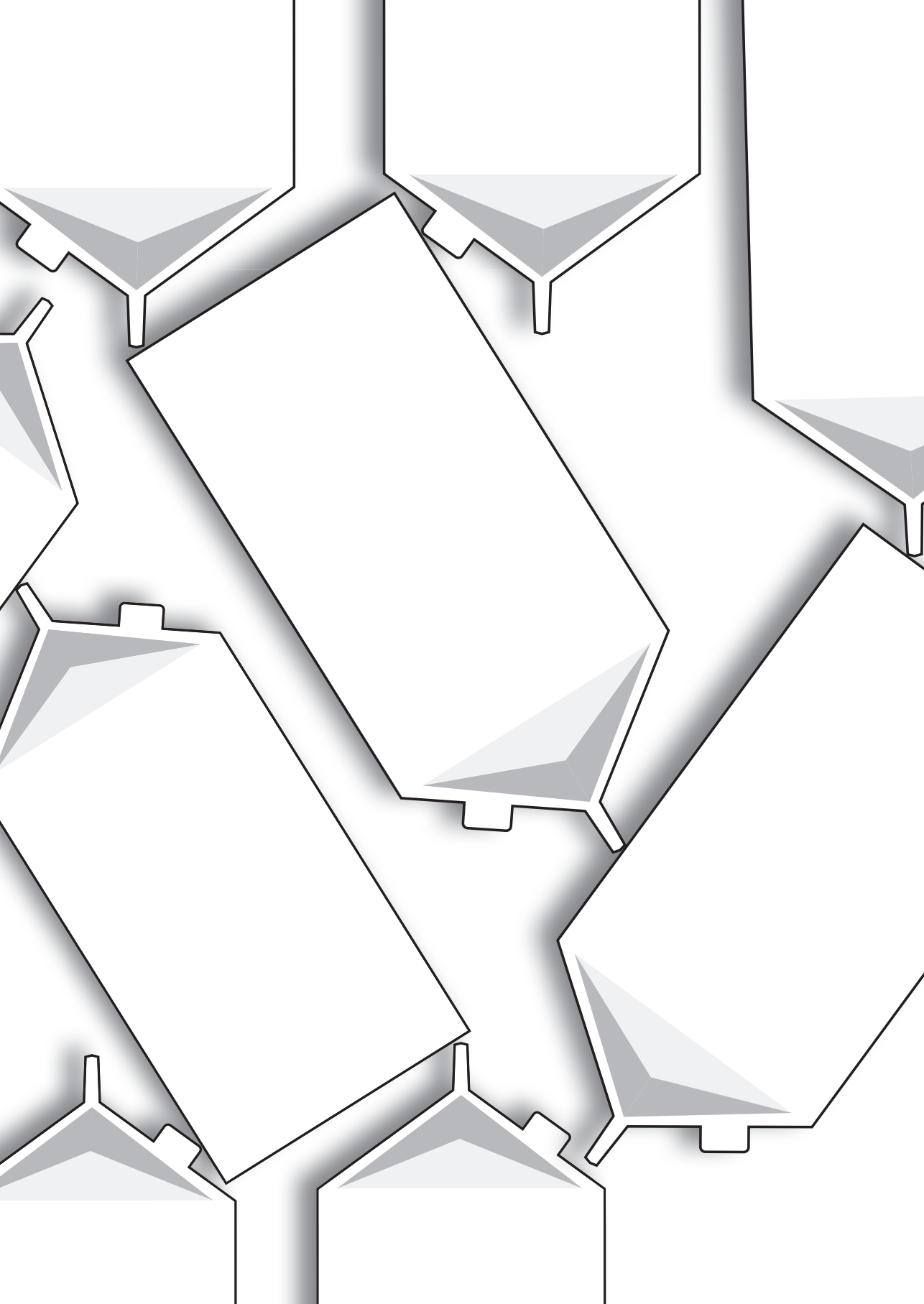
1. Osborne NJ, Koplin JJ, Martin PE, Gurrin LC, Lowe AJ, Matheson MC, Ponsonby AL, Wake M, Tang ML, Dharmage SC, Allen KJ, and HealthNuts I. Prevalence of challenge-proven ige-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. J Allergy Clin Immunol 2011. 127(3): p. 668-76 e1-2.
2. Beck C, Koplin J, Dharmage S, Wake M, Gurrin L, McWilliam V, Tang M, Sun C, Foskey R, Allen KJ, and HealthNuts I. Persistent food allergy and food allergy coexistent with eczema is associated with reduced growth in the first 4 years of life. J Allergy Clin Immunol Pract 2016. 4(2): p. 248-56 e3.
3. Vermeulen EM, Koplin JJ, Dharmage SC, Gurrin LC, Peters RL, McWilliam V, Ponsonby AL, Dwyer T, Lowe AJ, Tang MLK, Allen KJ, and HealthNuts i. Food allergy is an important risk factor for childhood asthma, irrespective of whether it resolves. J Allergy Clin Immunol Pract 2017: p. S2213-2198(17)30857-7.
4. Sicherer SH and Sampson HA. Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention and management. J Allergy Clin Immunol 2017: p. 141(1):41-58.
5. Vazquez-Ortiz M and Turner PJ. Improving the safety of oral immunotherapy for food allergy. Pediatr Allergy Immunol 2016. 27(2): p. 117-25.
6. Nurmatov U, Dhami S, Arasi S, Pajno GB, Fernandez-Rivas M, Muraro A, Roberts G, Akdis C, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, du Toit G, Ebisawa M, Eigenmann P, Knol E, Makela M, Nadeau KC, O'Mahony L, Papadopoulos N, Poulsen LK, Sackesen C, Sampson H, Santos AF, van Ree R, Timmermans F, and Sheikh A. Allergen immunotherapy for ige-mediated food allergy: A systematic review and meta-analysis. Allergy 2017. 72(8): p. 1133-1147.
7. Chiang D and Berin MC. An examination of clinical and immunologic outcomes in food allergen immunotherapy by route of administration. Curr Allergy Asthma Rep 2015. 15(6): p. 35.
8. Freeland DMH, Manohar M, Andorf S, Hobson BD, Zhang W, and Nadeau KC. Oral immunotherapy for food allergy. Semin Immunol 2017. 30: p. 36-44.
9. McKenzie C, Tan J, Macia L, and Mackay CR. The nutrition-gut microbiome-physiology axis and allergic diseases. Immunol Rev 2017. 278(1): p. 277-295.
10. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, and Marsland BJ. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 2014. 20(2): p. 159-66.
11. Tang ML, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, and Donath S. Administration of a probiotic with peanut oral

immunotherapy: A randomized trial. J Allergy Clin Immunol 2015. 135(3): p. 737-44 e8.
12. Vonk MM, Diks MAP, Wagenaar L, Smit JJ, Pieters RHH, Garssen J, van Esch B, and Knippels LMJ. Improved efficacy of oral immunotherapy using non-digestible oligosaccharides in a murine cow's milk allergy model: A potential role for foxp3+ regulatory t cells. Front Immunol 2017. 8: p. 1230.
13. Niki T, Tsutsui S, Hirose S, Aradono S, Sugimoto Y, Takeshita K, Nishi N, and Hirashima M. Galectin-9 is a high affinity ige-binding lectin with anti-allergic effect by blocking ige-antigen complex formation. Journal of Biological Chemistry 2009. 284(47): p. 32344-32352.
14. Koh A, De Vadder F, Kovatcheva-Datchary P, and Backhed F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell 2016. 165(6): p. 1332-1345.
15. Sun M, Wu W, Liu Z, and Cong Y. Microbiota metabolite short chain fatty acids, gpcr, and inflammatory bowel diseases. J Gastroenterol 2017. 52(1): p. 1-8.
16. Richards LB, Li M, van Esch BCAM, Garssen J, and Folkerts G. The effects of short-chain fatty acids on the cardiovascular system. PharmaNutrition 2016. 4.
17. Torrero MN, Larson D, Hubner MP, and Mitre E. Cd200r surface expression as a marker of murine basophil activation. Clin Exp Allergy 2009. 39(3): p. 361-9.
18. de Theije CG, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J, Garssen J, Kraneveld AD, and Oozeer R. Altered gut microbiota and activity in a murine model of autism spectrum disorders. Brain Behav Immun 2014. 37: p. 197-206.
19. Castillo-Courtade L, Han S, Lee S, Mian FM, Buck R, and Forsythe P. Attenuation of food allergy symptoms following treatment with human milk oligosaccharides in a mouse model. Allergy 2015. 70(9): p. 1091-102.
20. Fantini MC, Dominitzki S, Rizzo A, Neurath MF, and Becker C. In vitro generation of cd4+ cd25+ regulatory cells from murine naive t cells. Nat Protoc 2007. 2(7): p. 1789-94.
21. Vonk MM, Wagenaar L, Pieters RHH, Knippels LMJ, Willemsen LEM, Smit JJ, van Esch B, and Garssen J. The efficacy of oral and subcutaneous antigen-specific immunotherapy in murine cow's milk- and peanut allergy models. Clin Transl Allergy 2017. 7: p. 35.
22. van de Veen W, Wirz OF, Globinska A, and Akdis M. Novel mechanisms in immune tolerance to allergens during natural allergen exposure and allergen-specific immunotherapy. Curr Opin Immunol 2017. 48: p. 74-81.
23. Novak N, Mete N, Bussmann C, Maintz L, Bieber T, Akdis M, Zumkehr J, Jutel M, and Akdis C. Early suppression of basophil activation during allergen-specific immunotherapy by histamine receptor 2. J Allergy Clin Immunol 2012. 130(5): p. 1153-1158 e2.
24. Zhang H, Du M, Yang Q, and Zhu

MJ. Butyrate suppresses murine mast cell proliferation and cytokine production through inhibiting histone deacetylase. J Nutr Biochem 2016. 27: p. 299-306.

25. Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, Urbanek R, and Szepfalusi Z. Prebiotic oligosaccharides: In vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatr Allergy Immunol 2010. 21(8): p. 1179-88.
26. Goehring KC, Kennedy AD, Prieto PA, and Buck RH. Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS One 2014. 9(7): p. e101692.
27. Syed A, Garcia MA, Lyu SC, Bucayu R, Kohli A, Ishida S, Berglund JP, Tsai M, Maecker H, O'Riordan G, Galli SJ, and Nadeau KC. Peanut oral immunotherapy results in increased antigen-induced regulatory t-cell function and hypomethylation of forkhead box protein 3 (foxp3). J Allergy Clin Immunol 2014. 133(2): p. 500-10.
28. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, Viola A, Odom S, Rivera J, Colombo MP, and Pucillo CE. Cd4+cd25+ regulatory t cells suppress mast cell degranulation and allergic responses through ox40-ox40l interaction. Immunity 2008. 29(5): p. 771-81.
29. Takeda I, Ine S, Killeen N, Ndhlovu LC, Murata K, Satomi S, Sugamura K, and Ishii N. Distinct roles for the ox40-ox40 ligand interaction in regulatory and nonregulatory t cells. J Immunol 2004. 172(6): p. 3580-9.
30. Jin CJ, Sellmann C, Engstler AJ, Ziegenhardt D, and Bergheim I. Supplementation of sodium butyrate protects mice from the development of non-alcoholic steatohepatitis (nash). Br J Nutr 2015. 114(11): p. 1745-55.
31. Lee C, Kim BG, Kim JH, Chun J, Im JP, and Kim JS. Sodium butyrate inhibits the nf-kappa b signaling pathway and histone deacetylation, and attenuates experimental colitis in an il-10 independent manner. Int Immunopharmacol 2017. 51: p. 47-56.
32. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, Richter F, Dusel G, and Kasper H. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterology 1992. 103(1): p. 51-6.
33. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, and Mackay CR. Dietary fiber and bacterial scfa enhance oral tolerance and protect against food allergy through diverse cellular pathways. Cell Rep 2016. 15(12): p. 2809-24.
34. Candy DCA, Van Ampting MTJ, Nijhuis MMO, Wopereis H, Butt AM, Peroni DG, Vandenplas Y, Fox AT, Shah N, West CE, Garssen J, Harthoorn LF, Knol J, and Michaelis LJ. A synbiotic-containing amino acid-based formula improves gut microbiota in non-ige-mediated

allergic infants. Pediatr Res 2017: p. 26(4):316-22.
35. Jiminez JA, Uwiera TC, Abbott DW, Uwiera RRE, and Inglis GD. Butyrate supplementation at high concentrations alters enteric bacterial communities and reduces intestinal inflammation in mice infected with citrobacter rodentium. mSphere 2017. 2(4): p. e00243-17.
36. van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, and Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev 2017. 75(4): p. 286-305.
37. de Kivit S, Kraneveld AD, Garssen J, and Willemsen LE. Glycan recognition at the interface of the intestinal immune system: Target for immune modulation via dietary components. Eur J Pharmacol 2011. 668 Suppl 1: p. S124-32.
38. de Kivit S, Kraneveld AD, Knippels LM, van Kooyk Y, Garssen J, and Willemsen LE. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. J Innate Immun 2013. 5(6): p. 625-38.
39. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BC, Knol J, Sprikkelman AB, van der Aa LB, Knippels LM, Garssen J, van Kooyk Y, and Willemsen LE. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012. 67(3): p. 343-52.
40. Blau CA, Constantoulakis P, Shaw CM, and Stamatoyannopoulos G. Fetal hemoglobin induction with butyric acid: Efficacy and toxicity. Blood 1993. 81(2): p. 529-37.
41. Sher GD, Ginder GD, Little J, Yang S, Dover GJ, and Olivieri NF. Extended therapy with intravenous arginine butyrate in patients with beta-hemoglobinopathies. N Engl J Med 1995. 332(24): p. 1606-10.
42. Burks AW, Harthoorn LF, Van Ampting MT, Oude Nijhuis MM, Langford JE, Wopereis H, Goldberg SB, Ong PY, Essink BJ, Scott RB, and Harvey BM. Synbiotics-supplemented amino acid-based formula supports adequate growth in cow's milk allergic infants. Pediatr Allergy Immunol 2015. 26(4): p. 316-22.



CHAPTER 7

ALTERED MICROBIAL COMMUNITY STRUCTURE AND METABOLISM IN COW'S MILK ALLERGIC MICE TREATED WITH ORAL IMMUNOTHERAPY AND FRUCTO-OLIGOSACCHARIDES

Marlotte M. Vonk[1,2]
Phillip A. Engen[3]
Ankur Naqib[4]
Stefan J. Green[4,5]
Ali Keshavarzian[3,6,7,1]
Bart R.J. Blokhuis[1]
Frank A. Redegeld[1]
Johan Garssen[1,2]
Léon M.J. Knippels[1,2]
Betty C.A.M. van Esch[1,2]

1. Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
2. Immunology Platform, Danone Nutricia Research, Utrecht, the Netherlands
3. Department of Internal Medicine, Division of Digestive Diseases and Nutrition, Section of Gastroenterology, Rush University Medical Center, Chicago, IL USA
4. DNA Services Facility, Research Resources Center, University of Illinois at Chicago, Chicago, IL USA
5. Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL USA
6. Department of Pharmacology, Rush University Medical Center, Chicago, IL USA
7. Department of Physiology, Rush University Medical Center, Chicago, IL USA

Submitted for publication

ABSTRACT

Background: Previously, we showed a reduction in clinically-related symptoms and mast cell degranulation in cow's milk allergic mice after oral immunotherapy (OIT) and co-administration with fructo-oligosaccharides (FOS, prebiotics). Prebiotics are fermented by gut bacteria, affecting both bacterial composition and availability of metabolites [i.e., short-chain fatty acids (SCFA)].

Aim: To explore potential changes in the microbiota composition and availability of SCFA induced by OIT+FOS and to study changes in bone marrow-derived mast cell (BMMC) development.

Materials and methods: C3H/HeOuJ mice were sensitized and received OIT with or without a FOS supplemented diet. After three weeks, faecal samples were collected to analyze gut microbiota composition using 16S rRNA sequencing. SCFA concentrations were determined in cecum content. Bone marrow cells from treated mice were differentiated into mast cells and expression of the FcεRI and c-Kit receptor was analyzed. BMMC functionality was assessed using an IgE-mediated degranulation assay.

Results: FOS supplementation in sensitized mice changed the overall microbial community structure in faecal samples compared to sensitized mice fed the control diet ($p=0.03$). In contrast, a high level of resemblance in bacterial community structure was observed between the non-sensitized control mice and the OIT+FOS treated mice. OIT mice showed an increased relative abundance of the dysbiosis-associated phylum Proteobacteria compared to the OIT+FOS mice. FOS supplementation increased the relative abundance of genus *Allobaculum* (Firmicutes), putative butyrate-producing bacteria. OIT+FOS reduced the abundances of the genera's unclassified *Rikenellaceae* (Bacteroidetes, putative pro-inflammatory bacteria) and unclassified *Clostridiales* (Firmicutes) compared to sensitized controls and increased the abundance of *Lactobacillus* (Firmicutes, putative beneficial bacteria) compared to FOS. OIT+FOS mice had increased butyric acid and propionic acid concentrations. BMMC from FOS-exposed mice showed reduced FcεRI and c-Kit expression and degranulation.

Conclusion: OIT+FOS induced a microbial profile closely linked to non-allergic mice and increased concentrations of butyric acid and propionic acid. FOS increased the butyrate-producing *Allobaculum* and affected *in vitro* development of BMMC, suggesting epigenetic changes in mast cell progenitors via bacterial metabolites. Future research should confirm whether there is a causal relationship between microbial modulation and the reduction in allergic symptoms induced by OIT+FOS.

INTRODUCTION

IgE-mediated food allergies embody a serious public health concern affecting up to 5% of children and 8% of adults in Westernized countries [1]. Prevalence rates in a birth cohort study were shown to peak in early childhood with partial natural resolution during late childhood. However, late adolescence was associated with acquirement of novel food sensitizations and was accompanied by an increased prevalence [2]. To date, food allergy management relies on strict allergen avoidance and symptomatic treatment to control adverse clinical events upon accidental exposure. A significant amount of antigen-specific immunotherapy trials to treat IgE-mediated food allergies shows promising results, especially using the oral route of administration, i.e., oral immunotherapy (OIT) [3]. A recent review discussing advances in OIT to treat peanut, hen's egg and cow's milk allergies concluded that overall achievement of clinical desensitization was the most successful in OIT compared to sublingual immunotherapy (SLIT) and epicutaneous immunotherapy (EPIT) and the highest tolerable dose of antigen could be consumed upon OIT [4]. Nevertheless, OIT causes the highest numbers of adverse events during and after treatment compared to SLIT and EPIT and thus needs to be improved [4].

The use of immunomodulatory adjuvants with beneficial effects on gut health or immune regulatory processes might support skewing of the allergic immune response during OIT. Co-administration of a probiotic, *Lactobacillus rhamnosus* CGMCC, during an 18 month OIT trial in peanut allergic subjects resulted in a high percentage of potential sustained unresponsiveness in the treatment group (82.1%) compared to the placebo group (3.6%) during a food challenge conducted 2-5 weeks post-treatment [5]. In previous work, we showed the added value of a diet supplemented with fructo-oligosaccharides (FOS, prebiotics) in supporting OIT efficacy to treat cow's milk allergy (CMA) in mice [6]. OIT+FOS treatment improved desensitization shown by a reduction in clinically-related symptoms and mast cell degranulation [6]. It has been described in literature that prebiotic fibers protect against peanut allergy in mice via bacterial fermentation into short-chain fatty acids (SCFA) which enhance oral tolerance induction to peanut [7]. However, the contribution of dietary fiber-mediated modulation of the microbial composition and metabolism in the gut with respect to OIT-induced suppression of food allergy, and more specifically CMA, remains to be elucidated.

The human gut is colonized with bacterial species that belong to the following main phyla: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and the Fusobacteria [8]. Bacterial communities feature complex metabolic activities and their interaction with the human host ensures both states of health and disease [9]. Firmicutes and

Bacteroidetes are the two major bacterial phyla that comprise the majority of the normal gut microbiota of healthy humans. Altered ratio of Firmicutes to Bacteroidetes has been linked to a variety of pathologies associated with aging or disease [10, 11]. In addition, the gut microbial composition of healthy humans is recognized by a low abundance of Proteobacteria [12]. Expansion of Proteobacteria members is linked to gut dysbiosis and a pro-inflammatory state in the host as shown for inflammatory bowel disease and Parkinson's disease [13, 14]. Furthermore, airway microbial dysbiosis was shown to be related to exacerbations in severe asthmatics and was dominated by increased abundance of Proteobacteria in the lower airways [15].

Considering (food) allergy development, the importance of the microbiome has been recognized in the concept of the 'hygiene hypothesis' [16]. *In utero* or post-natal exposure to environmental factors such as maternal or infant diet and invading pathogens, shapes the gut microbiota composition and functionality with long-term effects on allergy or asthma development [17, 18]. Differences in microbial diversity and abundance of specific taxa were observed in infants showing early life sensitization against egg proteins or actual development of egg allergy [19]. Furthermore, resolution of CMA at 8 years of age was associated with enrichment of the phylum Firmicutes in infant gut microbiota samples collected at 3 to 6 months of age [20]. Manipulation of the bacterial composition has gained interest in the field of food allergy management: cow's milk allergic infants fed an extensively hydrolyzed casein formula with the probiotic *Lactobacillus rhamnosus* GG showed accelerated tolerance induction after 6 months [21]. Tolerant infants showed a significant increase in the abundance of *Oscillospira* (Firmicutes) compared to allergic infants and had elevated butyric acid levels in faecal samples [21].

The interplay between the gut microbiome and the host immune system stretches beyond local communication, since bacterial metabolites like SCFA can enter the systemic circulation and exert anti-inflammatory properties at distinct sites in the organism [22]. It has been shown that symptoms of allergic asthma in mice could be suppressed by a dietary intervention with fermentable fibers which increased systemic concentrations of SCFA [23]. In addition, the authors showed that oral supplementation with propionate affected bone marrow hematopoiesis leading to generation of dendritic cells (DC) with reduced ability to initiate T helper 2 (Th2) cell-driven allergic inflammation [23]. We hypothesized that alteration of the gut microbial composition and/or function, via OIT and FOS supplementation, increases the availability of immunogenic bacterial metabolites that might influence the development of immune precursors in the bone marrow. We focused on mast cell progenitors, based on previous results of reduced mast cell degranulation

after OIT+FOS [6], and since mast cells are main drivers of allergic inflammation. Modification of mast cell precursors might yield substantial (long-term) benefit in food allergy therapies. Moreover, development of mast cells is highly dependent on the micro-environment [24].

The aim of the current experiment was to analyze the gastrointestinal tract's microbiota and SCFA profiles in cow's milk allergic mice subjected to OIT and FOS supplementation that had previously shown reduced acute allergic symptoms upon food challenge (i.e., reduced acute allergic skin response, reduced symptoms of anaphylaxis and reduced mucosal mast cell degranulation) [6]. Furthermore, we sought to investigate if OIT and FOS could mediate their effects through changes in bone marrow hematopoiesis leading to potential altered mast cell development.

MATERIALS AND METHODS

Animals

Female specific-pathogen free mice (6-week-old) were purchased (Charles River Laboratories, Erkrath, Germany) and randomly allocated to the following groups: sham-sensitized control, sham (n=5); whey-sensitized control, sens (n=8); FOS supplementation group, FOS (n=8); OIT group, OIT (n=8); and the combination group of OIT and FOS supplementation, OIT+FOS (n=8). All mice were acclimatized for six days prior to the first sensitization and were housed in filter-topped macrolon cages (one cage/group) at the animal facility of Utrecht University on a 12 h light/dark cycle with unlimited access to food and water. All experimental procedures were approved by the Ethical Committee of Animal research of Utrecht University and conducted according to the principles of good laboratory animal care as stated in the European Directive for the protection of animals used for scientific purposes.

Control and experimental diets

Upon arrival and during acclimatization and oral sensitization, all mice received the semi-purified cow's milk protein-free pelleted AIN-93G control diet. After oral sensitization, mice in the FOS and OIT+FOS groups were fed the AIN-93G diet supplemented with a specific mixture of plant-derived short-chain fructo-oligosaccharides (scFOS: oligofructose, Raftilose P95, degree of polymerization (DP) <6) and long-chain fructo-oligosaccharides (lcFOS: long-chain inulin, Raftiline HP, average DP of 23 or higher with <1% DP of 5 or lower) in a 9:1 scFOS/lcFOS ratio (1% w/w) produced by Ssniff Spezialdiäten GmbH, Soest, Germany as specified previously [6]. Both scFOS and lcFOS were provided by Orafti (Wijchen, the Netherlands).

Sensitization and oral immunotherapy

According to the experimental set-up depicted in **Figure 1**, all mice were intragastrically (i.g.) sensitized against the cow's milk protein whey (DMV International, Veghel, the Netherlands) on day 0, 7, 14, 21 and 28 [20 mg whey in 0.5 ml PBS with 15 µg cholera toxin (CT, List Biological Laboratories Inc., Campbell, CA, USA) per mouse]. The sham-sensitized control group received CT in PBS alone. From D42-D59, mice in the OIT and OIT+FOS groups were subjected to oral dosing with 10 mg whey in 0.5 ml PBS (5x/week for 3 consecutive weeks). After three weeks of FOS supplementation and OIT, faecal samples were collected and the mice were sectioned in order to isolate bone marrow and cecum content at D63.

Microbiota profiling and bioinformatics analysis

Total DNA was extracted from mice faeces collected after three weeks of experimental interventions utilizing the FastDNA bead-beating Spin Kit for Soil (MP Biomedicals, Solon, OH, USA), and verified with fluorometric quantitation (Qubit, Life Technologies, Grand Island, NY, USA). Primers 515FB/806RB (515FB:GTGYCAGCMGCCGCGGTAA; 806RB:GGACTACNVGGGTWTCTAAT) targeting the V4 variable region of microbial small subunit (SSU or 16S) ribosomal RNA (rRNA) genes were used for PCR [25], and prepared for high-throughput amplicon sequencing using a modified two-step targeted amplicon sequencing (TAS) approach, as described previously [26]. Negative controls were used with each set of amplifications, which indicated no contamination. Samples were pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter, Brea, CA, USA) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq mid-output flow cell (2x153 paired-end reads) and sequenced using Fluidigm sequencing primers. Based on the distribution of reads per barcode, the amplicons (before purification) were re-pooled to generate a more balanced distribution of reads. The re-pooled and re-purified libraries were then sequenced on a high-output MiniSeq run. Library preparation, pooling, and MiniSeq sequencing were performed at the DNA Services (DNAS) facility, Research Resources Center (RRC), University of Illinois at Chicago (UIC). Raw sequence data (FASTQ files) were deposited in the NCBI Sequence Read Archive under project PRJNA434262.

Raw FASTQ files for each sample were merged using the software package PEAR (Paired-end-read merger) (v0.9.8) [27, 28]. Merged reads were quality trimmed and sequences shorter than 250 bases were discarded (CLC Genomics Workbench, v10.0, CLC Bio, Qiagen, Boston, MA). Sequences were screened for chimeras (usearch8.1 algorithm) [29], and putative chimeric sequences were removed from the dataset (QIIME v1.8) [30]. Each sample was rarefied (47,000 sequences/sample) and data were pooled, re-named, and clustered into operational taxonomic units (OTU) at 97% similarity (usearch8.1 algorithm). Representative sequences from each OTU were extracted and classified using the uclust consensus taxonomy assigner (Greengenes 13_8 reference database). A biological observation matrix (BIOM) [31] was generated at each taxonomic level from phylum to species ("make OTU table" algorithm) and analyzed and visualized using the software packages Primer7 [32] and the R programming environment [33].

Alpha-diversity (α-diversity) indices (within-sample) and Beta-diversity (β-diversity) (between-sample) were used to examine changes in microbial community structure between mice faecal group samples. Alpha-diversity indices (i.e., Shannon, Simpson, richness, and evenness) were generated using the package 'vegan' implemented in the R programming language [34]. To examine β-diversity differences in microbial community composition between samples, pairwise Bray-Curtis dissimilarity (non-phylogenetic) metric was generated using the Primer7 software package and used to perform analysis of similarity (ANOSIM) calculations. ANOSIM was performed at the taxonomic level of genus, using square-root transformed data. Also, Primer7 was used to conduct both non-metric multi-dimensional scaling (nMDS) and Bootstrapping (average values and dispersion within each sample's group) plots to visualize each mice group's overall microbial differences, at the genus level.

Beta-diversity differences in relative abundance of individual taxa, between mice faecal group samples, were assessed for significance using Kruskal-Wallis test controlling for false-discovery rate (FDR), implemented within the software package QIIME [30]. Furthermore, mice faecal group sample's community functional predictions were performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) [35] and differences in Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) abundances between groups were identified [36].

SCFA analysis in cecum content

SCFA analysis was conducted as described elsewhere [37]. In short, cecum content was collected and stored at -80°C until further processing. Samples were defrosted, diluted in ice cold PBS (1:10) and homogenized by vortexing. Supernatant was collected after centrifugation at 13,000 rpm for 10 min and analyzed using the Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) and concentrations of acetic acid, butyric acid, propionic acid, valeric acid, iso-butyric acid and iso-valeric acid were quantitated based on 2-ethylbutyric acid internal standard.

Bone marrow isolation and mast cell culture

The femurs and tibiae of five mice per control and experimental group were collected and bone marrow cells were isolated by flushing the bones under sterile conditions using BMMC culture medium (RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% FCS, 26 mM Hepes, 0.12 mM MEM non-essential amino acids, 2.4 mM Glutamax, 1.2 mM sodium pyruvate (all from Gibco, Thermo Fisher Scientific, Waltham, MA, USA), penicillin-streptomycin (100 U/ml-100 µg/ml, Sigma-Aldrich, Zwijndrecht, the Netherlands). Pooled cell suspensions were flushed through a 70-µm cell strainer and centrifuged (1200 rpm for 6 min) and washed. Lysis of red blood cells was performed with filter sterilized lysis buffer consisting of 1.5 M NH_4Cl, 0.1 M $NaHCO_3$, 10 mM EDTA (all from Merck, Darmstadt, Germany) in demineralized water. White blood cells were dissolved in freeze medium consisting of 40% BMMC culture medium, 10% DMSO (Sigma-Aldrich) and 50% FCS and aliquots were frozen at -80°C for at least 24 h and subsequently stored in liquid nitrogen until culture. Cells were cultured for 8 weeks in BMMC culture medium supplemented with IL-3 and stem cell factor (SCF), (both 10 ng/ml, Prospec, Ness-Ziona, Israel) at 37°C with 5% CO_2 in 175 cm^2 culture flasks (Costar, Corning Incorporated, Corning, NY, USA) to induce BMMC differentiation (**Figure 1**). Half of the BMMC culture medium was refreshed once a week and aliquots of cells were collected to measure expression of specific mast cell receptors (P1-8, **Figure 1**).

Flow cytometry

The BMMC phenotype is recognized by surface expression of the FcεRI (IgE receptor) and the c-Kit receptors (SCF receptor, CD117). Aliquots of cells were harvested during passage once a week and plated (400,000 cells/well) in 96-wells Falcon plates (BD Biosciences, Alphen a/d Rijn, the Netherlands). The cells were incubated with 1:100 anti-mouse CD16/CD32 (mouse Fc block, BD Biosciences) for 10 min on ice to block non-specific binding sites. After centrifugation (1400 rpm for 5 min), cells

were stained with anti-FcεRI-PE-Cy7 and anti-c-Kit-APC (1:100 in BMMC medium, from Thermo Fisher Scientific) and incubated for 45 min on ice. After centrifugation and washing in FACS buffer (PBS with 1% bovine serum albumin, BSA), cells were stained with viability dye (1:1000 YO-PRO-1 in FACS buffer, Thermo Fisher Scientific). Fluorescence was measured using a FACS Canto II (BD Biosciences) and data was analyzed with Flowlogic software (Inivai Technologies, Mentone, Australia). Single cells were distinguished from aggregates based on forward/sideward scatter properties. Cut-off gates for positivity of FcεRI and c-Kit expression were determined with the fluorescence-minus-one technique (FMO) and isotype controls were used.

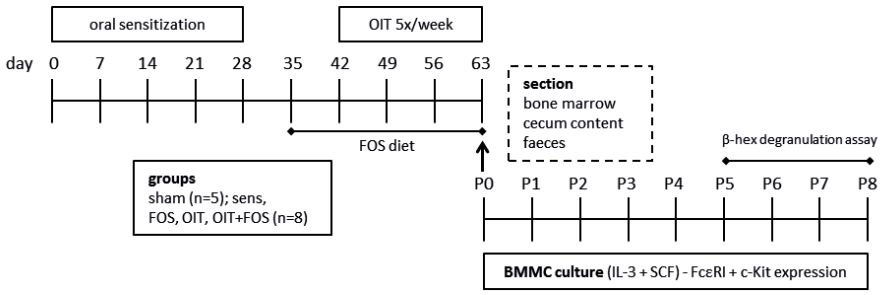


Figure 1. Experimental timeline of *in vivo* and *in vitro* experiments. Female C3H/HeOuJ mice were fed the AIN-93G control diet and grouped as depicted: sham, sham-sensitized control (n=5); sens, whey-sensitized control (n=8); FOS, FOS supplementation group (n=8); OIT, OIT group (n=8); OIT+FOS, combination of OIT and FOS supplementation group (n=8). On experimental day 0, 7, 14, 21 and 28, mice were i.g. sensitized to the cow's milk protein whey using CT as an adjuvant (20 mg whey in 0.5 ml PBS with 15 μg CT/mouse). Sham-sensitized mice received 0.5 ml PBS with 15 μg CT alone. From day 35, FOS and OIT+FOS mice were fed the AIN-93G diet supplemented with 1% FOS until the end of the protocol. OIT was provided per oral gavage from day 42-59 (10 mg whey in 0.5 ml PBS, 5x/week for 3 weeks). On day 63, faecal samples were collected and mice were sectioned to collect bone marrow and cecum content. Pooled bone marrow samples were used to culture BMMC in the presence of mast cell-stimulating factors IL-3 and SCF (10 ng/ml). An aliquot of cells was collected during passage (P1 to P8) to measure expression of FcεRI and c-Kit with flow cytometry. From P5 to P8, β-hex degranulation assays were performed to measure mast cell functionality. CT, cholera toxin; OIT, oral immunotherapy; FOS, fructo-oligosaccharides; β-hex, β-hexosaminidase; i.g., intragastric; BMMC, bone marrow-derived mast cells.

Beta-hexosaminidase degranulation assay

In vitro release of β-hexosaminidase (β-hex) can be used as a marker for mast cell degranulation. At culture week 5 to 8 (**Figure 1**), BMMC were harvested and washed with assay medium (RPMI-1640 with 2.4 mM L-Glutamine and without phenol red (Lonza) supplemented with 1% FCS) and sensitized using 20% anti-dinitrophenol (DNP)-IgE from hybridoma clone 26.82 at 37°C and 5% CO_2 for 2 h. IgE-sensitized BMMC were transferred to a sterile 96-wells plate (Costar, Corning Incorporated, 25,000 cells/well). Cells were activated upon incubation with DNP hapten conjugated to human serum albumin (DNP-HSA) in a dose-response concentration range at 37°C and 5% CO_2 for 1 h. The maximal level of degranulation was determined by adding 10% Trition (TX100, Merck) and background values were measured by adding assay medium without antigen. Subsequently, cells were centrifuged (1500 rpm for 5 min) and the supernatant was transferred to a new 96-wells plate together with 4-methylumbelliferyl-N-acetyl-ß-D-glucosaminide solution (4MUG, Sigma-Aldrich) in 0.1 M citrate buffer (pH=4.7, Merck) as a substrate for β-hex. The reaction was stopped with 0.2 M glycine-HCl buffer (Merck). The release of β-hex was measured by fluorescence (excitation 351 nm, emission 462 nm) with a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Fisher Scientific). Release of β-hex in percentages was calculated with the following formula:

$$\% \text{ release} = \left(\frac{\text{stimulated cells} - \text{unstimulated cells}}{\text{maximum release TX100} - \text{unstimulated cells}} \right) * 100$$

Data analysis and statistics

Alpha- and β-diversity were measured at each taxonomic level from phylum to genus. In SPSS (v.22, IBM, Chicago, IL, USA), all mice variables were checked for normality assumptions. The Shapiro-Wilk-Normality test was performed across the five control and experimental mice groups. Parametric one-way ANOVA, with Bonferroni's post-hoc test, or non-parametric Kruskal-Wallis test, with Dunn's post-hoc test, were used to compare the following mice groups: sham with sens; sens with FOS, OIT and OIT+FOS; FOS with OIT+FOS; OIT with OIT+FOS. Data with regard to α-diversity (within-sample) calculated indices (richness, evenness, Simpson and Shannon) [38, 39], β-diversity (between-sample) microbial compositions (relative abundance of bacterial taxa) [40, 41], SCFA concentrations and BMMC degranulation were exported, analyzed, and graphically depicted as mean ± standard error mean (SEM) with GraphPad Prism (v.7) software (GraphPad Software, La Jolla, CA, USA).

Alpha-diversity indices were calculated such as: Shannon index ($H' = -\sum \text{sum}(Pi/\log(Pi))$ where Pi = the relative abundance of each taxon), Pielou's evenness ($J' = H'/\log(S)$)

where S = number of taxa present in each sample), richness (number of taxa present in each sample), and Simpson's index (D=∑(Pi2)) where Pi = the relative abundance of each taxon.

Beta-diversity stacked histograms represent the percent relative abundance of individual taxa per mice group. Furthermore, the Firmicutes to Bacteroidetes (F/B) ratio between mice groups was studied. Correlations between SCFA concentrations (total SCFA, acetic acid, propionic acid, butyric acid and total butyric acid-to-total SCFA) and individual microbial taxa (phylum, family, and genus) were analyzed by Pearson's correlation analysis. Calculated p-values were corrected for the number of comparisons and statistical significance was set at $p<0.05$. Additionally, PICRUSt significance was accepted at $p<0.05$ based on the experiment's hypothesis derived a priori functional pathways.

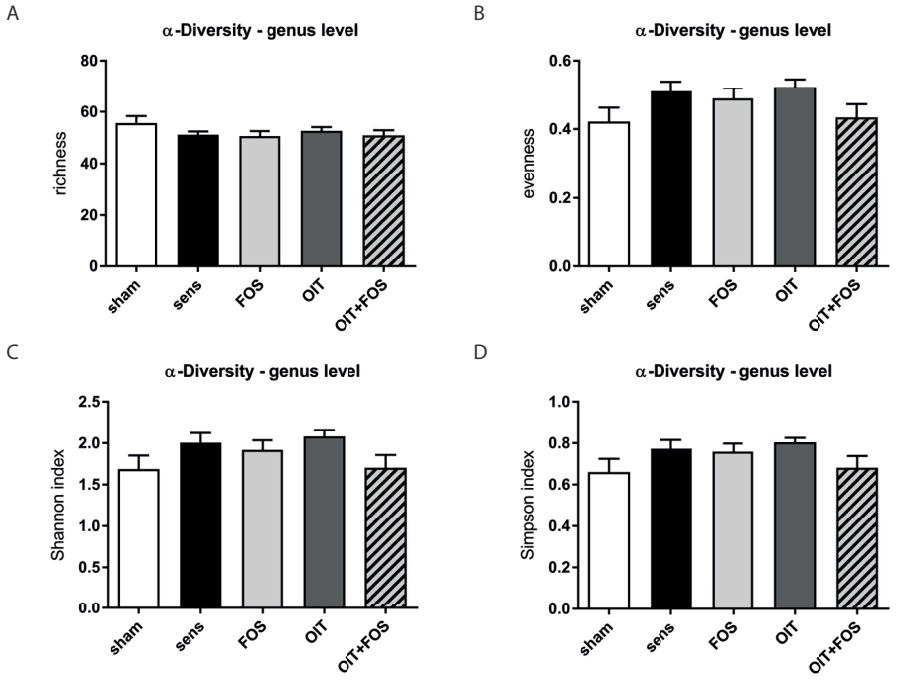


Figure 2. Alpha-diversity scores in faecal samples derived from treated mice, at the taxonomic level of genus. (A) Richness, **(B)** evenness, **(C)** Shannon Index and **(D)** Simpson Index indicate no differences in (within-sample) diversity between the control and experimental groups of mice. Data are represented as mean ± SEM, n=5-8/group. Statistical analysis was performed between groups using one-way ANOVA for parametric data with Bonferroni's post-hoc test for richness data; Kruskal-Wallis test for non-parametric data with Dunn's post-hoc test for evenness, Shannon Index and Simpson Index data.

RESULTS

Microbiota analysis revealed no differences in α-diversity index across the groups

Alpha-diversity represents the microbial diversity (richness and evenness) within a sample or, in this case, group of mice. The faecal microbiota analysis indicated no significant changes in α-diversity indices across the control and experimental groups at all taxonomic levels. Depicted are the results of the taxonomic level of genus (**Figures 2A-D**).

Bacterial community structures in sensitized mice treated with the combination of OIT+FOS showed a high level of resemblance with non-allergic mice

Beta-diversity measures the variety in bacterial communities between samples or groups of mice. ANOSIM was performed at the taxonomic level of genus to investigate the level of similarity in bacterial community structures between the control and experiment groups (**Table 1**, **Figure 3**). FOS supplementation significantly impacted microbiota community structure in allergic mice. Calculated p-values indicated a significant difference (global r=0.203; p=0.03) in overall bacterial community structure in the allergic mice receiving the FOS supplemented diet (shown in green) compared to the allergic mice receiving the control diet (shown in red). Interestingly, a high level of similarity in bacterial community structure was observed between the sham-sensitized control group (shown in dark blue) and the OIT+FOS group (shown in light blue) (global r=0.05; p=0.24) (**Figure 3**).

OIT and/or FOS supplementation induced specific differences in relative abundance of bacterial communities at the taxonomic level of phylum and genus

Figure 4A shows the relative abundance of the four main phyla (Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria) in the specific groups of mice. **Figure 4B** specifies the relative abundance distribution per group at the taxonomic level of genus. Group comparisons of the relative abundance data were analyzed at all taxonomic levels and only the significantly differing phylum and genera are depicted in **Figure 5**. As shown in **Figure 5A**, the relative abundance of the putative pro-inflammatory phylum Proteobacteria was increased in the OIT group compared to the OIT+FOS group. The ratio of the Firmicutes to Bacteroidetes was determined, but no significant differences between the groups were observed (data not shown). An

increase in relative abundance of the genus *Allobaculum* (Firmicutes) was observed in sensitized mice fed the FOS supplemented diet (FOS group) compared to the control diet (sens group) (**Figure 5B**). Three genera were shown to be different between the sensitized control mice fed the control diet and the OIT+FOS group: unclassified *Rikenellaceae* (Bacteroidetes, **Figure 5C**), unclassified *Clostridiales* (Firmicutes, **Figure 5D**) and *Oscillaspira* (Firmicutes, **Figure 5E**). In addition, the relative abundance of the putative pro-inflammatory *Oscillaspira* was reduced in the OIT+FOS group compared to the OIT group (**Figure 5E**). The genus *Lactobacillus* (Firmicutes) was increased in faecal samples of OIT+FOS mice compared to FOS mice, almost reaching the level measured in the non-allergic control mice (sham) as shown in **Figure 5F**. It is important to note that the relative abundance of the genus *Lactobacillus* is proportionately higher compared to the other genera depicted.

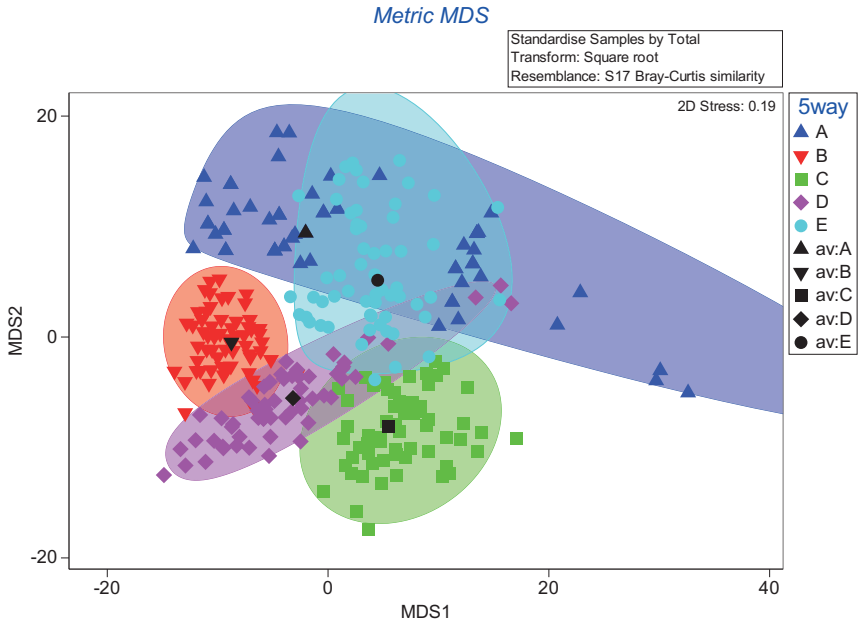


Figure 3. Overall microbial community structures in a metric multi-dimensional scaling plot, at the taxonomic level of genus. The bacterial community structure in sensitized control mice fed the control diet (shown in red) was significantly different from sensitized mice fed the FOS diet (shown in green) (ANOSIM: global r=0.203; p=0.03). A high level of resemblance was observed in the community structures of the sham-sensitized control mice (shown in dark blue) and the sensitized mice treated with the combination OIT+FOS (shown in light blue). A: sham, B: sens, C: FOS, D: OIT and E: OIT+FOS; MDS, multi-dimensional scale; Av, average value per group (based on Bootstrapping procedure in PRIMER 7).

Table 1. Across-group analysis of similarity (ANOSIM) results for mice faecal microbiota compositions.

Comparisons - genus taxonomic level	n	global r	p value
(A) PBS Sens_PBS Tx_Control Diet vs. **(B)** Whey Sens_PBS Tx_Control Diet	12	0.104	0.18
(B) Whey Sens_PBS Tx_Control Diet vs. **(C)** Whey Sens_PBS Tx_FOS Diet	15	0.203	**0.03**
(B) Whey Sens_PBS Tx_Control Diet vs. **(D)** Whey Sens_OIT 10mg Whey_Control Diet	14	-0.007	0.46
(B) Whey Sens_PBS Tx_Control Diet vs. **(E)** Whey Sens_OIT 10mg Whey_FOS Diet	15	0.071	0.15
(C) Whey Sens_PBS Tx_FOS Diet vs. **(E)** Whey Sens_OIT 10mg Whey_FOS Diet	16	0.064	0.18
(D) Whey Sens_OIT 10mg Whey_Control Diet vs. **(E)** Whey Sens_OIT 10mg Whey_FOS Diet	15	0.076	0.12

(Sens) = sensitivity; (Tx) = treatment; (n) = number of samples; global r comparison was based on ANOSIM performed within the software package Primer7. P values were calculated based on a permutational analysis, employing 999 permutations; square-root transformation analysis.

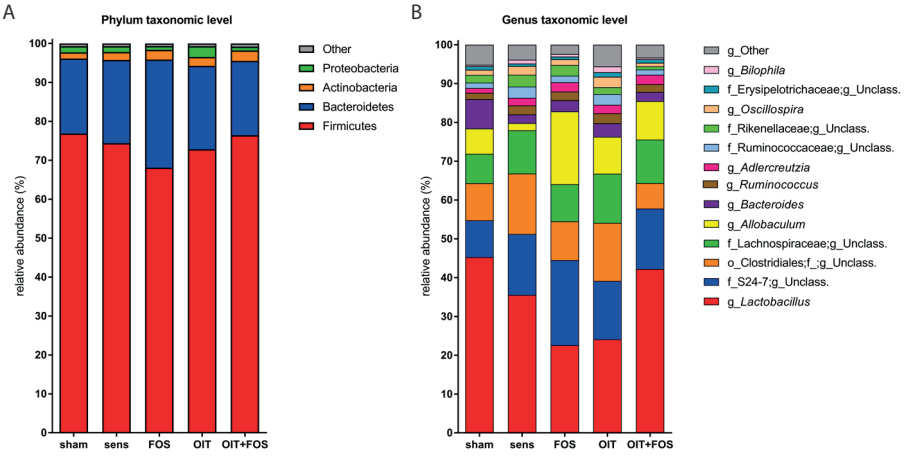


Figure 4. Stacked column plots showing the average relative abundance, at the taxonomic levels of phylum and genus. (A) Relative abundances (%) of the four main phyla (Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria) in each control and experimental group of mice. **(B)** Relative abundances (%) of the twelve most enriched bacterial genera in faecal samples in each control and experimental group of mice.

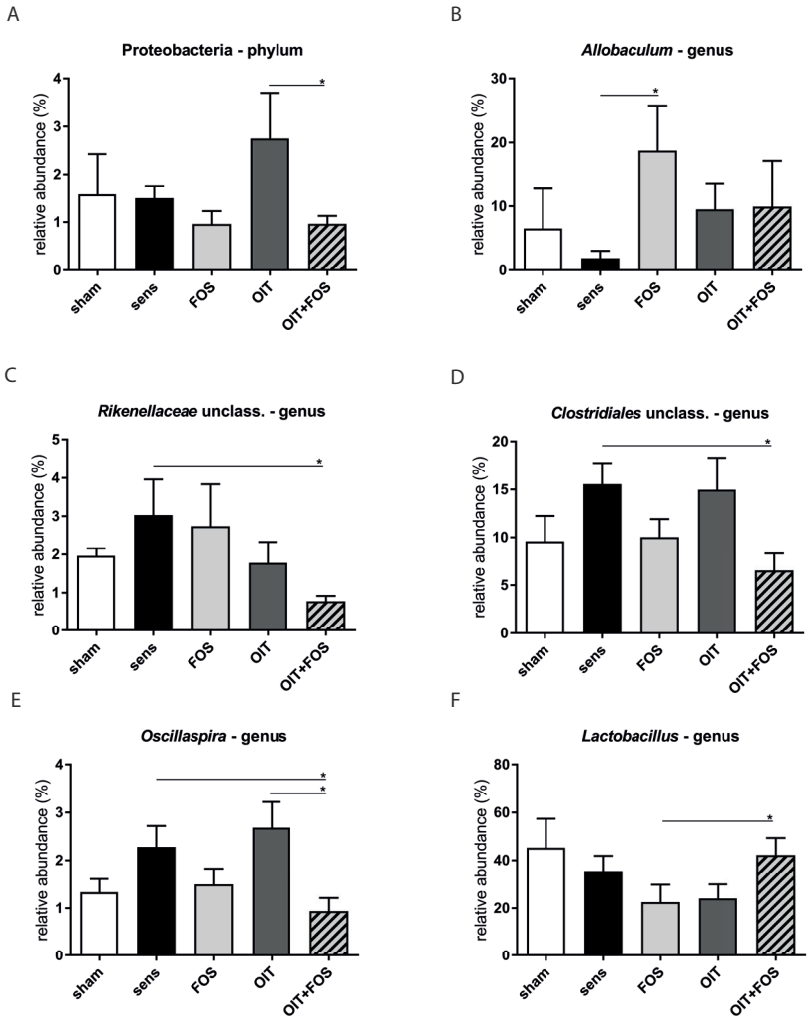


Figure 5. Significant differences in β-diversity in faecal samples derived from treated mice, at the taxonomic levels of phylum and genus. (A) OIT mice showed increased relative abundance of the phylum Proteobacteria compared to OIT+FOS mice. **(B)** FOS supplementation in sensitized mice significantly increased the relative abundance of the genus *Allobaculum* (Firmicutes) compared to sensitized control mice fed the control diet. OIT+FOS treatment in cow's milk allergic mice reduced the relative abundance of the genera **(C)** unclassified *Rikenellaceae* (Bacteroidetes) **(D)** unclassified *Clostridiales* (Firmicutes) and **(E)** *Oscillospira* (Firmicutes) compared to the sensitized control mice. In addition, the latter genus was significantly different from OIT mice. **(F)** OIT+FOS treatment increased the relative abundance of the genus *Lactobacillus* (Firmicutes) compared to FOS mice with levels approximating the abundance observed in sham-sensitized controls. Data are represented as mean ± SEM, n=5-8/group. Statistical analysis was performed using Kruskal-Wallis test for non-parametric data with Dunn's post-hoc test to compare pre-selected combinations as indicated. *p<0.05.

Predictive functional assessment showed up-regulation of metabolic pathways in allergic mice compared to non-allergic mice

Predictive assessment of the microbial community functional potential (PICRUSt analysis) was used to infer whether functional differences exist between resident microbial communities in the control and experimental groups. The level of expression of the amplified 16S rRNA subunit genes was predicted and assigned to relevant metabolic pathways. Group comparisons suggested significant differences in the predicted functional potential in a subset of metabolic pathways. We focused on the pathways which were associated with fatty acid metabolism or fiber fermentation, according to the a priori defined hypothesis that these pathways are involved in modulation of the allergic response. In **Figure 6A**, significant pathways were suggested to differ between the sham-sensitized controls and the sensitized controls: all pathways showed an increase in gene expression upon oral food sensitization compared to non-allergic mice. It should be noted that the overall amount of significantly altered pathways in the PICRUSt analysis was the highest in the analysis of non-allergic vs allergic mice. Interestingly, comparison of the control diet with the FOS supplemented diet in sensitized mice suggested reduced expression of genes associated to fiber fermentation and fatty acid and retinol metabolism in FOS supplemented mice (**Figure 6B**). Additional group comparisons (sens vs OIT+FOS, FOS vs OIT+FOS and OIT vs OIT+FOS) showed differences in only a limited number of (non-relevant) metabolic pathways (data not shown).

OIT+FOS treated mice showed increased concentrations of butyric acid and propionic acid in cecum content

Cecum content was collected and processed to measure concentrations of the fermentation-derived bacterial metabolites SCFA and branched-chain FA (BCFA) to investigate functional aspects of the microbiota. OIT+FOS treatment increased butyric acid levels compared to sensitized controls and OIT mice (**Figure 7A**). A similar result was observed for propionic acid (**Figure 7C**). No differences were observed in the amount of acetic acid present in cecum content (**Figure 7B**). Concentrations of valeric acid were increased in OIT+FOS samples compared to the FOS group and the sensitized control group (**Figure 7D**). The BCFA iso-butyric acid and iso-valeric acid were increased in the OIT+FOS samples compared to the FOS samples (**Figures 7E,F**). However, valeric acid and BCFA levels showed a limited contribution to the total SCFA load.

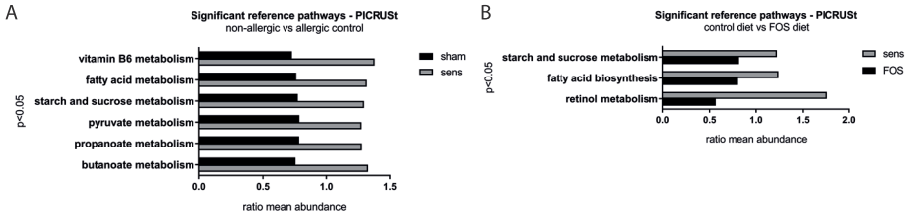


Figure 6. PICRUSt community functional predictions in relevant metabolic pathways. (A) Depicted are the inferred significantly increased metabolic pathways in whey-sensitized mice compared to sham-sensitized mice which can be linked to starch and fatty acid metabolism. **(B)** Significantly increased metabolic pathways in sensitized control mice compared to FOS supplemented mice. PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

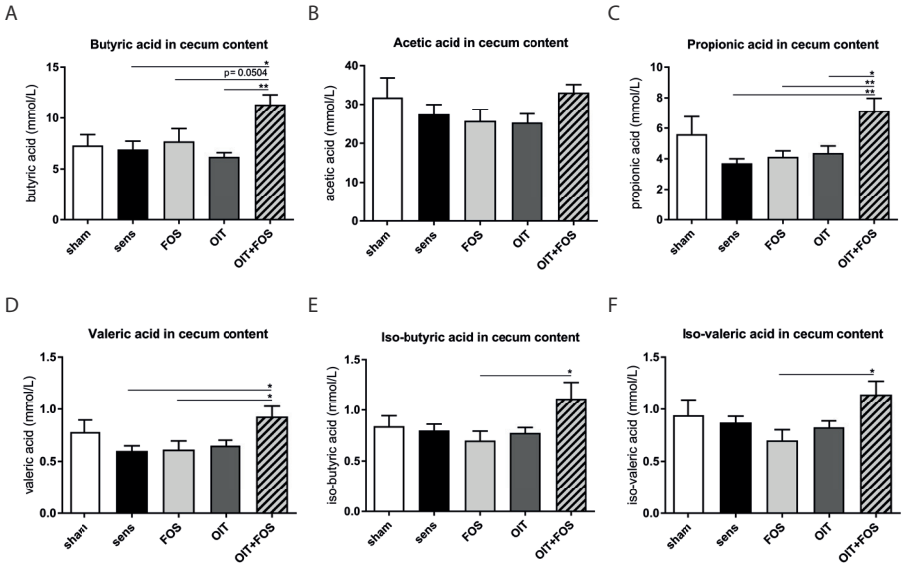


Figure 7. SCFA and BCFA concentrations in cecum content of treated mice. (A) Butyric acid was increased in OIT+FOS mice compared to sensitized controls and OIT mice and a trend was observed compared to FOS (p=0.0504). **(B)** No differences in acetic acid concentrations were observed. **(C)** OIT+FOS increased propionic acid concentrations compared to sensitized controls, FOS and OIT mice. **(D)** Valeric acid concentrations were increased in OIT+FOS mice compared to sensitized controls and FOS mice. The BCFA **(E)** iso-butyric acid and **(F)** iso-valeric acid were increased in OIT+FOS mice compared to FOS mice. Data are represented as mean ± SEM, n=5-8/group. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, **p<0.01. SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids.

The relative abundance of specific bacterial genera positively correlated to levels of propionic acid and acetic acid, but not butyric acid

Pearson's correlation analysis was conducted at the taxonomic level of phylum, family and genus to investigate a potential link between the relative abundance of specific bacteria in faecal samples and the main SCFA (butyric acid, acetic acid and propionic acid) concentrations in cecum content. In **Table 2**, only the correlations with ($p<0.05$) are depicted at the taxonomic level of family and genus, since no significant correlations were observed at phylum level. The total SCFA concentration in cecum content showed a positive correlation with the abundance of the taxonomic family Bacteroidaceae (Bacteroidetes) and the following genera of bacteria: 1) Bacteroides (Bacteroidetes), 2) unclassified Erysipelotrichaceae (Firmicutes), 3) Blautia (Firmicutes), 4) unclassified Coriobacteriaceae (Actinobacteria), 5) Proteus (Proteobacteria) and 6) Dorea (Firmicutes) (**Table 2**). The relative abundance of a total of 7 bacterial genera was positively correlated to acetic acid concentrations in mice. In addition, propionic acid concentrations were positively correlated to the relative abundance of 6 bacterial genera in faecal samples. Interestingly, none of the observed correlations between either bacterial families or genera and butyric acid concentrations were positive; in fact there was a negative correlation between butyric acid levels and butyrate-producing Roseburia. This could potentially be due to the relatively low abundance of these bacteria in the cecal content in these mice. However, the ratio of butyric acid to total SCFA levels was positively correlated to the relative abundance of two bacterial genera within the order Lactobaccillales (Firmicutes): Lactococcus and unclassified Leuconostocaceae, both lactic acid-producing bacteria. It is well known that lactic acid could be used by butyrate-producing bacteria (i.e., cross-feeding), resulting in increased production of butyric acid without increasing the abundance of butyrate-producing bacteria [42-44].

BMMC cultured from FOS supplemented mice showed reduced FcεRI and c-Kit expression and IgE-mediated degranulation after 6 weeks of culture

Bone marrow cells were cultured in the presence of IL-3 and SCF for 8 weeks and expression of the mast cell-specific IgE receptor (FcεRI) and the c-Kit receptor (SCF receptor) was analyzed with flow cytometry to monitor development of BMMC. During the first passages (P1-4), no differences in receptor expression were observed (only P1 plots are depicted in **Figure 8**). At P6, a double-negative cell population appeared in the culture derived from FOS supplemented and sensitized mice (FOS). Assessment of the BMMC at P7 and P8 showed a further increase in the percentage

of cells that lost expression of the specific mast cell receptors in the FOS culture. It is important to note that all cultures showed signs of double-negative cells at P7 and P8, but with a lower percentage compared to the FOS group. Functional analysis of BMMC at P5 showed decreased IgE-mediated degranulation (measured as β-hex release at the optimal antigen concentration) in the FOS-derived BMMC compared to sham-derived BMMC that were used as a reference for degranulation capacity (**Figure 9A**). BMMC cultured from sensitized control mice and OIT+FOS mice showed increased degranulation capacity at P5 compared to the control (**Figure 9A**). In accordance with the reduced FcεRI expression, IgE-mediated degranulation levels were decreased in the FOS-derived BMMC compared to the control at P6 and P8 (**Figures 9B,D**). At P7, all BMMC cultures showed increased degranulation capacity compared to the control, except for the FOS-derived BMMC (**Figure 9C**).

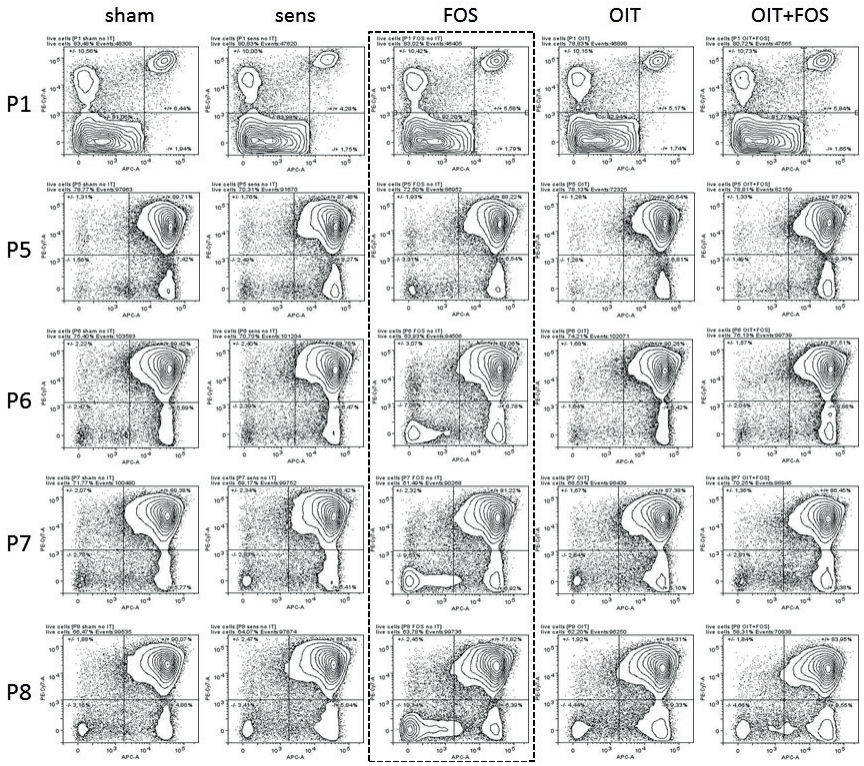


Figure 8. Expression of FcεRI and c-Kit on BMMC cultured from treated mice. BMMC cultured in the presence of IL-3 and SCF for 8 weeks (P1-8). Aliquots of cells were stained with anti-FcεRI-PE-Cy7 and anti-c-Kit-APC at every passage (once a week). No differences were observed from P1 until P5 (only P1 data shown). At P6, FOS-derived BMMC showed a reduced percentage of FcεRI and c-Kit double positive cells. At P7 and P8, all cultures showed signs of cells that lost receptor expression. BMMC, bone marrow-derived mast cells; SCF, stem cell factor; P, passage.

Table 2. Significant correlations comparing microbiota and SCFA concentrations.

Taxa	SCFA	acetic acid	propionic acid	butyric acid	butyric acid/ SCFA
	r, p value	r, p value	r, p value	r, p value	r, p value
Family					
Bacteroidaceae	+0.38, 0.02	+0.45, 0.01	+0.39, 0.02	-	-
Peptococcaceae	-0.39, 0.02	-0.34, 0.04	-0.39, 0.02	-0.36, 0.03	-
Ruminococcaceae	-0.34, 0.04	-	-0.40, 0.02	-	-
Alcaligenaceae	-	+0.36, 0.03	-	-	-0.36, 0.03
o_Clostridiales;f_Unclassified	-	-	-0.39, 0.02	-	-
Genus					
Bacteroides	+0.38, 0.02	+0.45, 0.01	+0.39, 0.02	-	-
f_Erysipelotrichaceae;g_Unclassified	+0.40, 0.02	+0.47, <0.00	+0.37, 0.03	-	-
Ruminococcus	-0.37, 0.03	-	-0.40, 0.02	-0.35, 0.04	-
Blautia	+0.45, 0.01	+0.52, <0.00	+0.47, <0.00	-	-
f_Coriobacteriaceae;g_Unclassified	+0.44, 0.01	+0.47, <0.00	+0.53, <0.00	-	-
Proteus	+0.42, 0.01	+0.39, 0.02	+0.50, <0.00	-	-
Dorea	+0.37, 0.03	+0.43, 0.01	+0.38, 0.02	-	-
f_Peptococcaceae;g_Unclassified	-0.39, 0.02	-0.34, 0.04	-0.39, 0.02	-0.36, 0.03	-
Roseburia	-0.34, 0.04	-	-0.37, 0.03	-0.37, 0.03	-
Sutterella	-	+0.36, 0.03	-	-	-0.36, 0.03
o_Clostridiales;f_;g_Unclassified	-	-	-0.13, 0.02		
f_Ruminococcaceae;g_Unclassified	-	-	-0.40, 0.02	-	-
Oscillospira	-	-	-0.37, 0.03	-0.35, 0.04	-
f_Ruminococcaceae;Other	-	-	-0.39, 0.02	-	-
f_Leuconostocaceae;g_Unclassified	-	-	-	-	+0.34, 0.04
Lactococcus	-	-	-	-	+0.34, 0.04
r = Pearson r value; p value = (p<0.05); no significant differences at phylum level.					

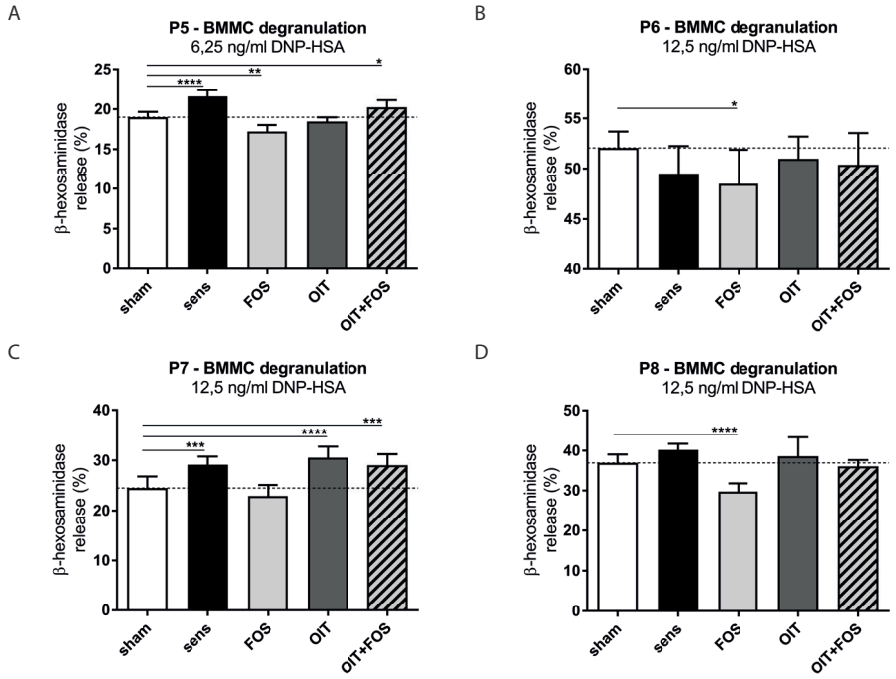


Figure 9. Functional analysis of BMMC cultured from treated mice using β-hex degranulation assay. β-hex release by anti-DNP-IgE sensitized BMMC upon activation with the optimal DNP-HSA concentration. **(A)** P5 and **(B)** P6 showed reduced degranulation of FOS-derived BMMC compared to control BMMC derived from sham-sensitized mice. **(C)** All groups showed increased degranulation capacity compared to control BMMC, except for FOS-derived BMMC. **(D)** At P8, FOS-derived BMMC showed reduced degranulation capacity compared to controls. Data are represented as mean ± SD, duplicate measurements per concentration DNP-HSA. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. β-hex, β-hexosaminidase; BMMC, bone marrow-derived mast cells; DNP-HSA, dinitrophenol hapten conjugated to human serum albumin; P, passage.

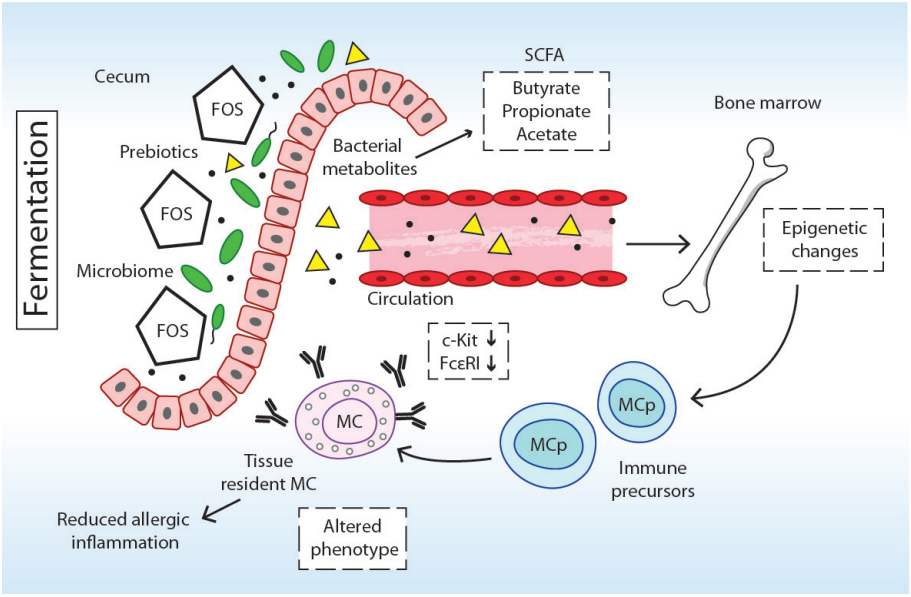


Figure 10. Proposed mechanism of action in cow's milk allergic mice treated with OIT+FOS. Based on the current data, it was hypothesized that alterations in microbial diversity and metabolic activity lead to increased availability of specific metabolites that can enter the systemic circulation and affect mast cell precursors resident in the bone marrow via epigenetic changes. Altered mast cell phenotypes might contribute to reduced allergic inflammation and long-term efficacy of treatment strategies. FOS, fructo-oligosaccharides; SCFA, short-chain fatty acids; MC, mast cell; MCp, mast cell precursor.

DISCUSSION

In a previous study, we showed that dietary supplementation using FOS improved OIT-induced desensitization in cow's milk allergic mice as demonstrated by enhanced suppression of acute allergic symptoms (i.e., acute allergic skin response and mast cell degranulation) upon food challenge. The suppressed clinical response was accompanied by control of specific IgE levels, reduced Th2-mediated cytokine production in splenocytes and an increased percentage of functionally active regulatory T cells (Tregs) in lymphoid organs of OIT+FOS mice [6]. In the current article, we performed additional analyses on faeces, cecum content and bone marrow samples collected from these mice, to investigate potential alterations in microbial composition and metabolism, underlying the allergy suppressive effects.

Our current study indicated that 16S rRNA sequencing of microbial communities resident in faecal samples revealed no differences in microbial diversity within each group of mice (α-diversity). Evenness and richness of bacterial communities were unaltered after induction of CMA and treatment with either OIT or FOS or the combination of both. In humans, the establishment of atopic disorders like asthma and allergies is characterized by alterations in microbial diversity and functionality in early infancy; however, there is an inconsistent association between allergy development and changes in α-diversity [21, 45-47]. Upon analyzing between-group bacterial diversity, clustering of bacterial communities in whey-sensitized mice (multi-dimensional plot) appeared to be different from the profile observed in sham-sensitized mice. However, specification of relative abundances of bacterial phyla and genera did not show significant differences between whey-sensitized and sham-sensitized mice.

A significant difference in the overall bacterial community structure was observed in sensitized mice receiving the FOS supplemented diet compared to sensitized mice receiving the control diet. In particular, the genus Allobaculum (family Erysipelotrichaceae, phylum Firmicutes) was increased in faecal samples of FOS supplemented mice. Furthermore, the abundance of a member of the same family of Erysipelotrichaceae (genus unclassified) positively correlated with the total SCFA, acetic- and propionic acid levels in cecum content of the mice. Allobaculum have been suggested to be putative SCFA-producers [48-52], and might benefit host-health via the control of inflammatory responses.

OIT+FOS treatment in cow's milk allergic mice induced a bacterial profile with a high level of resemblance with the profile observed in non-allergic mice. Differences in β-diversity of specific genera were observed in OIT+FOS faecal samples compared to whey-sensitized control samples: OIT+FOS decreased the abundance of a member of the potentially pro-inflammatory unclassified *Rikenellaceae* (family Rikenellaceae, phylum Bacteroidetes). Food allergy-prone mice were shown to have a specific microbial signature recognized by changes in abundance of, amongst others, the family Rikenellaceae after sensitization to ovalbumin compared to wild-type mice [53]. Transfer of gut microbiota to germ-free wild-type mice resulted in induction of the allergic phenotype, suggesting the involvement of the family Rikenellaceae in the development of food allergies [53]. In the current study, two genera derived from the order Clostridiales (phylum Firmicutes), *Oscillospira* (family Ruminococcaceae) and unclassified *Clostridiales* (family unclassified), were both significantly reduced in abundance in OIT+FOS samples compared to sensitized controls. Moreover, the relative abundance of *Oscillospira* was decreased in OIT+FOS compared to OIT.

Both genera approximated the relative abundance observed in the sham-sensitized controls. Even though Clostridiales are recognized as the main commensals in the healthy murine colon [54] and *Oscillospira* have the ability to produce butyrate from host glycans or animal-derived sugar structures [55], our observations suggest that an increased abundance is associated with the allergic phenotype induced by sensitization and sustained by OIT. The use of host glycans, a major component of the mucus layer in the gut, represents a potential mechanism for involvement of *Oscillospira* in promoting the allergic phenotype, since disruption of the mucus layer might result in increased intestinal permeability and activation of mucosal immune cells including mast cells. In addition, negative correlations were observed between the abundance of *Oscillospira* and butyric- and propionic acid concentrations in the mice. Our observations are potentially in contrast with previously reported data obtained from an experimental food allergy model showing a pronounced role for Clostridiales clusters XIVa, IV, and XVIII in dampening inflammatory and allergic responses [56]. In addition, high levels of peanut-specific and total IgE and symptoms of anaphylaxis upon challenge in sensitized germ-free mice could be abrogated in mice colonized with commensal Clostridiales [57]. Nevertheless, the current study is the first to describe microbial changes in relation to OIT and FOS supplementation in allergic mice. To date, the opinion on Clostridiales with regard to favorable outcomes in food allergy studies in humans states 'mostly beneficial'; however, heterogeneity in study protocols, diagnostic techniques and dietary composition restrict clear recommendations to date [58]. In future experiments, shotgun metagenomics will be required to detect specific genus and species taxonomic annotation, to better understand microbial changes associated with unclassified *Clostridiales*, as observed in the current study.

The strategy to combine OIT and FOS supplementation to treat cow's milk allergic mice induced a specific microbial profile in faecal samples, since limited similarity in bacterial community structures was observed compared to either OIT-treated mice or FOS-treated mice. An increase in abundance of the genus Lactobacillus (family Lactobacillaceae, phylum Firmicutes) was observed in OIT+FOS faecal samples compared to FOS faecal samples, with levels approximating the abundance in non-allergic mice. Amongst others, Lactobacillus species are used as probiotics in experimental models for the prevention of food allergy or allergic asthma [59, 60] and were shown to support natural resolution of CMA in infants fed extensively hydrolyzed casein formula in a randomized trial [61]. Presence of Lactobacilli in the gastrointestinal tract is associated with host-health and reduced development of allergies [62]. Interestingly, the relative abundance of the phylum Proteobacteria was significantly increased in faecal samples derived from mice subjected to OIT

compared to OIT+FOS. With regard to the available literature concerning gut dysbiosis and Proteobacteria [12-15], we hypothesize that a causal relationship exists between OIT and the suboptimal clinical outcomes we observed in cow's milk allergic mice [6] and the increased abundance of Proteobacteria. One of the underlying mechanisms triggering Proteobacteria flares includes colonic epithelial dysfunction. Colonic epithelial cells are a main source of cellular oxygen [63] in an otherwise anaerobic gut environment and any disruption in the amount of oxygen diffusing into the lumen can alter the anaerobiosis and thereby the growth of opportunistic facultative anaerobes, including the Proteobacteria, in expense of the obligate commensal anaerobes, including the Firmicutes and Bacteroidetes [64].

As observed upon PICRUSt analysis, the allergic phenotype, but not the FOS supplemented diet, appears to be associated with an increased functional potential of the microbial community in relation to several metabolic pathways linked to starch and fatty acid metabolism. Taken together, the predictive functionality assessment inferred that the induction of the food allergic immune response increased bacterial-driven metabolic pathways in the gut, since the majority of up-regulated pathways occurred in whey-sensitized mice. It has been described that resolution of CMA was associated with decreased expression of genes involved in fatty acid metabolism by the gut microbiome [20]. The current data showing decreased metabolic activity (i.e., fatty acid metabolism or starch fermentation) upon FOS supplementation in sensitized mice, suggest that prebiotic fibers help to restore a balanced state of the bacterial metabolome, rather than stimulating metabolic processes. However, future studies using shotgun metagenomics are required to confirm the results of the PICRUSt analysis.

Together, the composition of the gut microbiome and fermentation of both carbohydrates and dietary proteins determine the availability of specific bio-active compounds (e.g., SCFA) in local and peripheral tissues via uptake in the systemic circulation [65, 66]. In the current study, SCFA concentrations in cecum content were elevated in mice treated with the combination OIT+FOS, rather than FOS supplementation or OIT alone. We questioned whether the altered (allergic) immune response affected bacterial metabolic activity and thereby the availability of SCFA. Interestingly, correlation analyses revealed that none of the bacterial communities detected in murine faecal samples were positively correlated to butyric acid levels. It might point out that the increased butyric acid concentrations observed in OIT+FOS mice originate from indirect fermentative reactions that convert acetic acid into butyric acid [67]. Furthermore, a positive correlation was observed with the relative abundance of lactic acid-producing bacteria and the butyric acid/SCFA ratio. This

observation indicates another indirect mechanism of butyric acid production, since lactic acid can be converted into butyric acid by members of the Clostridiales cluster (Firmicutes) [43]. Concisely, OIT+FOS treatment in cow's milk allergic mice might stimulate the activity of bacteria that either produce acetic acid and lactic acid, and/or convert acetic acid and lactic acid, rather than butyrate-producing bacteria.

Bone marrow precursors derived from FOS supplemented mice and cultured *in vitro* for 6 weeks, showed a reduction in the percentage of mast cells: expression of the FcεRI and c-Kit receptors was lost in a subset of cultured cells with an increase in percentage observed upon continuation of the culture. In accordance, IgE-mediated degranulation upon DNP-HSA activation was reduced. These observations suggest a suboptimal development or lifespan of mast cells derived from precursors that have been exposed to bacterial metabolites upon FOS fermentation *in vivo*. FOS supplementation indeed caused an increase in the relative abundance of the butyrate-producing *Allobaculum* genus (Firmicutes). In addition, expression of fatty acid receptor GPR43 on mucosal mast cells has been observed in rat and human colon tissue [68, 69]. The bone marrow is an open system and systemically available bacterial metabolites and cytokines can influence the development of immune precursor cells, with wide-spread consequences for both health and disease of the host [70]. Bone marrow-derived DC (BMDC) from *Lactobacillus johnsonii*-supplemented mice showed altered cytokine production and reduced CD4+ T cell activation upon respiratory syncytial virus (RSV) infection. Moreover, co-incubation of BMDC with plasma derived from *L. johnsonii*-supplemented mice or docasahexanoic acid (DHA) could mimic this effect [71]. Although further research is necessary, impaired mast cell functionality via epigenetic changes might support long-term outcomes of immunotherapy strategies (**Figure 10**).

CONCLUSION

The combination of OIT+FOS to treat cow's milk allergic mice altered microbial diversity and metabolism and induced a microbial profile closely linked to non-allergic control mice. Moreover, correlation analysis revealed potential indirect butyric acid pathways via acetic acid and lactic acid, rather than stimulation of butyric acid-producing bacteria upon OIT+FOS treatment. Improved treatment efficacy of OIT by FOS supplementation was previously only shown by reduced allergic symptoms upon food provocation, and the current study contributes to our understanding of the involvement of the gut microbiota. In addition, we showed impaired development of mast cells from bone marrow of FOS supplemented mice. Further research, using metagenomics, will be required to better understand the composition of the gut bacteria and it's functional/mechanistic roles and to link microbial profiles and availability of bacterial metabolites to the development of immune precursors originating from bone marrow that potentially influence long-term efficacy of OIT and FOS.

ACKNOWLEDGEMENTS

We would like to thank D. Verschoor for her valuable input and effort in the BMMC *in vitro* project.

REFERENCES

1. Nwaru BI, Hickstein L, Panesar SS, Roberts G, Muraro A, Sheikh A, Allergy EF, and Anaphylaxis Guidelines G. Prevalence of common food allergies in europe: A systematic review and meta-analysis. Allergy 2014. 69(8): p. 992-1007.
2. Venkataraman D, Erlewyn-Lajeunesse M, Kurukulaaratchy RJ, Potter S, Roberts G, Matthews S, and Arshad SH. Prevalence and longitudinal trends of food allergy during childhood and adolescence: Results of the isle of wight birth cohort study. Clin Exp Allergy 2018.
3. Pajno GB, Fernandez-Rivas M, Arasi S, Roberts G, Akdis CA, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, Ebisawa M, Eigenmann P, Knol E, Nadeau KC, Poulsen LK, van Ree R, Santos AF, du Toit G, Dhami S, Nurmatov U, Boloh Y, Makela M, O'Mahony L, Papadopoulos N, Sackesen C, Agache I, Angier E, Halken S, Jutel M, Lau S, Pfaar O, Ryan D, Sturm G, Varga EM, van Wijk RG, Sheikh A, Muraro A, and Group EAIG. Eaaci guidelines on allergen immunotherapy: Ige-mediated food allergy. Allergy 2017. p. 28(8):728-745.
4. Burks AW, Sampson HA, Plaut M, Lack G, and Akdis CA. Treatment for food allergy. J Allergy Clin Immunol 2018. 141(1): p. 1-9.
5. Tang ML, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, and Donath S. Administration of a probiotic with peanut oral immunotherapy: A randomized trial. J Allergy Clin Immunol 2015. 135(3): p. 737-44 e8.
6. Vonk MM, Diks MAP, Wagenaar L, Smit JJ, Pieters RHH, Garssen J, van Esch B, and Knippels LMJ. Improved efficacy of oral immunotherapy using non-digestible oligosaccharides in a murine cow's milk allergy model: A potential role for foxp3+ regulatory t cells. Front Immunol 2017. 8: p. 1230.
7. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, and Mackay CR. Dietary fiber and bacterial scfa enhance oral tolerance and protect against food allergy through diverse cellular pathways. Cell Rep 2016. 15(12): p. 2809-24.
8. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, and Relman DA. Diversity of the human intestinal microbial flora. Science 2005. 308(5728): p. 1635-8.
9. Peterson CT, Sharma V, Elmen L, and Peterson SN. Immune homeostasis, dysbiosis and therapeutic modulation of the gut microbiota. Clin Exp Immunol 2015. 179(3): p. 363-77.
10. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, Dore J, Corthier G, and Furet JP. The firmicutes/bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol 2009. 9: p. 123.
11. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, Cosnes J, Corthier G, Marteau P, and Dore J. Low

counts of faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis 2009. 15(8): p. 1183-9.
12. Shin NR, Whon TW, and Bae JW. Proteobacteria: Microbial signature of dysbiosis in gut microbiota. Trends Biotechnol 2015. 33(9): p. 496-503.
13. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB, Bousvaros A, Korzenik J, Sands BE, Xavier RJ, and Huttenhower C. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol 2012. 13(9): p. R79.
14. Keshavarzian A, Green SJ, Engen PA, Voigt RM, Naqib A, Forsyth CB, Mutlu E, and Shannon KM. Colonic bacterial composition in parkinson's disease. Mov Disord 2015. 30(10): p. 1351-60.
15. Huang YJ, Nariya S, Harris JM, Lynch SV, Choy DF, Arron JR, and Boushey H. The airway microbiome in patients with severe asthma: Associations with disease features and severity. J Allergy Clin Immunol 2015. 136(4): p. 874-84.
16. Strachan DP. Hay fever, hygiene, and household size. BMJ 1989. 299(6710): p. 1259-60.
17. McKenzie C, Tan J, Macia L, and Mackay CR. The nutrition-gut microbiome-physiology axis and allergic diseases. Immunol Rev 2017. 278(1): p. 277-295.
18. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, Ledford JG, Marques Dos Santos M, Anderson RL, Metwali N, Neilson JW, Maier RM, Gilbert JA, Holbreich M, Thorne PS, Martinez FD, von Mutius E, Vercelli D, Ober C, and Sperling AI. Innate immunity and asthma risk in amish and hutterite farm children. N Engl J Med 2016. 375(5): p. 411-421.
19. Fazlollahi M, Chun Y, Grishin A, Wood RA, Burks AW, Dawson P, Jones SM, Leung DYM, Sampson HA, Sicherer SH, and Bunyavanich S. Early-life gut microbiome and egg allergy. Allergy 2018.
20. Bunyavanich S, Shen N, Grishin A, Wood R, Burks W, Dawson P, Jones SM, Leung DYM, Sampson H, Sicherer S, and Clemente JC. Early-life gut microbiome composition and milk allergy resolution. J Allergy Clin Immunol 2016. 138(4): p. 1122-1130.
21. Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, Calignano A, Khan AA, Gilbert JA, and Nagler CR. Lactobacillus rhamnosus gg-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J 2016. 10(3): p. 742-50.
22. Koh A, De Vadder F, Kovatcheva-Datchary P, and Backhed F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell 2016. 165(6): p. 1332-1345.
23. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, and Marsland BJ. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 2014. 20(2): p. 159-66.

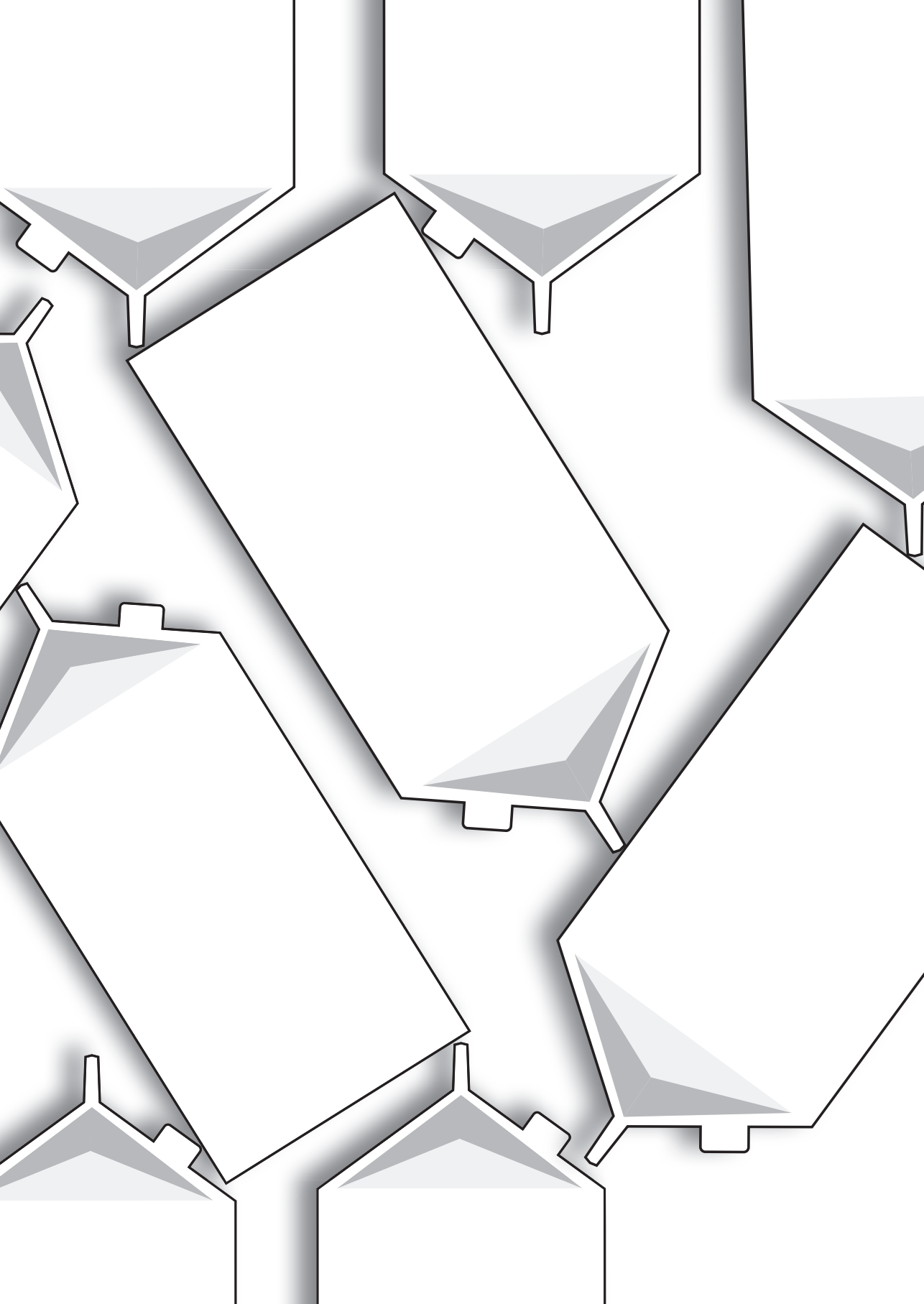
24. Wiener Z, Andrasfalvy M, Pallinger E, Kovacs P, Szalai C, Erdei A, Toth S, Nagy A, and Falus A. Bone marrow-derived mast cell differentiation is strongly reduced in histidine decarboxylase knockout, histamine-free mice. Int Immunol 2002. 14(4): p. 381-7.
25. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, Gilbert JA, Jansson JK, Caporaso JG, Fuhrman JA, Apprill A, and Knight R. Improved bacterial 16s rrna gene (v4 and v4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems 2016. 1(1).
26. Green SJ, Venkatramanan R, and Naqib A. Deconstructing the polymerase chain reaction: Understanding and correcting bias associated with primer degeneracies and primer-template mismatches. PLoS One 2015. 10(5): p. e0128122.
27. Schmieder R and Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics 2011. 27(6): p. 863-4.
28. Zhang J, Kobert K, Flouri T, and Stamatakis A. Pear: A fast and accurate illumina paired-end read merger. Bioinformatics 2014. 30(5): p. 614-20.
29. Edgar RC. Search and clustering orders of magnitude faster than blast. Bioinformatics 2010. 26(19): p. 2460-1.
30. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, and Knight R. Qiime allows analysis of high-throughput community sequencing data. Nat Methods 2010. 7(5): p. 335-6.
31. McDonald D, Clemente JC, Kuczynski J, Rideout JR, Stombaugh J, Wendel D, Wilke A, Huse S, Hufnagle J, Meyer F, Knight R, and Caporaso JG. The biological observation matrix (biom) format or: How i learned to stop worrying and love the ome-ome. Gigascience 2012. 1(1): p. 7.
32. Clarke KR. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 1993: p. 18, 117–143.
33. Team RDC, R: A language and environment for statistical computing. 2013: Vienna, Austria.
34. Oksanen JB, Kindt, R. , Package 'vegan', community ecology package. 2016.
35. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, and Huttenhower C. Predictive functional profiling of microbial communities using 16s rrna marker gene sequences. Nat Biotechnol 2013. 31(9): p. 814-21.
36. Kanehisa M and Goto S. Kegg: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000. 28(1): p. 27-30.
37. de Theije CG, Wopereis H, Ramadan M, van Eijndthoven T, Lambert J, Knol J, Garssen J, Kraneveld AD, and Oozeer R. Altered gut microbiota and activity

in a murine model of autism spectrum disorders. Brain Behav Immun 2014. 37: p. 197-206.
38. Jost L. Partitioning diversity into independent alpha and beta components. Ecology 2007. 88(10): p. 2427-39.
39. Morris EK, Caruso T, Buscot F, Fischer M, Hancock C, Maier TS, Meiners T, Muller C, Obermaier E, Prati D, Socher SA, Sonnemann I, Waschke N, Wubet T, Wurst S, and Rillig MC. Choosing and using diversity indices: Insights for ecological applications from the german biodiversity exploratories. Ecol Evol 2014. 4(18): p. 3514-24.
40. Lozupone CA, Hamady M, Kelley ST, and Knight R. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol 2007. 73(5): p. 1576-85.
41. Mandal RS, Saha S, and Das S. Metagenomic surveys of gut microbiota. Genomics Proteomics Bioinformatics 2015. 13(3): p. 148-58.
42. Belenguer A, Duncan SH, Calder AG, Holtrop G, Louis P, Lobley GE, and Flint HJ. Two routes of metabolic cross-feeding between bifidobacterium adolescentis and butyrate-producing anaerobes from the human gut. Appl Environ Microbiol 2006. 72(5): p. 3593-9.
43. Louis P and Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. Fems Microbiology Letters 2009. 294(1): p. 1-8.
44. Riviere A, Selak M, Lantin D, Leroy F, and De Vuyst L. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. Front Microbiol 2016. 7: p. 979.
45. Abrahamsson TR, Jakobsson HE, Andersson AF, Bjorksten B, Engstrand L, and Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. Clin Exp Allergy 2014. 44(6): p. 842-50.
46. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, Kuzeljevic B, Gold MJ, Britton HM, Lefebvre DL, Subbarao P, Mandhane P, Becker A, McNagny KM, Sears MR, Kollmann T, Investigators CS, Mohn WW, Turvey SE, and Finlay BB. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med 2015. 7(307): p. 307ra152.
47. Thompson-Chagoyan OC, Vieites JM, Maldonado J, Edwards C, and Gil A. Changes in faecal microbiota of infants with cow's milk protein allergy--a spanish prospective case-control 6-month follow-up study. Pediatr Allergy Immunol 2010. 21(2 Pt 2): p. e394-400.
48. Greetham HL, Gibson GR, Giffard C, Hippe H, Merkhoffer B, Steiner U, Falsen E, and Collins MD. Allobaculum stercoricanis gen. Nov., sp. Nov., isolated from canine feces. Anaerobe 2004. 10(5): p. 301-7.

49. Everard A, Lazarevic V, Gaia N, Johansson M, Stahlman M, Backhed F, Delzenne NM, Schrenzel J, Francois P, and Cani PD. Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. ISME J 2014. 8(10): p. 2116-30.
50. Raza GS, Putaala H, Hibberd AA, Alhoniemi E, Tiihonen K, Makela KA, and Herzig KH. Polydextrose changes the gut microbiome and attenuates fasting triglyceride and cholesterol levels in western diet fed mice. Sci Rep 2017. 7(1): p. 5294.
51. Zhang X, Zhao Y, Xu J, Xue Z, Zhang M, Pang X, Zhang X, and Zhao L. Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. Sci Rep 2015. 5: p. 14405.
52. Zhang X, Zhao Y, Zhang M, Pang X, Xu J, Kang C, Li M, Zhang C, Zhang Z, Zhang Y, Li X, Ning G, and Zhao L. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. PLoS One 2012. 7(8): p. e42529.
53. Rivas MN, Burton OT, Wise P, Zhang YQ, Hobson SA, Lloret MG, Chehoud C, Kuczynski J, DeSantis T, Warrington J, Hyde ER, Petrosino JF, Gerber GK, Bry L, Oettgen HC, Mazmanian SK, and Chatila TA. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. Journal of Allergy and Clinical Immunology 2013. 131(1): p. 201-212.
54. Nagano Y, Itoh K, and Honda K. The induction of treg cells by gut-indigenous clostridium. Curr Opin Immunol 2012. 24(4): p. 392-7.
55. Gophna U, Konikoff T, and Nielsen HB. Oscillospira and related bacteria - from metagenomic species to metabolic features. Environ Microbiol 2017. 19(3): p. 835-841.
56. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, and Honda K. Treg induction by a rationally selected mixture of clostridia strains from the human microbiota. Nature 2013. 500(7461): p. 232-6.
57. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, Tjota MY, Seo GY, Cao S, Theriault BR, Antonopoulos DA, Zhou L, Chang EB, Fu YX, and Nagler CR. Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci U S A 2014. 111(36): p. 13145-50.
58. Huang YJ, Marsland BJ, Bunyavanich S, O'Mahony L, Leung DY, Muraro A, and Fleisher TA. The microbiome in allergic disease: Current understanding and future opportunities-2017 practall document of the american academy of allergy, asthma & immunology and the european academy of allergy and clinical immunology. J Allergy Clin Immunol 2017. 139(4): p. 1099-1110.
59. Shin HS, Eom JE, Shin DU, Yeon SH, Lim

SI, and Lee SY. Preventive effects of a probiotic mixture in an ovalbumin-induced food allergy model. J Microbiol Biotechnol 2017. 28;28(1): p. 65-76

60. Wang X, Hui Y, Zhao L, Hao Y, Guo H, and Ren F. Oral administration of lactobacillus paracasei l9 attenuates pm2.5-induced enhancement of airway hyperresponsiveness and allergic airway response in murine model of asthma. PLoS One 2017. 12(2): p. e0171721.
61. Canani RB, Nocerino R, Terrin G, Coruzzo A, Cosenza L, Leone L, and Troncone R. Effect of lactobacillus gg on tolerance acquisition in infants with cow's milk allergy: A randomized trial. Journal of Allergy and Clinical Immunology 2012. 129(2): p. 580-U482.
62. Sepp E, Julge K, Vasar M, Naaber P, Bjorksten B, and Mikelsaar M. Intestinal microflora of estonian and swedish infants. Acta Paediatr 1997. 86(9): p. 956-61.
63. Rivera-Chavez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, Xu G, Velazquez EM, Lebrilla CB, Winter SE, and Baumler AJ. Depletion of butyrate-producing clostridia from the gut microbiota drives an aerobic luminal expansion of salmonella. Cell Host Microbe 2016. 19(4): p. 443-54.
64. Litvak Y, Byndloss MX, Tsolis RM, and Baumler AJ. Dysbiotic proteobacteria expansion: A microbial signature of epithelial dysfunction. Curr Opin Microbiol 2017. 39: p. 1-6.
65. Wypych TP and Marsland BJ. Diet hypotheses in light of the microbiota revolution: New perspectives. Nutrients 2017. 9(6).
66. Windey K, De Preter V, and Verbeke K. Relevance of protein fermentation to gut health. Mol Nutr Food Res 2012. 56(1): p. 184-96.
67. Louis P, Scott KP, Duncan SH, and Flint HJ. Understanding the effects of diet on bacterial metabolism in the large intestine. J Appl Microbiol 2007. 102(5): p. 1197-208.
68. Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, and Kuwahara A. Short-chain fatty acid receptor, gpr43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res 2006. 324(3): p. 353-60.
69. Karaki S, Tazoe H, Hayashi H, Kashiwabara H, Tooyama K, Suzuki Y, and Kuwahara A. Expression of the short-chain fatty acid receptor, gpr43, in the human colon. J Mol Histol 2008. 39(2): p. 135-42.
70. Lloyd CM and Marsland BJ. Lung homeostasis: Influence of age, microbes, and the immune system. Immunity 2017. 46(4): p. 549-561.
71. Fonseca W, Lucey K, Jang S, Fujimura KE, Rasky A, Ting HA, Petersen J, Johnson CC, Boushey HA, Zoratti E, Ownby DR, Levine AM, Bobbit KR, Lynch SV, and Lukacs NW. Lactobacillus johnsonii supplementation attenuates respiratory viral infection via metabolic reprogramming and immune cell modulation. Mucosal Immunol 2017. 10(6): p. 1569-1580.



CHAPTER 8

IN VITRO EVALUATION OF ALLERGEN-SPECIFIC IGE AS A BIOMARKER TO PREDICT THE ALLERGIC EFFECTOR RESPONSE AFTER ORAL IMMUNOTHERAPY WITH FRUCTO-OLIGOSACCHARIDE SUPPLEMENTATION IN A MURINE COW'S MILK ALLERGY MODEL

Marlotte M. Vonk[1,2]
Bart R.J. Blokhuis[1]
Daniëlle Verschoor[1]
Frank A. Redegeld[1]
Johan Garssen[1,2]
Léon M.J. Knippels[1,2]
Betty C.A.M. van Esch[1,2]

1. Department of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands
2. Immunology Platform, Danone Nutricia Research, Utrecht, the Netherlands

ABSTRACT

Background: In previous work, we showed that supplementation with prebiotics (fructo-oligosaccharides, FOS) improved the efficacy of oral immunotherapy (OIT) in cow's milk allergic mice. Thorough investigation of relevant biomarkers supports future application of the current treatment approach in food allergy patients.

Aim: To evaluate the capacity of the gold-standard biomarker allergen-specific IgE to monitor changes in the acute allergic effector response induced by OIT+FOS in an antigen-specific *in vitro* degranulation system.

Materials and methods: Female C3H/HeOuJ mice were sensitized intragastrically (i.g.) to the cow's milk protein whey and received either a control diet or a diet supplemented with a specific mixture of plant-derived FOS (1% w/w). OIT with 10 mg whey was provided 5x/week for three consecutive weeks per oral gavage. An intradermal (D64) and i.g. challenge (D70) were performed to measure acute allergic symptoms and mast cell degranulation. Serum was collected at three time points in the experiment [halfway IT (D50), after IT (D63) and after i.g. challenge (D71)] from a subgroup of each control and experimental group prior to sectioning. Serum samples were used *in vitro* to perform antigen-specific degranulation assays with a RBL-2H3 cell line and primary bone marrow-derived mast cells (BMMC). Total IgE, whey-specific IgE and IgG1, galectin-9 and galectin-9-IgE complex levels were determined by means of ELISA. The involvement of galectin-9 in effector cell suppression was studied with BMMC.

Results: Sensitization of RBL-2H3 cells with experimental mouse serum enabled whey-induced degranulation. A positive correlation was shown between release of β-hexosaminidase by whey-stimulated RBL-2H3 cells and the abundance of whey-specific IgE present in serum collected at D50 ($r=0.684$, $p<0.0001$) and D71 ($r=0.605$, $p=0.0004$). Incubation of DNP-specific IgE sensitized BMMC with experimental serum lowered activation with DNP-HSA and enabled degranulation with whey, indicating the difference in affinity for the FcεRI between DNP-specific IgE and whey-specific IgE. Mucosal mast cell degranulation induced by oral food provocation (D70) corresponded with *in vitro* degranulation levels of both RBL-2H3 and BMMC using experimental serum collected at D71. Galectin-9-IgE complexes were detected in experimental serum and recombinant galectin-9 was shown to reduce BMMC degranulation.

Conclusion: The current data demonstrate that the abundance of whey-specific IgE present in experimental mouse serum determined the whey-induced degranulation capacity of RBL-2H3 cells and BMMC. *In vitro* degranulation assays were shown to be a suitable model to monitor OIT+FOS induced (intermediate) changes in the *in vivo* allergic effector response induced by oral food provocation.

INTRODUCTION

The prevalence of food allergies has increased in Western countries over the last decades [1]. Food allergies have a severe impact on quality of life in patients and constitute a growing health, economic and social problem. Moreover, a recent review reported on the increased prevalence of food sensitization and oral food challenge-proven food allergies in developing countries in Africa and Asia, potentially mediated by economic growth and adaptation to a Western lifestyle [2]. Current options in food allergy management are limited to strict allergen avoidance and symptomatic treatment in case of adverse events.

To date, antigen-specific immunotherapy (AIT) is an effective treatment strategy for patients suffering from allergic rhinitis or asthma. International consensus exist on the safety and efficacy of subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) to induce tolerance toward allergens causing respiratory allergies and venom hypersensitivities [3]. In contrast, concerns with regard to the application of AIT in food allergies or atopic dermatitis remain, due to the high incidence of adverse local and systemic reactions and the need for long-term continuous exposure to the allergen [4]. Oral immunotherapy (OIT) to treat food allergies has been studied extensively and substantial benefit for patients suffering from hen's egg, peanut or cow's milk allergy has been shown upon continuous treatment [5]. However, a lack of high-quality randomized controlled trials and limited evidence on effectiveness post-discontinuation of therapy hamper clear recommendations for clinical use [6]. Thus far, OIT for food allergy should be performed in specialized centers with access to rescue medication in case of systemic anaphylaxis.

Attempts to improve the efficacy and safety profile of AIT include the identification of new biomarkers that can be used to predict or monitor effectiveness of the therapy [7]. Quantitative and validated biomarkers which rely on non-invasive methods can contribute to the development of safe and well-defined AIT protocols. A consensus statement described six domains of potential biomarkers for allergen immunotherapy: 1) antibodies (IgE, IgG4 and IgA), 2) serum inhibitory activity of IgE [IgE-Facilitated Allergen Binding (FAB)], 3) basophil activation, 4) cytokines and chemokines, 5)

cellular factors (T, B and dendritic cells) and 6) *in vivo* biomarkers upon provocation [8]. The gold-standard biomarker IgE (specific IgE, total IgE and/or specific IgE/total IgE ratio) has been used as an inclusion criterion for AIT studies and serves as a primary outcome for efficacy in respiratory allergies [9]. In addition, assessment of specific IgE in serum prior to SLIT in allergic patients was shown to correlate with responsiveness to the therapy [10]. In a cohort of children and teenagers aged 5-18 years with a diagnosis of IgE-mediated egg allergy, the ovalbumin-specific IgE/total IgE ratio appeared to be the most suitable biomarker to predict natural tolerance development [11]. Furthermore, baseline and endpoint allergen-specific IgE levels were lowered in peanut allergic subjects with successful sustained unresponsiveness (12 out of 24) after 5 years of OIT [12]. Specific tolerance induction to cow's milk proteins was successful in 30 out of 30 allergic subjects after one year of treatment and in 15 out of these 30 subjects cow's milk-specific IgE was significantly lowered compared to baseline [13]. In addition, the baseline cow's milk-specific IgE/total IgE ratio correlated to sustained unresponsiveness outcomes in a placebo-controlled OIT trial with cow's milk allergic subjects either or not pre-treated with omalizumab [14, 15].

Combinations of OIT and adjunct therapies (e.g., omalizumab, probiotics, prebiotics) to treat food allergies are currently under investigation. Supplementation with *Lactobacillus rhamnosus* CGMCC during peanut OIT induced sustained unresponsiveness in 82.1% of the treated individuals (1-10 years of age) and a decrease in peanut-specific IgE was observed [16]. In previous work, we showed enhanced desensitization in cow's milk allergic mice subjected to OIT in combination with a diet supplemented with a specific mixture of short-chain and long-chain fructo-oligosaccharides (scFOS/lcFOS, 9:1, 1% w/w, FOS) [17]. An initial increase in whey-specific IgE was shown during OIT independent of the dietary intervention with FOS. Oral food provocation extensively increased whey-specific IgE in serum of sensitized control mice, but not in mice treated with OIT and FOS supplementation [17]. In addition, mucosal mast cell degranulation measured as serum mMCP-1 (mucosal mast cell protease-1) was significantly decreased after OIT+FOS. In the current chapter, we aimed to examine whether changes in whey-specific IgE levels correspond to the reduced *in vivo* allergic effector response observed after OIT+FOS in mice and thus functions as a predictive biomarker. Confirmation of the predictive value of allergen-specific IgE for treatment efficacy might support future translation of the current treatment strategy to a human setting. Moreover, a functional IgE-mediated degranulation assay could be used in addition to the gold-standard biomarker specific IgE to monitor (intermediate) responses during immunotherapy strategies.

We used two *in vitro* degranulation models with either bone marrow-derived mast cells (BMMC) or rat basophil leukemia cells (RBL-2H3) and collected experimental serum at three time points during an animal experiment in which mice were treated with OIT and FOS supplementation: halfway immunotherapy (D50), after immunotherapy (D63) and after intragastric (i.g.) food challenge (D71). The current *in vitro* approach is based on antigen-specific degranulation mediated by specific IgE and allows mimicking of the *in vivo* acute allergic degranulation response [18]. Moreover, the effect of OIT+FOS on serum factors with allergy protective capacities in cow's milk allergic mice can be studied into more detail with this method. Specifically, we investigated the potential involvement of galectin-9 in suppression of mast cell activation. Galectin-9, a β-galactoside-binding protein, is a member of the lectin-family with glycan recognition capacities and is described to be involved in regulation of mast cell functionality [19, 20]. Previous studies performed by our group and others have shown a potential role for galectin-9 in enhanced efficacy of immunotherapy strategies for both food allergy and asthma treatment in mice [17, 21].

MATERIALS AND METHODS

Mice

6-week-old female specific-pathogen-free C3H/HeOuJ mice were purchased at Charles River Laboratories (Erkrath, Germany). All mice were randomly assigned to the following groups: sham-sensitized control, sham (n=5/subgroup); whey-sensitized control, sens (n=8/subgroup); FOS supplemented group, FOS (n=8/subgroup); OIT group, OIT (n=8/subgroup) and the combination group of OIT and FOS supplementation, OIT+FOS (n=8/subgroup). The mice were housed in filter-topped macrolon cages at the animal facility of Utrecht University (one cage/subgroup) and acclimatized for 6 days. All mice had unlimited access to food and water and were on a 12 h light/dark cycle. The experimental procedures were approved by the Ethical Committee of Animal research of Utrecht University and conducted according to the principles of good laboratory animal care as stated in the European Directive for the protection of animals used for scientific purposes.

Diet

All mice were fed the semi-purified cow's milk protein-free pelleted AIN-93G control diet during acclimatization and sensitization. From D35, the FOS and OIT+FOS groups received the AIN-93G diet supplemented with a specific mixture of plant-derived short-chain fructo-oligosaccharides (scFOS: oligofructose, Raftilose P95, degree of

polymerization (DP) <6) and long-chain fructo-oligosaccharides (lcFOS: long-chain inulin, Raftiline HP, average DP of 23 or higher with <1% DP of 5 or lower) in a 9:1 ratio (scFOS/lcFOS, 1%, w/w, Ssniff Spezialdiäten GmbH, Soest, Germany) [17]. Fructo-oligosaccharides were provided by Orafti (Wijchen, the Netherlands).

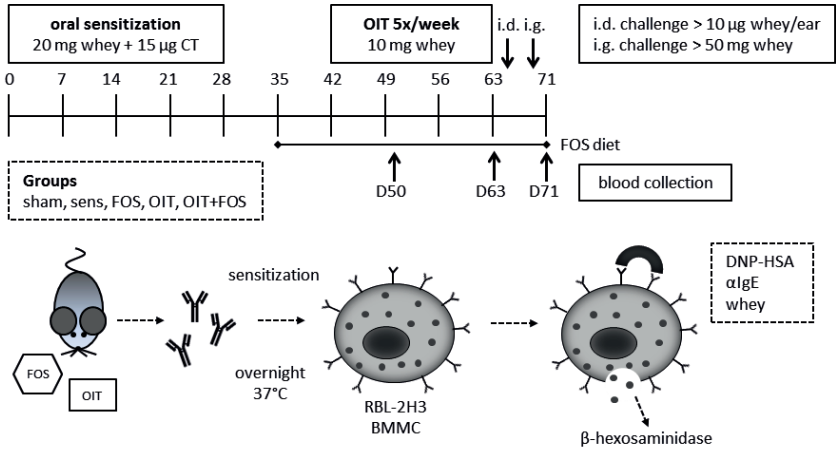


Figure 1. Experimental timeline, blood sample collection and schematic representation of *in vitro* degranulation assays. Female C3H/HeOuJ mice were randomly assigned to the following groups: sham-sensitized control, sham (n=5/subgroup); whey-sensitized control, sens (n=8/subgroup); FOS supplemented group, FOS (n=8/subgroup); OIT group, OIT (n=8/subgroup) and the combination group of OIT and FOS supplementation, OIT+FOS (n=8/subgroup). Mice were i.g. sensitized to the cow's milk protein whey (D0-D28) and fed either a control diet or a diet supplemented with a specific mixture of FOS from D35 to the end of the protocol. OIT was provided from D42-D59 (5x/week for 3 weeks). All mice were i.d. challenged at D64 to measure the acute allergic skin response, body temperature and severity of anaphylaxis symptoms. At D70, all mice were subjected to an oral food challenge and serum samples were collected 30 min post-challenge to measure mMCP-1 concentrations as a marker for mucosal mast cell degranulation. At three time points in the animal experiment (D50, D63 and D71), serum samples were collected from subgroups of each control and experimental group prior to sectioning to perform *in vitro* antigen-specific degranulation assays as depicted in the schematic representation. RBL-2H3 cells and BMMC were sensitized overnight with experimental mouse serum and subsequently activated with antigens to measure release of β-hex as a marker for the degranulation response. CT, cholera toxin; OIT, oral immunotherapy; i.g., intragastric; i.d., intradermal; FOS, fructo-oligosaccharides; RBL-2H3, rat basophil leukemia cell line, BMMC, bone marrow-derived mast cells; β-hex, β-hexosaminidase; DNP-HSA, dinitrophenol hapten conjugated to human serum albumin; mMCP-1, mucosal mast cell protease-1.

Experimental procedures and blood sample collection

Sensitization was conducted per oral gavage on experimental day 0, 7, 14, 21 and 28 using 20 mg whey (DMV International, Veghel, the Netherlands) in 0.5 ml PBS (Lonza, Verviers, Belgium) with 15 µg cholera toxin (CT, List Biological Laboratories Inc., Campbell, CA, USA) per mouse (**Figure 1**). Sham-sensitized control mice received CT dissolved in PBS. OIT (10 mg whey in 0.5 ml PBS) was provided 5x/week per oral gavage for three consecutive weeks (D42-D59) in the OIT and OIT+FOS groups. On D64, all mice were intradermally (i.d.) challenged to determine the acute allergic skin response, body temperature and symptom scores during anaphylaxis. All mice were anesthetized using inhalation of isoflurane to determine basal ear thickness (in duplicate in both ears) using a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). Subsequently, both ears were i.d. injected with 10 µg whey in 20 µl PBS. One hour after i.d. injection, ear thickness was again measured under anesthesia and Δ ear swelling was determined by subtracting mean basal ear thickness from mean ear thickness 1 h post-challenge. Body temperature was measured with a rectal thermometer 30 min after challenge and the severity of anaphylaxis symptoms was scored according to the method described previously [22]. On D70, all mice were i.g. challenged (50 mg whey in 0.5 ml PBS) and blood samples were collected via cheek puncture 30 min after challenge to measure mucosal mast cell degranulation (serum concentration of mMCP-1). Subgroups of mice from each control and experimental group were sectioned at D50 (halfway immunotherapy), D63 (after immunotherapy) and D71 (after i.g. challenge) and serum samples were collected and stored at -20°C until further analysis and *in vitro* experiments. Additional subgroups of mice were included in the animal experiment to perform histological analysis on the intestines and were treated in parallel to the control and experimental groups of the current study. To increase sample size, all mice participated in the i.d. and i.g. challenge. Depicted are the results of n=5-14 mice/group. In addition, serum samples used in the galectin-9 experiments were derived from a follow-up experiment with identical groups.

RBL-2H3 cell line culture and β-hexosaminidase degranulation assay

Rat basophil leukemia cells (RBL-2H3) were cultured in a 75 cm^2 culture flask with filter cap (Cellstar, Greiner Bio-One, Frickenhausen, Germany) in culture medium consisting of EMEM (Lonza) supplemented with 10% FCS and penicillin-streptomycin (100 U/ml-100 µg/ml, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) at 37°C and 5% CO_2. Release of β-hexosaminidase (β-hex) by RBL-2H3 cells was used as a marker for *in vitro* mast cell degranulation. Optimization experiments to

determine concentrations of experimental serum, antibodies [DNP-specific IgE (2,4 dinitrophenol-IgE) and αIgE] and antigens [DNP-HSA (DNP hapten conjugated to human serum albumin) and whey] were conducted previously to ensure optimal degranulation (data not shown). Concisely, RBL-2H3 cells were incubated overnight with 20% v/v experimental serum per individual mouse collected at D50, D63 or D71 in the animal experiment. Hereafter, cells were washed with assay medium (RPMI-1640 with 2.4 mM L-Glutamine and without phenol red (Lonza) supplemented with 1% FCS) and transferred to a sterile 96-wells plate (Costar, Corning Incorporated, Corning, NY, USA; 25,000 cells/well). Activation of IgE-sensitized RBL-2H3 cells with αIgE (1 µg/ml, eBioscience, Breda, the Netherlands) or whey (1 mg/ml, DMV international) for 3 h at 37°C and 5% CO_2 resulted in release of β-hex. Incubation with Triton X100 (TX100, Merck, Darmstadt, Germany) was used as 100% degranulation control and incubation with only assay medium was used to determine background levels of degranulation. After centrifugation (5 min 1500 rpm), supernatant was transferred to a new 96-wells culture plate and incubated for 1 h with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide solution (4MUG, Sigma-Aldrich) in 0.1 M citrate buffer (pH=4.7, Merck). The reaction was stopped with 0.2 M glycine-HCl buffer (Merck). The release of β-hex was measured by fluorescence (excitation 351 nm, emission 462 nm) with a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Release of β-hex in percentages was calculated with the following formula:

$$\%\ \text{release} = \left(\frac{\text{stimulated cells} - \text{unstimulated cells}}{\text{maximum release TX100} - \text{unstimulated cells}} \right) * 100$$

BMMC isolation, culture and β-hexosaminidase degranulation assay

Bone marrow cells were isolated from naïve female C3H/HeOuJ mice by flushing femurs and tibiae under sterile conditions with BMMC culture medium (RPMI-1640 medium (Lonza) supplemented with 10% FCS, 26 mM Hepes, 0.12 mM MEM non-essential amino acids, 2.4 mM Glutamax, 1.2 mM sodium pyruvate (all from Gibco, Thermo Fisher Scientific), penicillin-streptomycin (100 U/ml-100 µg/ml, Sigma-Aldrich). Cell suspensions were flushed through a 70-µm cell strainer and subsequently centrifuged (6 min 1200 rpm) and washed. Red blood cells were lysed (lysis buffer consisted of 1.5 M NH_4Cl, 0.1 M $NaHCO_3$, 10 mM EDTA (all from Merck) in demineralized water, filter sterilized) and remaining cells were dissolved in freeze medium consisting of 40% BMMC culture medium, 10% DMSO (Sigma-Aldrich) and 50% FCS. Aliquots of

cells were frozen at -80°C for at least 24 h and stored in liquid nitrogen until culture. BMMC were cultured in a 175 cm^2 culture flask with filter cap (Costar) in BMMC culture medium supplemented with IL-3 and Stem Cell Factor (SCF, both 10 ng/ml, Prospec, Ness-Ziona, Israel) at 37°C with 5% CO_2. After 5 weeks of differentiation and proliferation, BMMC were harvested and used in β-hex degranulation assays as described for RBL-2H3 cells. Prior to sensitization of BMMC, Fcγ receptors were blocked with non-specific IgG (Oxazolone) antibodies to avoid unwanted antigen-mediated crosslinking with IgE bound to Fcε receptors. In contrast to the RBL-2H3 experiments, BMMC were overnight sensitized with 10% v/v IgE specific for DNP from hybridoma clone 26.82. Subsequently, cells were incubated with 10% v/v pooled experimental serum collected at D50, D63 or D71 from all control and experimental groups for an additional 24 h at 37°C with 5% CO_2. After serum incubation, sensitized BMMC were activated with DNP-HSA, αIgE or whey in a concentration range to induce release of β-hex. In addition, a BMMC experiment was conducted to investigate the inhibitory effect of recombinant galectin-9 on degranulation capacity. Ionomycin stimulation (1 µM, Sigma-Aldrich) was used as a positive control for BMMC degranulation next to DNP-HSA activation (25 ng/ml). Recombinant galectin-9 (1 µM, R&D systems, Oxon, UK) was added prior to DNP-HSA activation. Galectin-9 was neutralized by adding lactose (20 mM, Acros Organics, Thermo Fisher Scientific) in the culture medium.

ELISA

Whole blood samples collected via cheek puncture prior to sectioning of the mice were centrifuged (10 min at 10,000 rpm) and serum was stored at -20°C until analysis of mMCP-1, galectin-9, galectin-9-IgE complexes, whey-specific IgE and IgG1 and total IgE by means of ELISA. Concentrations of mMCP-1 were determined in serum collected 30 min after i.g. challenge according to the manufacturer's instructions (Mouse MCPT-1 ELISA Ready-SET-Go kit, eBioscience). Galectin-9 and whey-specific antibodies were measured according to the method described previously [17]. Total IgE was measured according to the protocol described for whey-specific IgE except for coating. Concisely, 96-wells high-binding plates (Costar) were incubated overnight (4°C) with 100 µl of coating antibody (purified rat anti-mouse IgE, 1 µg/ml, BD Biosciences, Alphen a/d Rijn, the Netherlands) in coating buffer. To measure galectin-9 bound to IgE in serum, anti-galectin-9 coating antibody (0.75 µg/ml, mouse galectin-9 affinity purified polyclonal goat IgG antibody, R&D systems) in combination with anti-IgE detection antibody (1 µg/ml, BD Biosciences) was used.

Data analysis and statistics

Data are depicted as mean ± SEM and were analyzed using GraphPad Prism software version 7 (GraphPad software, La Jolla, CA, USA). We used one-way ANOVA and Bonferroni's post-hoc test to compare sham with sens; sens with FOS, OIT and OIT+FOS; FOS with OIT+FOS; OIT with OIT+FOS. The anaphylaxis symptom scores were analyzed using Kruskal-Wallis test for nonparametric data with Dunn's post-hoc test to compare the above mentioned combinations. Antibody data were log-transformed prior to testing. Pearson's correlation analysis was conducted with whey-specific IgE data and RBL-2H3 degranulation data per time point of serum collection. Calculated p-values were corrected for the number of comparisons and were considered statistically significant when $p<0.05$.

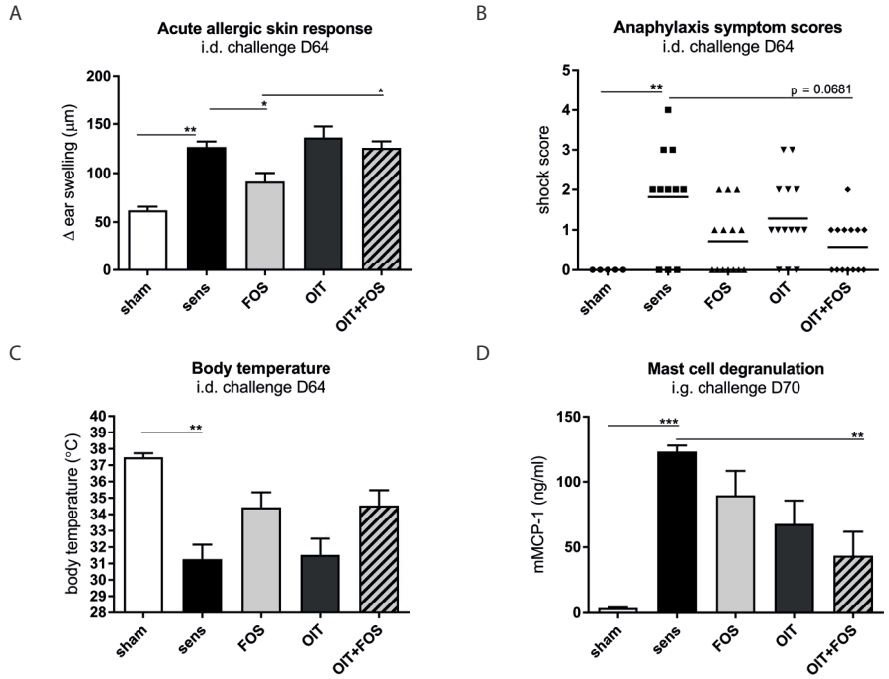


Figure 2. Acute allergic symptoms upon i.d. and i.g. food challenges. (A) Acute allergic skin responses measured as Δ ear swelling 1 h after i.d. injection of allergen in the ear (D64). **(B)** Anaphylaxis scores and **(C)** body temperature measured 30 min after i.d. challenge. **(D)** Serum mMCP-1 concentrations measured by means of ELISA as a marker for mucosal mast cell degranulation upon i.g. challenge at D70. Data are depicted as mean ± SEM, n=5-14 mice/subgroup due to the inclusion of additional groups of mice with similar treatments in the challenges. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. Anaphylaxis symptom scores in **(B)** were analyzed using Kruskal-Wallis test for nonparametric data with Dunn's post hoc test. *p<0.05, **p<0.01, ***p<0.001. OIT, oral immunotherapy; FOS, fructo-oligosaccharides; i.d., intradermal; i.g., intragastric; mMCP-1, mucosal mast cell protease-1.

RESULTS

The combination of OIT+FOS effectively reduced mucosal mast cell degranulation upon oral food provocation

I.g. sensitization to the cow's milk protein whey induced a significant increase in the acute allergic skin response (Δ ear swelling) (**Figure 2A**), severity of anaphylaxis symptoms (**Figure 2B**) and decreased body temperature levels (**Figure 2C**) in sensitized control mice compared to sham-sensitized control mice upon i.d. challenge. FOS supplementation without OIT, but not the combination of OIT+FOS, reduced the ear swelling response in sensitized mice in the current experiment (**Figure 2A**). A trend ($p=0.0681$) toward a decrease in anaphylaxis symptom scores was observed in the OIT+FOS mice compared to sensitized controls (**Figure 2B**). The anaphylaxis-associated drop in body temperature was not significantly altered in the experimental groups compared to the sensitized controls; however, FOS and OIT+FOS mice showed a less severe temperature drop (**Figure 2C**). Only OIT+FOS effectively reduced mucosal mast cell degranulation (mMCP-1 concentration in serum collected 30 min after challenge) compared to sensitized control mice upon oral food provocation (**Figure 2D**).

OIT and OIT+FOS prevented the challenge-induced increase in whey-specific IgE observed in sensitized controls

Serum samples were collected at three time points in the animal experiment: halfway immunotherapy (D50), after immunotherapy (D63) and after i.g. challenge (D71). Total IgE levels and whey-specific IgE and IgG1 levels were measured by means of ELISA to gain more insight into changes in antibody profiles during and after immunotherapy. At D50 (halfway immunotherapy), OIT+FOS mice showed elevated total and whey-specific IgE levels (**Figures 3A,B**), but no difference in whey-specific IgG1 was observed (**Figure 3C**). After three weeks of OIT (D63), treated mice showed increased serum levels of whey-specific IgE and IgG1 independent of FOS supplementation (**Figures 3E,F**). Total IgE levels in serum collected at D63 corresponded with whey-specific IgE and were elevated in sensitized controls, OIT and OIT+FOS mice (**Figure 3D**). All mice were subjected to an i.d. and i.g. challenge and serum levels of whey-specific IgE were significantly increased in sensitized control mice (**Figure 3H**). OIT and OIT+FOS prevented the observed increase in whey-specific IgE upon food provocation (**Figure 3H**). Differences in total IgE levels were less pronounced at time point D71 (**Figure 3G**) compared to whey-specific IgE (**Figure 3H**). Whey-specific IgG1 levels were similar to the previous time point (D63, after immunotherapy); however, levels seemed increased in sensitized control mice after challenge (**Figure 3I**).

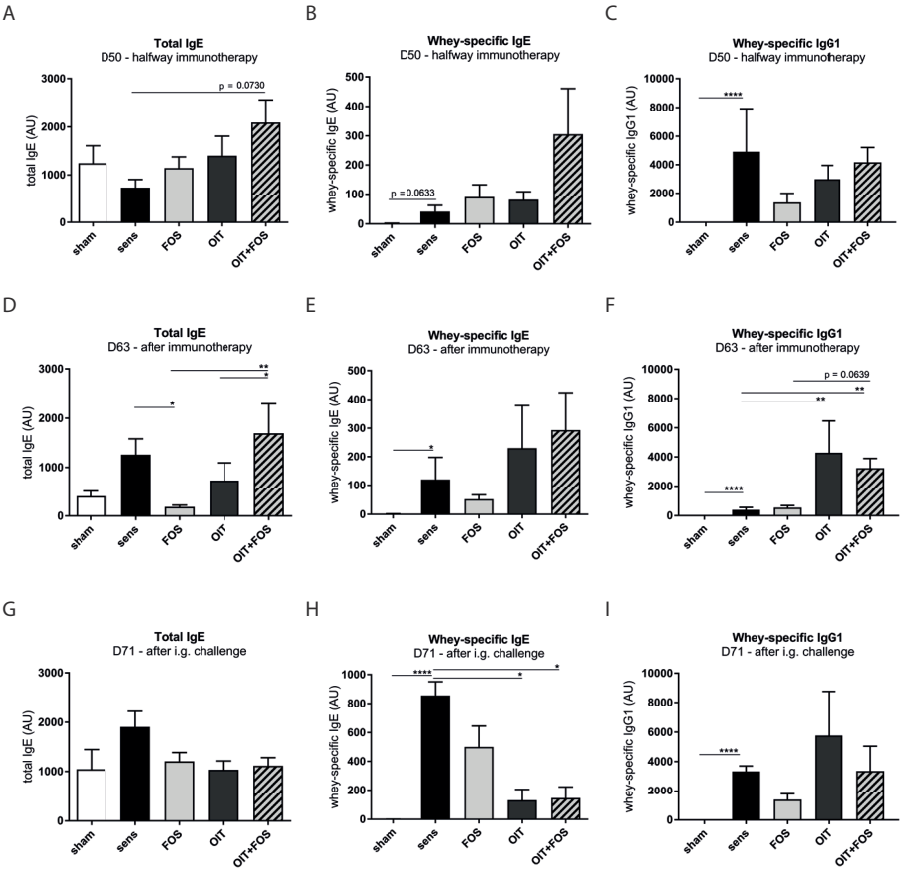


Figure 3. Levels of total IgE, whey-specific IgE and whey-specific IgG1 in serum collected at D50, D63 and D71 in the animal experiment. (A) Total IgE, **(B)** whey-specific IgE and **(C)** whey-specific IgG1 measured in serum collected halfway immunotherapy (D50). **(D)** Total IgE, **(E)** whey-specific IgE and **(F)** whey-specific IgG1 measured in serum collected after immunotherapy (D63). **(G)** Total IgE, **(H)** whey-specific IgE and **(I)** whey-specific IgG1 measured in serum collected after i.g. challenge (D71). Data are depicted as mean ± SEM, n=5-8 mice/subgroup. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. Antibody data was log-transformed prior to testing. *p<0.05, **p<0.01, ****p<0.0001. AU, arbitrary units; i.g., intragastric.

Sensitization of RBL-2H3 with experimental serum enabled whey-induced degranulation

Experimental serum collected during and after immunotherapy and challenges was used *in vitro* to sensitize RBL-2H3 overnight. IgE-sensitized RBL-2H3 were activated with αIgE or whey and release of β-hex was measured (**Figure 4**). Serum collected at D50 enabled RBL-2H3 degranulation upon activation with αIgE in all groups (**Figure 4A**); however, degranulation was significantly lowered with OIT+FOS serum compared to sensitized controls or FOS (**Figure 4A**). Whey activation increased RBL-2H3 degranulation after OIT and OIT+FOS serum sensitization (**Figure 4B**). A positive correlation ($r=0.684$, $p<0.0001$) was shown between whey-specific IgE levels in serum collected at D50 and the percentage β-hex release by RBL-2H3 upon whey stimulation (**Figure 4C**). No pronounced differences in either αIgE- or whey-induced RBL-2H3 degranulation were observed after sensitization with serum collected at D63 (after immunotherapy, **Figures 4D-F**). D71 serum (collected after i.g. challenge) induced a significant reduction in degranulation upon activation with αIgE in sensitized controls compared to sham-sensitized controls, OIT and OIT+FOS (**Figure 4G**). In contrast, whey-specific activation of RBL-2H3 sensitized with serum derived from sensitized controls significantly increased β-hex release compared to all groups (**Figure 4H**). As shown in **Figure 4I**, whey-specific IgE levels in serum collected at D71 were positively correlated to degranulation of RBL-2H3 upon whey stimulation ($r=0.605$, $p=0.0004$). Direct sensitization of BMMC with experimental serum collected at D71 resulted in similar β-hex concentrations upon whey stimulation as observed with RBL-2H3 cells, indicating that both cell types are suitable to study antigen-specific degranulation with the described approach (data not shown).

DNP-specific IgE sensitized BMMC incubated with experimental serum showed efficient degranulation upon whey stimulation

We sought to investigate whether serum factors (i.e., galectin-9) interfere with IgE-mediated degranulation of BMMC. Therefore, BMMC were sensitized with DNP-specific IgE prior to 24 h incubation with experimental serum collected at D50, D63 and D71. Subsequently, BMMC were activated with DNP-HSA, αIgE or whey and release of β-hex was measured (**Figure 5**). We hypothesized that the level of potential inhibitory factors in serum would differ between the control and experimental groups. However, incubation with serum for 24 h reduced DNP-HSA-induced BMMC degranulation in all groups, including the sham-sensitized control group which was

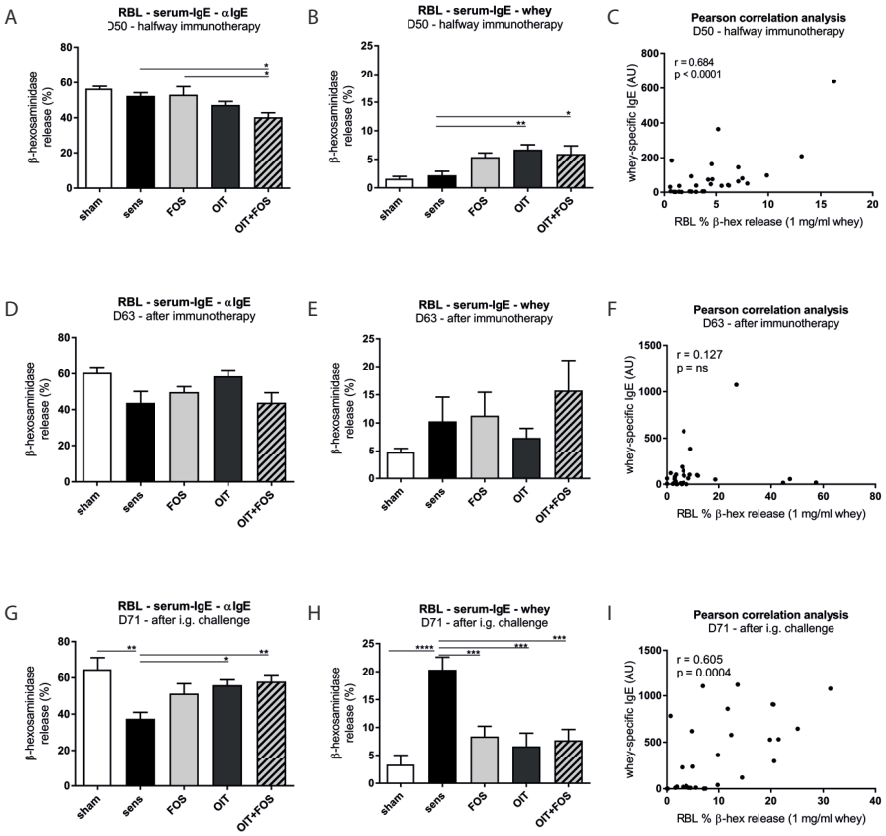


Figure 4. Beta-hexosaminidase release by RBL-2H3 sensitized overnight with experimental serum and activated with αIgE and whey. Beta-hex levels after overnight sensitization of RBL-2H3 using experimental mouse serum collected at D50 (halfway immunotherapy) and activated with **(A)** αIgE and **(B)** whey. **(C)** Pearson's correlation analysis with whey-specific IgE in serum (D50) and degranulation levels of RBL-2H3 sensitized with serum (D50) and activated with whey. Beta-hex degranulation assay using serum collected at D63 (after immunotherapy) upon activation with **(D)** αIgE and **(E)** whey. **(F)** Pearson's correlation analysis with whey-specific IgE in serum (D63) and degranulation levels of RBL-2H3 sensitized with serum (D63) and activated with whey. Beta-hex degranulation assay using serum collected at D71 (after i.g. challenge) upon activation with **(G)** αIgE and **(H)** whey. **(I)** Pearson's correlation analysis with whey-specific IgE in serum (D71) and degranulation levels of RBL-2H3 sensitized with serum (D71) and activated with whey. Data are depicted as mean ± SEM, serum samples per individual mouse were tested separately, n=5-8 mice/subgroup. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. β-hex, β-hexosaminidase; RBL, rat basophil leukemia cell line; i.g., intragastric.

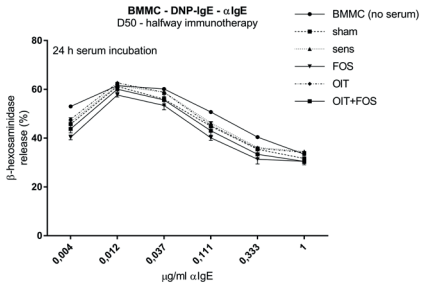
used as a reference, compared to the control condition without serum incubation (data not shown). BMMC activation with αIgE showed no difference with experimental serum collected at D50 (**Figure 5A**). Interestingly, whey-specific activation led to increased β-hex release in OIT+FOS samples (**Figure 5B**). All groups showed reduced β-hex release after incubation with serum collected at D63 and activation with αIgE (**Figure 5C**). Whey stimulated BMMC degranulation after incubation with serum from OIT and OIT+FOS mice (**Figure 5D**). Serum collected from sensitized control animals at D71 reduced αIgE-mediated degranulation of BMMC (**Figure 5E**). Whey stimulation led to increased degranulation of BMMC after incubation with serum derived from sensitized control and FOS mice (**Figure 5F**).

To circumvent the observed suppressive effect of 24 h serum incubation on DNP-HSA-mediated BMMC degranulation compared to the control condition without serum, we tested 1.5 h incubation with serum collected at D71 in DNP-specific IgE sensitized BMMC and again activated the cells with DNP-HSA, αIgE and whey. DNP-HSA activation did not show differences in degranulation capacity of sensitized BMMC exposed to experimental serum (**Figure 5G**). In accordance, no differences were observed with αIgE activation (data not shown). In contrast to 24 h serum incubation, whey stimulation was not able to activate BMMC after 1.5 h serum incubation (**Figure 5H**).

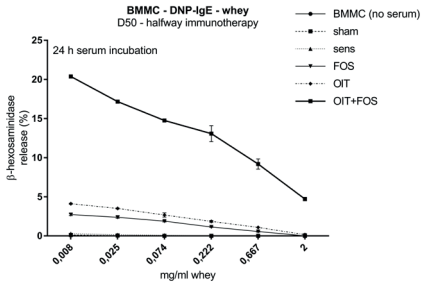
Galectin-9-IgE complexes were present in serum samples of cow's milk allergic mice

Galectin-9 concentrations in serum collected at D70 in a follow-up experiment were measured by means of ELISA. No significant differences between the groups were shown; however, galectin-9 levels had a tendency to be higher in OIT and OIT+FOS mice (**Figure 6A**). As shown in **Figure 6B**, galectin-9-IgE complexes were present in serum samples derived from all sensitized groups, except for sham-sensitized control samples. A significant difference between sham-sensitized controls and whey-sensitized controls was observed (**Figure 6B**). To investigate the biological effect of galectin-9 on DNP-HSA activation of DNP-specific IgE sensitized BMMC, recombinant galectin-9 was added at 1 μM. The presence of recombinant galectin-9 resulted in decreased degranulation capacity, and the inhibitory effect was abrogated by adding lactose to the culture medium (**Figure 6C**).

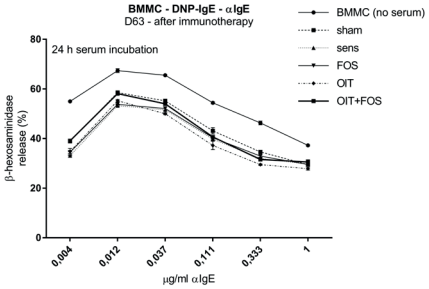
A


BMMC - DNP-IgE - αIgE
D50 - halfway immunotherapy
24 h serum incubation
β-hexosaminidase release (%)
μg/ml αIgE
0,004
0,012
0,037
0,111
0,333
1
BMMC (no serum)
sham
sens
FOS
OIT
OIT+FOS

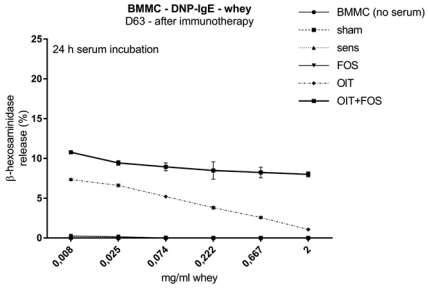
B


BMMC - DNP-IgE - whey
D50 - halfway immunotherapy
24 h serum incubation
β-hexosaminidase release (%)
mg/ml whey
0,008
0,025
0,074
0,222
0,667
2
BMMC (no serum)
sham
sens
FOS
OIT
OIT+FOS

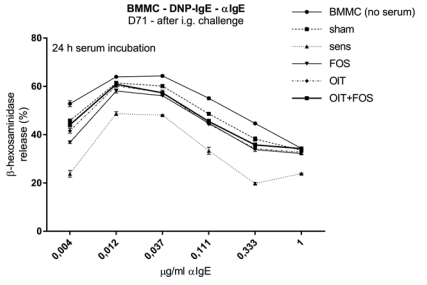
C


BMMC - DNP-IgE - αIgE
D63 - after immunotherapy
24 h serum incubation
β-hexosaminidase release (%)
μg/ml αIgE
0,004
0,012
0,037
0,111
0,333
1
BMMC (no serum)
sham
sens
FOS
OIT
OIT+FOS

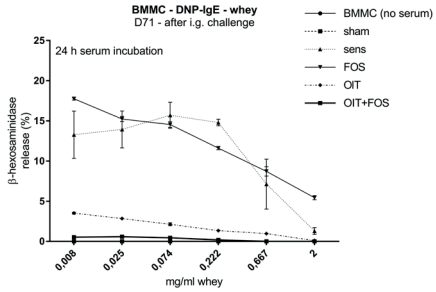
D


BMMC - DNP-IgE - whey
D63 - after immunotherapy
24 h serum incubation
β-hexosaminidase release (%)
mg/ml whey
0,008
0,025
0,074
0,222
0,667
2
BMMC (no serum)
sham
sens
FOS
OIT
OIT+FOS

E


BMMC - DNP-IgE - αIgE
D71 - after i.g. challenge
24 h serum incubation
β-hexosaminidase release (%)
μg/ml αIgE
0,004
0,012
0,037
0,111
0,333
1
BMMC (no serum)
sham
sens
FOS
OIT
OIT+FOS

F


BMMC - DNP-IgE - whey
D71 - after i.g. challenge
24 h serum incubation
β-hexosaminidase release (%)
mg/ml whey
0,008
0,025
0,074
0,222
0,667
2
BMMC (no serum)
sham
sens
FOS
OIT
OIT+FOS

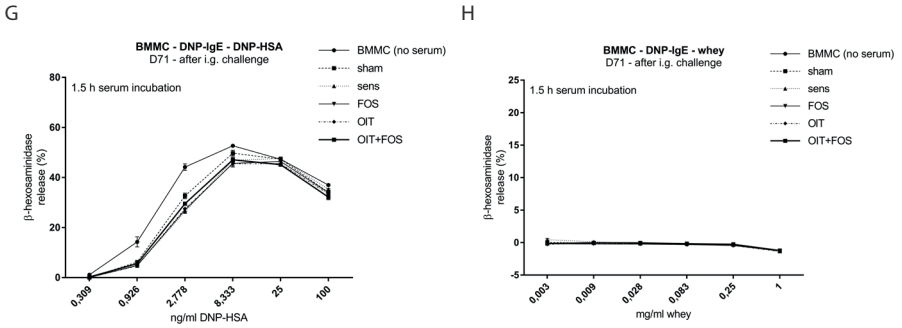


Figure 5. Beta-hexosaminidase release by DNP-specific IgE sensitized BMMC incubated 24 h with experimental serum and activated with αIgE and whey. Beta-hex release by DNP-specific IgE sensitized BMMC after 24 h incubation with experimental mouse serum collected at D50 (halfway immunotherapy) upon activation with **(A)** αIgE and **(B)** whey. Beta-hex degranulation assay after 24 h incubation with serum collected at D63 (after immunotherapy) and activation with **(C)** αIgE and **(D)** whey. Beta-hex degranulation assay after 24 h incubation with serum collected at D71 (after i.g. challenge) and activation with **(E)** αIgE and **(F)** whey. Beta-hex release by DNP-specific IgE sensitized BMMC after 1.5 h incubation with experimental serum collected at D71 (after i.g. challenge) upon activation with **(G)** DNP-HSA and **(H)** whey. Data are depicted as mean ± SEM, serum samples per subgroup were pooled and measurements were performed in duplicate. BMMC, bone marrow-derived mast cells; β-hex, β-hexosaminidase; DNP-HSA, dinitrophenol hapten conjugated to human serum albumin; i.g., intragastric.

DISCUSSION

With the current *in vitro* approach we sought to evaluate the predictive value of the gold-standard biomarker allergen-specific IgE in relation to the acute allergic effector response in mice treated with OIT and a FOS supplemented diet. The abundance of whey-specific IgE in serum collected at D50 and D71 positively correlated with *in vitro* whey-induced degranulation of RBL-2H3 cells. In addition, *in vitro* degranulation was aligned with the observed *in vivo* degranulation response upon oral food provocation. Previous results have shown that repeated food challenges unintentionally affect whey-specific antibody patterns in sensitized control animals [23]. Moreover, the current study showed increased whey-specific IgG1 levels in sensitized control animals

upon dual food challenge. Hence, murine models of OIT might benefit from *ex vivo* functional assays that can either replace or reduce the number allergen challenges. Functional IgE-mediated assays, as described in the current chapter, can be used in addition to the gold-standard parameter allergen-specific IgE to monitor mast cell degranulation responses during and after immunotherapy strategies. Furthermore, functional read-outs linked to the specific IgE response might contribute to the improvement of (pre-)clinical OIT protocols, since immunotherapy efficacy can often only be evaluated upon food challenge after treatment.

We showed previously that FOS supplementation enhanced the efficacy of OIT shown by a reduction in the acute allergic skin response upon i.d. challenge and in mucosal mast cell degranulation after oral food provocation [17]. In the current study, FOS supplementation without OIT reduced the acute allergic skin response induced by i.d. challenge. Although these observations were not in agreement with previous results, they were aligned with the low IgE levels observed in serum collected from FOS supplemented mice one day prior to the i.d. challenge conducted at D64.

Sensitization using experimental serum derived from cow's milk allergic mice resulted in effective degranulation upon whey stimulation in both RBL-2H3 and BMMC cultures, though with lower efficiency compared to the combination of DNP-specific IgE and DNP-HSA-induced degranulation. Whey-induced degranulation patterns were linked to whey-specific IgE abundance in serum samples. Interestingly, overnight incubation of DNP-specific IgE sensitized BMMC with experimental serum showed similar whey-induced degranulation patterns, despite the fact that the FcεRI were saturated in advance. No β-hex release upon whey activation was observed after 1.5 h incubation with experimental serum. Hence, we hypothesized that the affinity of whey-specific IgE for the FcεRI may be higher compared to DNP-specific IgE and it has the ability to bind to the FcεRI in expense of DNP-specific IgE during the (longer) incubation of 24 h. Another possible explanation might be that simply the excess of whey-specific IgE in serum leads to replacement of DNP-specific IgE by whey-specific IgE. These phenomena in combination with the observation that serum incubation decreased DNP-HSA-mediated degranulation in all groups, including the sham-sensitized control that was used as a reference, hindered our attempts to investigate the effect of inhibitory factors (i.e., intestinal epithelium-derived galectin-9) present in serum samples. It was shown that BMMC degranulation was primarily dependent on whey-specific IgE abundance in serum.

We aimed to study the role of galectin-9 in inhibition of BMMC degranulation, since galectin-9 has been described to bind strongly and specifically to IgE via glycosylated binding sites and can thus prevent IgE-antigen complex formation [24]. We confirmed by means of ELISA that galectin-9-IgE complexes are present in serum of cow's milk allergic mice regardless of treatment and hypothesized that galectin-9 interferes with *in vivo* mast cell degranulation via this mechanism. With the current approach, we were not able to distinguish between IgE and whey-specific IgE; however, the absence of galectin-9-IgE complexes in sham-sensitized serum samples suggests that galectin-9 is bound to whey-specific IgE. Interestingly, OIT+FOS samples showed reduced levels of galectin-9-IgE complexes and higher levels of galectin-9, suggesting that the majority of the galectin-9 proteins was present in free form. Investigation of functionality of galectin-9 and/or galectin-9-IgE complexes present in murine serum samples was hampered by the relatively low (free) galectin-9 concentrations. Galectin-9-mediated inhibition of RBL-2H3 activation was previously shown within the 0.1-1 μM range [24], and we observed galectin-9 concentrations within a 20-fold lower molarity range in experimental mouse serum. A pilot experiment using plate-bound DNP-HSA and DNP-specific IgE sensitized BMMC to ensure suboptimal degranulation, could not show serum galectin-9-mediated inhibition of degranulation. Nevertheless, we confirmed the inhibitory effect of recombinant galectin-9 (1 μM) on BMMC degranulation and showed that neutralization of galectin-9 using lactose abrogated this effect, as described in previous studies [24, 25]. Recombinant galectin-9-mediated protection against asthma development and passive cutaneous anaphylaxis was observed in animal models [24], suggesting the use of galectin-9 as a therapeutic strategy in food allergy management.

CONCLUSION

In vitro degranulation assays using experimental mouse serum were shown to be a suitable method to monitor OIT+FOS-induced changes in the *in vivo* allergic effector response upon oral food provocation. The abundance of allergen-specific IgE positively correlated with degranulation capacity of RBL-2H3 activated with allergen. Serum galectin-9 is a potential candidate in OIT+FOS-induced reduction in mucosal mast cell degranulation; however, mechanistic studies should confirm this observation.

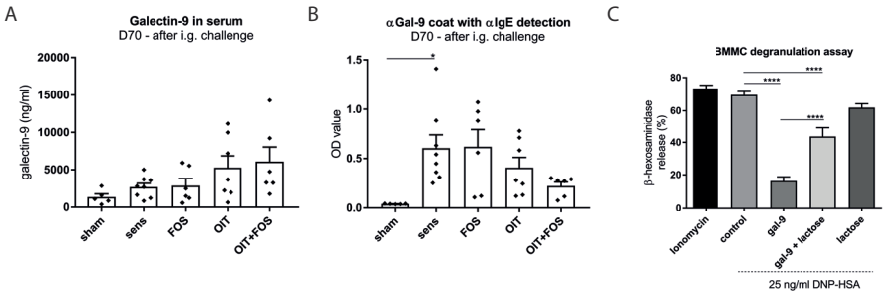


Figure 6. Galectin-9 concentrations and galectin-9-IgE complex levels in serum. (A) Galectin-9 concentrations measured by means of ELISA in serum samples collected at D70 in a follow-up experiment. **(B)** Galectin-IgE complex levels measured with αGalectin-9 coating antibody and αIgE detection antibody depicted in OD values. **(C)** Beta-hex degranulation assay with recombinant galectin-9 in the presence or absence of the neutralizing factor lactose. Data are depicted as mean ± SEM, n=5-8 samples/subgroup for ELISA and BMMC measurements were performed in duplicate. Statistical analysis was performed using one-way ANOVA and Bonferroni's post-hoc test to compare pre-selected combinations. *p<0.05, ****p<0.0001. OD, optical density; i.g., intragastric; gal-9, galectin-9; BMMC, bone marrow-derived mast cells.

REFERENCES

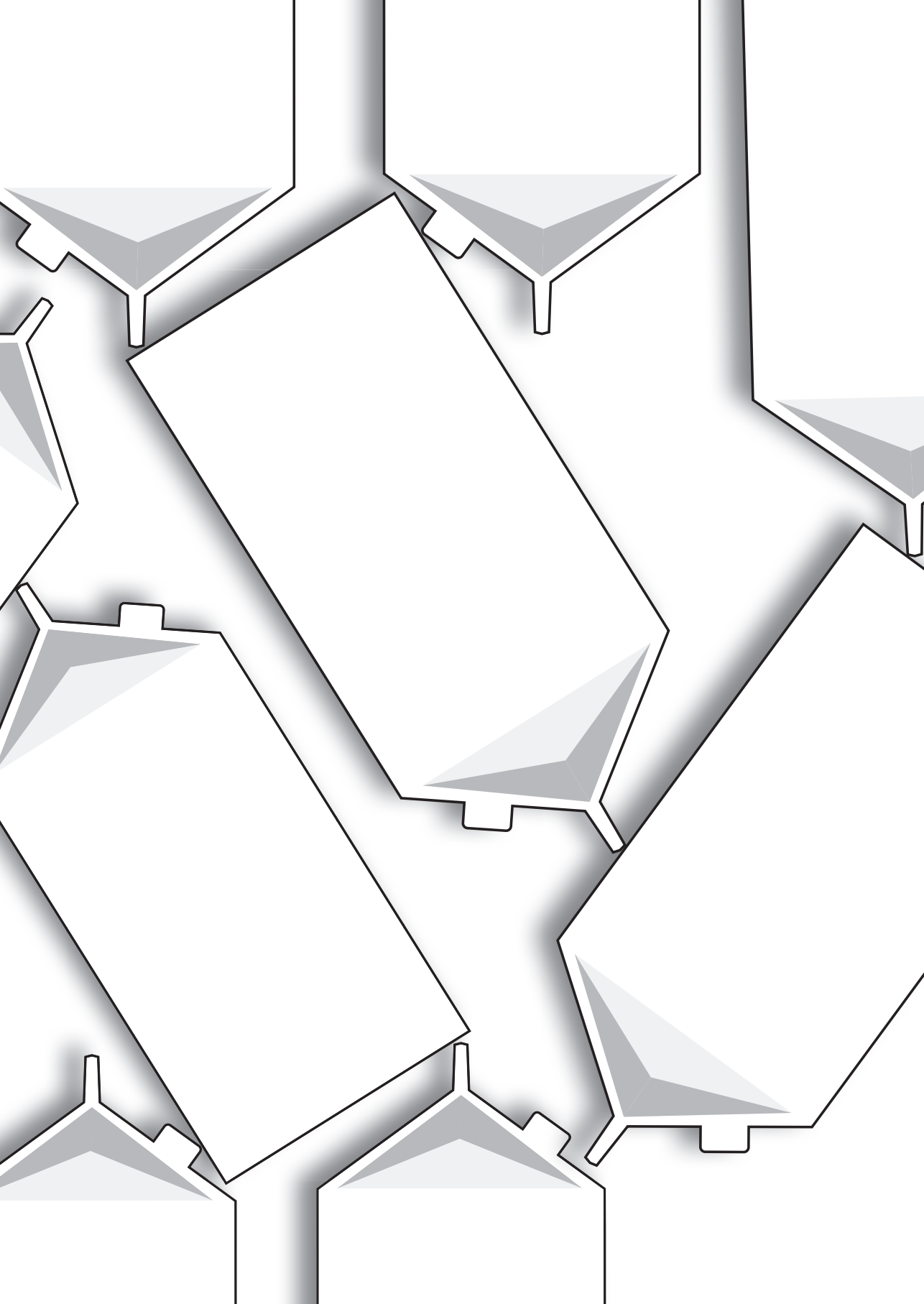
1. Sicherer SH and Sampson HA. Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention and management. J Allergy Clin Immunol 2017. 141(1): p. 41-58.
2. Leung AS, Wong GW, and Tang ML. Food allergy in the developing world. J Allergy Clin Immunol 2017. p. 141(1):76-78.e1.
3. Jutel M, Agache I, Bonini S, Burks AW, Calderon M, Canonica W, Cox L, Demoly P, Frew AJ, O'Hehir R, Kleine-Tebbe J, Muraro A, Lack G, Larenas D, Levin M, Nelson H, Pawankar R, Pfaar O, van Ree R, Sampson H, Santos AF, Du Toit G, Werfel T, Gerth van Wijk R, Zhang L, and Akdis CA. International consensus on allergy immunotherapy. J Allergy Clin Immunol 2015. 136(3): p. 556-68.
4. de Silva D, Geromi M, Panesar SS, Muraro A, Werfel T, Hoffmann-Sommergruber K, Roberts G, Cardona V, Dubois AE, Halken S, Host A, Poulsen LK, Van Ree R, Vlieg-Boerstra BJ, Agache I, Sheikh A, Allergy EF, and Anaphylaxis Guidelines G. Acute and long-term management of food allergy: Systematic review. Allergy 2014. 69(2): p. 159-67.
5. Pajno GB, Fernandez-Rivas M, Arasi S, Roberts G, Akdis CA, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, Ebisawa M, Eigenmann P, Knol E, Nadeau KC, Poulsen LK, van Ree R, Santos AF, du Toit G, Dhami S, Nurmatov U, Boloh Y, Makela M, O'Mahony L, Papadopoulos N, Sackesen C, Agache I, Angier E, Halken S, Jutel M, Lau S, Pfaar O, Ryan D, Sturm G, Varga EM, van Wijk RG, Sheikh A, Muraro A, and Group EAIG. Eaaci guidelines on allergen immunotherapy: Ige-mediated food allergy. Allergy 2017. p. 28(8):728-745.
6. Nurmatov U, Venderbosch I, Devereux G, Simons FE, and Sheikh A. Allergen-specific oral immunotherapy for peanut allergy. Cochrane Database Syst Rev 2012(9): p. CD009014.
7. Berings M, Karaaslan C, Altunbulakli C, Gevaert P, Akdis M, Bachert C, and Akdis CA. Advances and highlights in allergen immunotherapy: On the way to sustained clinical and immunologic tolerance. J Allergy Clin Immunol 2017. 140(5): p. 1250-1267.
8. Shamji MH, Kappen JH, Akdis M, Jensen-Jarolim E, Knol EF, Kleine-Tebbe J, Bohle B, Chaker AM, Till SJ, Valenta R, Poulsen LK, Calderon MA, Demoly P, Pfaar O, Jacobsen L, Durham SR, and Schmidt-Weber CB. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: An eaaci position paper. Allergy 2017. 72(8): p. 1156-1173.
9. Shamji MH and Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. J Allergy Clin Immunol 2017. 140(6): p. 1485-1498.
10. Ciprandi G and Silvestri M. Serum

specific ige: A biomarker of response to allergen immunotherapy. J Investig Allergol Clin Immunol 2014. 24(1): p. 35-9.

11. Vazquez-Ortiz M, Machinena-Spera A, Giner MT, Alvaro M, Piquer M, Dominguez O, Lozano J, Jimenez-Feijoo R, and Plaza AM. Ovalbumin-specific ige/total ige ratio improves the prediction of tolerance development in egg-allergic children aged >/=5 years. Pediatr Allergy Immunol 2015. 26(6): p. 580-3.
12. Vickery BP, Scurlock AM, Kulis M, Steele PH, Kamilaris J, Berglund JP, Burk C, Hiegel A, Carlisle S, Christie L, Perry TT, Pesek RD, Sheikh S, Virkud Y, Smith PB, Shamji MH, Durham SR, Jones SM, and Burks AW. Sustained unresponsiveness to peanut in subjects who have completed peanut oral immunotherapy. J Allergy Clin Immunol 2014. 133(2): p. 468-75.
13. Longo G, Barbi E, Berti I, Meneghetti R, Pittalis A, Ronfani L, and Ventura A. Specific oral tolerance induction in children with very severe cow's milk-induced reactions. J Allergy Clin Immunol 2008. 121(2): p. 343-7.
14. Frischmeyer-Guerrerio PA, Masilamani M, Gu W, Brittain E, Wood R, Kim J, Nadeau K, Jarvinen KM, Grishin A, Lindblad R, and Sampson HA. Mechanistic correlates of clinical responses to omalizumab in the setting of oral immunotherapy for milk allergy. J Allergy Clin Immunol 2017. 140(4): p. 1043-1053 e8.
15. Wood RA, Kim JS, Lindblad R, Nadeau K, Henning AK, Dawson P, Plaut M, and Sampson HA. A randomized, double-blind, placebo-controlled study of omalizumab combined with oral immunotherapy for the treatment of cow's milk allergy. J Allergy Clin Immunol 2016. 137(4): p. 1103-1110 e11.
16. Tang ML, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, and Donath S. Administration of a probiotic with peanut oral immunotherapy: A randomized trial. J Allergy Clin Immunol 2015. 135(3): p. 737-44 e8.
17. Vonk MM, Diks MAP, Wagenaar L, Smit JJ, Pieters RHH, Garssen J, van Esch B, and Knippels LMJ. Improved efficacy of oral immunotherapy using non-digestible oligosaccharides in a murine cow's milk allergy model: A potential role for foxp3+ regulatory t cells. Front Immunol 2017. 8: p. 1230.
18. Bogh KL, Kroghsbo S, Dahl L, Rigby NM, Barkholt V, Mills EN, and Madsen CB. Digested ara h 1 has sensitizing capacity in brown norway rats. Clin Exp Allergy 2009. 39(10): p. 1611-21.
19. de Kivit S, Kraneveld AD, Garssen J, and Willemsen LE. Glycan recognition at the interface of the intestinal immune system: Target for immune modulation via dietary components. Eur J Pharmacol 2011. 668 Suppl 1: p. S124-32.
20. Kojima R, Ohno T, Iikura M, Niki T, Hirashima M, Iwaya K, Tsuda H, Nonoyama S, Matsuda A, Saito H, Matsumoto K, and Nakae S. Galectin-9

enhances cytokine secretion, but suppresses survival and degranulation, in human mast cell line. Plos One 2014. 9(1).

21. Ikeda M, Katoh S, Shimizu H, Hasegawa A, Ohashi-Doi K, and Oka M. Beneficial effects of galectin-9 on allergen-specific sublingual immunotherapy in a dermatophagoides farinae-induced mouse model of chronic asthma. Allergology International 2017. 66(3): p. 432-439.
22. Li XM, Schofield BH, Huang CK, Kleiner GI, and Sampson HA. A murine model of ige-mediated cow's milk hypersensitivity. J Allergy Clin Immunol 1999. 103(2 Pt 1): p. 206-14.
23. Vonk MM, Wagenaar L, Pieters RHH, Knippels LMJ, Willemsen LEM, Smit JJ, van Esch B, and Garssen J. The efficacy of oral and subcutaneous antigen-specific immunotherapy in murine cow's milk- and peanut allergy models. Clin Transl Allergy 2017. 7: p. 35.
24. Niki T, Tsutsui S, Hirose S, Aradono S, Sugimoto Y, Takeshita K, Nishi N, and Hirashima M. Galectin-9 is a high affinity ige-binding lectin with anti-allergic effect by blocking ige-antigen complex formation. Journal of Biological Chemistry 2009. 284(47): p. 32344-32352.
25. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BC, Knol J, Sprikkelman AB, van der Aa LB, Knippels LM, Garssen J, van Kooyk Y, and Willemsen LE. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012. 67(3): p. 343-52.



CHAPTER 9

GENERAL DISCUSSION

Food allergies impact the quality of life of both patients and their families and cause limitations in dietary and social habits. Moreover, food allergies can lead to life-threatening situations in case of food-induced systemic anaphylaxis. The worldwide prevalence of IgE-mediated food allergies has been estimated to be 4% among children [1] and 1-3% in the adult population [2]; however, discrepancies remain between self-reported and oral food challenge-proven food allergy prevalence rates. To date, no curative treatments exist for food allergy patients and food allergy management relies primarily on avoidance of the offending food(s) [3].

Natural resolution of food allergies is observed in a subgroup of patients; however, risk factors associated with food allergy persistence have been identified [4, 5]. Furthermore, persistence of established food allergies can be triggered by genetic predisposition as was shown in school-aged Japanese children with filaggrin loss-of-function mutations [6]. Filaggrin regulates skin barrier integrity and filaggrin mutations are an important predisposing factor for the development of atopic dermatitis followed by sensitization to food antigens via the disrupted skin. In addition, persistence of egg allergy has been shown to originate from a specific innate immune profile in peripheral blood mononuclear cells (PBMC) derived from one-year-old egg allergic infants [7]. Infants with persistent egg allergy at the age of 2-4 years showed increased numbers of circulating monocytes and dendritic cells (DC) and increased *ex vivo* production of inflammatory cytokines. Natural resolution of egg allergy was associated with higher vitamin D levels in serum, indicating a key role of vitamin D in controlling innate responses [7]. Besides the influence of genetics and early life immune status, the composition and activity of the gut microbiome is potentially involved in persistence of food allergies in humans [8]. Persistence of cow's milk allergy (CMA) at the age of 8 years was associated with higher abundance of the families Bacteriodetes and Enterobacter in faecal samples compared to infants with natural resolution of CMA [9]. Patients suffering from persistent food allergies, either in childhood, during adolescence or in adulthood, benefit from therapeutic strategies aiming for specific induction of oral tolerance to the offending food(s). In particular oral immunotherapy (OIT) has been widely studied as a therapeutic approach to control (acute) allergic symptoms and enable reintroduction of the allergen into the diet. Human trials of OIT to treat IgE-mediated food allergies have delivered promising results to date; however, current recommendations are limited to the controlled application of OIT in specialized centers with access to rescue medication in case of (severe) adverse events [10].

Substantial evidence of efficacy has been observed in studies of OIT in hen's egg, peanut or cow's milk allergic subjects upon continuous treatment, in other words successful desensitization can be acquired [11]. Long-term maintenance dosing during a 6 year follow-up of OIT participants resulted in safe consumption of 2 g of protein during an oral food challenge, irrespective of low dosing or high dosing during maintenance [12]. It has been shown that individuals subjected to egg OIT with a long duration of 4 years that successfully achieved sustained unresponsiveness, were more likely to ingest both unbaked and baked egg after treatment [13]. Clinical efficacy post-discontinuation of therapy, i.e., sustained unresponsiveness without maintenance dosing, has been suggested in several food allergy trials; however, convincing evidence is limited [14]. Nevertheless, heterogeneity in study designs, sample size, clinical outcomes and dosing regimens restrict the current body of evidence in relation to long-term efficacy of OIT to treat food allergies. In addition, knowledge gaps remain with regard to safety of OIT protocols [15], especially in the severe food allergic individuals that suffer an even higher risk of adverse events during treatment. A history of severe (life-threatening) food-induced clinical responses is often used as an exclusion criterion in clinical trials, therefore efficacy and safety of OIT in this specific patient group is poorly investigated [16]. Overall, OIT for food allergy shows the most promising efficacy, but needs improvement of the safety profile and confirmation of long-term (off-therapy) beneficial effects [17].

Adjunct therapies may represent a new window of opportunity to support OIT-induced modulation of the food allergic immune response and long-term induction of oral tolerance [18]. Selection of potential adjuvants is based on either immunomodulatory properties or enhancement of the safety profile by blocking the functionality of specific IgE, e.g., omalizumab [19]. Nutritional adjuvants targeting the microbiome and/or the gut-associated immune system, including probiotics [20] and prebiotics [21], or bacterial products stimulating TLR-signaling or Th1 polarization, including CpG-oligodeoxynucleotides [22] are potential candidates. To date, one randomized placebo-controlled trial of peanut OIT has been conducted to investigate the beneficial effect of co-administration with the probiotic *Lactobacillus rhamnosus* on desensitization and allergen-specific antibody titers [23]. Despite the successful desensitization shown in the majority of the enrolled participants in the OIT with probiotics group, further confirmation of the added value of the adjuvant with respect to OIT alone is necessary.

The main focus of this thesis was to investigate novel nutrition-based interventions that can support the efficacy of OIT to treat food allergy. In this thesis, we presented pre-clinical data showing the improved efficacy of OIT via co-administration of prebiotics, i.e., a specific mixture of short-chain and long-chain fructo-oligosaccharides (scFOS/lcFOS, 9:1, 1% w/w, FOS), and the short-chain fatty acid (SCFA) butyrate in cow's milk allergic mice. We have shown enhanced desensitization of basophils and mast cells, effective modulation of the Th2-mediated allergic response, increased levels of Foxp3+ regulatory T cells (Tregs) with suppressive capacity toward mast cell degranulation and altered whey-specific antibody titers in mice treated with the combination of OIT and FOS or butyrate supplementation. Furthermore, we presented *in vitro* data i) confirming the predictive capacity of serum whey-specific IgE with regard to *in vitro* and *in vivo* degranulation of effector cells, ii) showing FOS-induced enhancement of the suppressive action of Tregs toward bone marrow-derived mast cells (BMMC) and iii) showing the potential epigenetic influence of FOS supplementation on bone marrow resident immune precursor cells. In addition, multi-parameter analysis using Bayesian networks and Topological plots yielded novel insight into potential biomarkers (i.e., the SCFA butyrate, acetate and propionate) with a close association with clinical outcomes in mice. Microbiome analysis in murine faecal samples using 16S rRNA sequencing revealed modulation of gut bacterial diversity and activity upon treatment with OIT and FOS supplementation. In the current chapter, we sought to highlight our main experimental findings and discuss their relevance with respect to the available literature of both murine and human food allergy treatment studies.

Clinical outcomes

A food allergic patient's primary concern is the (perceived) risk of allergic reactions accompanied by the dietary and social restrictions that require attention on a daily basis [24]. Furthermore, clinical side effects during OIT protocols occur frequently and can lead to withdrawal of participants [25]. Health-related quality of life in children subjected to peanut OIT [26] and egg OIT [27] was negatively influenced by adverse allergic symptoms during treatment. Therefore, controlling the severity and frequency of allergic symptoms related to OIT will contribute to treatment compliance and thereby efficacy of the therapeutic strategy. Several alternatives to control the occurrence of adverse reactions are under study: low-dose OIT was shown to effectively induce desensitization in severe egg-allergic patients [28], co-administration of anti-IgE during OIT protocols in peanut, egg and cow's milk allergic patients showed promising results with regard to severity of adverse events [29] and pre-treatment with a sublingual immunotherapy (SLIT) escalation protocol prior to OIT was studied in cow's milk allergic patients [30]. In chapter 3 of this thesis,

we presented the data of murine CMA and peanut allergy (PNA) models in which mice were subjected to OIT or subcutaneous immunotherapy (SCIT) with different allergen dose ranges. Treatment efficacy was determined with a series of food challenges conducted via different routes (i.e., intradermal, i.d.; intragastric, i.g.; or intraperitoneal, i.p.). In the CMA model, the influence of the allergen dose on clinical efficacy of OIT was primarily indicated by the dose-related prevention of the drop in body temperature during anaphylaxis. Oral administration of 100 mg whey (during a three week protocol with 5 oral gavages per week) induced a stronger protective effect compared to oral dosing with 0.1, 1 or 10 mg whey. In addition, drop in body temperature during anaphylaxis was decreased in a dose-related manner in PNA mice treated with OIT. Even though the pre-clinical data suggest the use of the highest (safe) dose of allergen to conduct OIT to achieve maximal protection against clinical symptoms, low(er) dose OIT was shown to substantially decrease the severity of acute allergic symptoms upon challenge in both the PNA and CMA models. To date, studies have been conducted to investigate the possibility to replace conventional OIT by low-dose OIT and low-dose oral food challenges [31]. Low-dose OIT in food allergic patients was shown to be as effective as conventional OIT and was accompanied by less severe symptoms [32]. Immunological changes induced by conventional OIT were similar in low-dose OIT in hen's egg allergic subjects shown by decreased specific IgE and increased specific IgG4 against hen's egg proteins [33]. However, the exact minimal dose needed to induce (long-term) tolerance remains to be elucidated in humans.

In the current OIT model, we treated cow's milk allergic mice with 10 mg whey during a 3 week intervention period either or not combined with oral supplementation of FOS or butyrate (chapter 4 and chapter 6 of this thesis). The rationale to choose a suboptimal whey dose to conduct OIT is based on the main hypothesis of this thesis in which we propose that nutritional adjuvants can improve OIT efficacy to treat food allergy. A maximal desensitization response would not allow further improvement by the dietary intervention in the primary clinical parameters. Both combinations of OIT+FOS and OIT+butyrate showed the most pronounced effect on the acute allergic skin response induced by i.d. challenge and could significantly decrease mast cell degranulation induced by i.g. challenge (chapter 6). Despite the observation that the additive effect of FOS or butyrate was not significant compared to OIT alone, OIT without dietary adjuvants did not result in a significant reduction in allergic symptoms upon challenge compared to non-treated allergic control mice. Our observations relate to the limited amount of studies that have been conducted to improve OIT efficacy with immunomodulatory (bacterial) adjuvants: symptoms of PNA decreased in mice exposed to heat/phenol killed *Escherichia coli* encapsulated

recombinant modified peanut proteins Ara h 1, Ara h 2 and Ara h 3 [34]; however, a phase I trial in humans was accompanied by severe adverse reactions in 5 out of 10 participants [35]. Tolerance induction was accelerated in cow's milk allergic infants fed an extensively hydrolyzed cow's milk-based formula compared to avoidance of cow's milk proteins in the diet and this effect could be enhanced by co-administration with *Lactobacillus rhamnosus GG* [36, 37]. Nevertheless, a significant added effect of the adjuvant strategy compared to conventional OIT was not shown in the above mentioned studies, with some studies lacking the conventional OIT group as a reference for treatment efficacy [23].

Desensitization of effector cells

Mast cells and basophils are the main drivers of acute allergic symptoms in patients suffering from food allergies. Desensitization of basophils and mast cells has been recognized as one of the earliest phenomena induced by antigen-specific immunotherapy (AIT) [38]. Upon continuous exposure to the allergen during the first months of therapy, the threshold of degranulation increases substantially. Moreover, desensitization of basophils and mast cells occurs in the presence of high specific IgE levels that tend to further increase during the first months of OIT [39]. Proposed underlying mechanisms as studied in humans and mice involve expression of histamine receptor 2 induced by allergen-IgE-mediated activation of basophils that promotes a negative feedback loop for activation [40] and the generation of antigen-specific IgG antibodies that bind to FcγRIIb leading to inhibition of IgE-mediated responses [41, 42]. With the current murine OIT model, the primary outcome for efficacy of the novel treatment approaches was the decreased degranulation capacity of effector cells like mast cells and basophils upon food challenge, i.e., the desensitization effect. OIT using 1, 10 and 100 mg whey reduced mucosal mast cell degranulation after i.g. challenge in cow's milk allergic mice (chapter 3). In chapter 4 and chapter 6, we showed a more pronounced reduction in mucosal mast cell degranulation induced by the combinations OIT+FOS and OIT+butyrate. Moreover, oral butyrate supplementation further improved OIT-induced suppression of basophil activation in an *ex vivo* antigen-specific stimulation assay (chapter 6). Limited evidence exists with regard to the direct influence of SCFA on mast cell and basophil functionality. SCFA are known to exert anti-inflammatory properties that support gut homeostasis via G-protein-coupled receptors (GPR) 41, GPR43 and GPR109A on immune and epithelial cells [43] and were shown to regulate the differentiation and functionality of colonic Tregs [44]. Mucosal mast cells resident in colon tissue of both rats and humans were shown to express GPR43 on their surface [45, 46]. In addition, butyrate suppressed *in vitro* murine mast cell proliferation and

FcεRI-mediated cytokine responses via inhibition of histone deacetylase (HDAC) [47]. Exposure to non-digestible oligosaccharides, including human milk oligosaccharides (HMOS), was shown to either directly suppress murine mast cell activation or enhance Treg-mediated suppression of mast cells *in vitro* [48]. Furthermore, non-digestible oligosaccharides were previously shown to interact with intestinal epithelial cells (IEC) and trigger the release of the soluble carbohydrate-binding protein galectin-9 [49]. Galectin-9 has the capacity to bind to IgE with high affinity and can thereby reduce antigen-IgE complex-mediated activation of mast cells [50] and allergic symptoms [51]. Galectin-9 levels were increased in serum samples of mice treated with OIT+FOS (chapter 4), suggesting the involvement of galectin-9 in protection against allergic symptoms induced by OIT+FOS. The exact contribution of FOS and/or bacterial metabolites including butyrate in control of IgE-mediated activation of mast cells and basophils needs further study; nevertheless, we have shown the added benefit of co-administration with immunomodulatory adjuvants on early OIT-induced desensitization of basophils and mast cells in cow's milk allergic mice.

Long-term AIT is associated with reduced infiltration of mast cells into the peripheral tissues, recognized as the late desensitization response [52]. Mast cell numbers were significantly reduced in colon tissue of mice subjected to OIT+FOS and it was hypothesized that this reduction contributed to the decreased mast cell-associated mMCP-1 levels in serum after oral challenge (chapter 4). Homing of mast cell progenitors to the gut is dependent on expression of specific integrins, chemotactic receptors or the interaction of stem cell factor (SCF) with the c-Kit receptor, and alterations in the micro-environment could interfere with mast cell recruitment [53]. The increased levels of the anti-inflammatory mediator butyric acid, shown in cecum content of OIT+FOS mice (chapter 6), might contribute to reduced gut inflammation and thereby reduced recruitment of inflammation-responsive mast cells.

Antibody responses

The majority of OIT trials to treat food allergies reports primarily on clinical outcomes including skin prick test (SPT) wheal size, clinical symptoms related to oral food challenges and antigen-specific antibody levels at baseline, during immunotherapy and after treatment. Levels of antigen-specific IgE, total IgE or the specific IgE/total IgE ratio are currently used as the gold-standard biomarker(s) to identify food allergic patients eligible for treatment, monitor treatment efficacy and determine successful modulation after discontinuation of treatment [54, 55]. In chapter 8 of this thesis, we sought to confirm the relation between whey-specific IgE in serum of treated mice and mast cell degranulation responses. We showed that the kinetics of whey-

specific IgE are associated with *in vitro* degranulation capacity of the RBL-2H3 cell line and BMMC exposed to experimental mouse serum, and corresponded with *in vivo* degranulation of mucosal mast cells in the gastrointestinal tract. Overall, whey-specific IgE responses observed in the current pre-clinical model were influenced by immunotherapy and this effect could be enhanced by the dietary intervention with FOS (chapter 4) in cow's milk allergic mice. In chapter 3, we showed a dose-related increase in whey- and peanut-specific IgE during OIT and SCIT in mice, as observed in food allergic patients subjected to immunotherapy [56]. Interestingly, specific IgE levels increased in sensitized control mice upon challenge and this rise was prevented in OIT and SCIT mice (chapter 3) as well as in OIT+FOS mice (chapter 4). This finding suggested early modulation of cellular responses (mediated by e.g., Tregs, regulatory B cells (Bregs) or de novo B cell proliferation [57]) responsible for antigen-specific antibody production induced by immunotherapy; however, at this stage, we could not determine the exact underlying mechanism.

Control of IgE-mediated allergies and anaphylaxis involves production of specific IgG subtypes during immunotherapy [58]. In particular, OIT elicits an antigen-specific IgG response, as observed after a 6 month OIT trial in cow's milk allergic children: a significant increase in cow's milk-specific IgG4 was measured after the intervention compared to baseline [59]. The presence of IgG blocking antibodies in blood is associated with achievement of sustained unresponsiveness and long-term protection in allergic patients [60]. In addition, plasma derived from either peanut-sensitized but tolerant children or OIT subjected peanut-allergic children, could suppress peanut-induced basophil activation, and this effect was dependent on detectable levels of peanut-specific IgG4 [61]. IgG4 as observed in humans is non-existing in mice; however, murine IgG1 and IgG2a responses can be studied to determine immunotherapy efficacy in murine CMA models [62]. In chapter 3, we showed a dose-related increase in whey- and peanut-specific IgG1 and IgG2a in OIT and SCIT mice, with a transient decrease in IgG titers upon discontinuation of the treatment. In chapter 4, OIT and OIT+FOS mice showed elevated levels of whey-specific IgG1 compared to non-treated sensitized controls, with no (significant) additive effect of the dietary intervention with FOS (chapter 4 and 6). FOS supplementation or oral butyrate administration did not result in altered whey-specific IgG subtype levels. Although the direct suppressive effect of whey-specific IgG subtypes on allergic phenomena in the current model remains to be confirmed, we have shown a similar AIT-induced increase of antigen-specific IgG subtypes in mice as described in human studies [63, 64].

In chapter 4, we observed increased levels of whey-specific IgA in OIT and OIT+FOS mice compared to non-treated sensitized controls during the treatment phase. Moreover, in chapter 6, whey-specific IgA levels were again higher in OIT and OIT+FOS mice at the time point of clinical challenge. It has been described that immunotherapy induces specific IgA [65]. Rush OIT in egg allergic children significantly increased serum levels of IgA, with a decrease observed during maintenance dosing [66]. Furthermore, children categorized as responders showed higher baseline IgA levels prior to OIT [66]. Low levels of IgA in early life were associated with an increased risk to develop allergies [67]. Overall, the changes in antibody levels presented in this thesis correspond to our current understanding of the important role of shifted antibody responses in effective treatment protocols. Supplementation with neither FOS nor butyrate in the absence of OIT could significantly affect whey-specific IgE, IgG or IgA levels, indicating that alteration of the humoral reponse during OIT can only be achieved via antigen-specific pathways.

Cellular responses

T helper 2 (Th2) cells are main drivers of allergic inflammation via production of IL-4, IL-5 and IL-13, leading to activation of IgE-producing B cells and recruitment of mast cells, basophils and eosinophils [68]. Evidence with regard to the AIT-mediated induction of Tregs and the suppression of Th2 immunity is abundant in experimental models of food allergy, but less studied in human trials [69]. AIT led to increased numbers of CD4+CD25+Foxp3+ Tregs and decreased numbers of Th2 cells in affected tissue biopsies, and was accompanied by decreased Th2-mediated late-phase skin responses in allergic patients [70]. We showed decreased %Th2 together with increased %Th1 in spleens derived from cow's milk allergic mice subjected to OIT and SCIT (chapter 3). Stimulation via an i.p. antigen challenge prior to organ collection induced proliferation and revealed differences in T helper subsets in treated mice versus control mice. In chapter 4, OIT+FOS and FOS supplementation alone effectively reduced %Th2 compared to sensitized control mice in the spleen. Functionality of effector T cells was assessed by *ex vivo* antigen-induced cytokine production. As observed in chapter 3, OIT significantly decreased IL-5, IL-13 and IL-10 production by spleen-derived lymphocytes in a dose-dependent manner upon whey stimulation, and this effect was absent in SCIT. OIT+FOS-derived lymphocytes collected halfway immunotherapy showed decreased production of IL-5 and IL-10 compared to splenocytes derived from sensitized control mice upon whey stimulation in chapter 4. These observations are in accordance with the reduced production of IL-5, IL-13 and IL-10 upon β-casein-specific stimulation of PBMC derived from cow's milk allergic infants showing successful OIT-induced desensitization [71]. IL-10 is

mostly described in the context of Treg and Breg induction [72] and, together with TGFβ, plays an important part in oral tolerance induction [73]. Nevertheless, our data suggest a reduction in Th2-mediated IL-10 production upon exposure to OIT and FOS supplementation. It has been described previously that IL-10 is essential in food allergy induction in mice via mucosal mast cell proliferation and activation: IL-10 deficient knock-out mice were unable to develop food allergic symptoms [74].

Induction of antigen-specific Tregs is currently considered as one of the key mechanisms in restoring oral tolerance against allergens [75]. In chapter 3, we observed an increase in %Foxp3+ Tregs in mesenteric lymph nodes (MLN) of mice treated with 10 mg whey during OIT. Addition of a FOS supplemented diet further improved the regulatory immune response shown by increased %Foxp3+ Tregs in MLN halfway immunotherapy (chapter 4). Functionality of OIT+FOS-derived and purified Tregs was shown in an adoptive transfer experiment in which recipients of CD25-depleted cell suspensions were more susceptible to challenge-induced mast cell degranulation compared to recipients of the non-depleted fraction (chapter 4). Tregs derived from mice exposed to OIT+FOS and OIT+butyrate showed enhanced suppression of IgE-mediated degranulation of BMMC *in vitro* (chapter 6).

Induction of Foxp3+ Tregs occurs in the MLN via tolerogenic CD103+ DC and involvement of TGFβ and vitamin A (i.e., retinoic acid (RA)) [76]. It has been described that dietary supplementation with non-digestible fibers increased the expression of the vitamin A-converting enzyme retinaldehyde dehydrogenase-1 (RALDH1) in IEC in mice, and this effect could me mimicked *in vitro* using SCFA [77]. Increased RALDH1 expression by IEC was accompanied by increased activity of vitamin A-converting enzymes in MLN-derived DC and increased numbers of intestinal Tregs [77]. In addition, in a murine peanut allergy model, high fiber feeding was associated with protection against food allergic symptoms via up-regulation of RALDH activity in mucosal CD103+ DC responsible for oral tolerance induction [78]. Mice lacking SCFA receptors (GPR43 or GPR109A) showed exacerbated allergic symptoms and lower numbers of tolerogenic DC [78]. In our studies, the %CD103+ DC was increased in small intestine lamina propria tissue of mice treated with OIT and OIT+FOS, but not after FOS supplementation alone, after exposure to an oral food challenge (chapter 4). In the future, it would be interesting to assess potential functional changes in CD103+ DC induced by OIT+FOS and to study whether altered DC responses are responsible for the observed Treg-mediated protection against allergic symptoms in the used pre-clinical model.

Synergy

Effective treatment of food allergies involves modulation of innate and adaptive immune responses with long-lasting memory to avoid recurrence of the allergic phenotype. In addition to the mucosal immune system, specific mediators derived from the diet, the intestinal epithelial lining and the resident gut microbiota influence regulatory responses [79]. Their involvement in modulation of the allergic immune response during antigen-specific immunotherapy is less well defined to date. It has been shown in mice that low dietary intake of fermentable fibers enhances secretion of TSLP by IEC, a cytokine known to induce Th2 immunity leading to development of allergy [78]. Next to prebiotic activity, structurally different HMOS were shown to exert distinct biological activities on intestinal epithelial cell lines in response to antigen-antibody complexes or pro-inflammatory cytokine stimulation, indicated by specific alteration of chemokine release (IL-8 and CCL20) and expression of transcription factor NF-κB and AP-1 [80]. As mentioned earlier, IEC-derived galectin-9 was increased in serum samples of cow's milk allergic mice treated with OIT+FOS (chapter 4). FOS supplementation in the absence of OIT did not significantly alter epithelial responses, underlining the complexity of the interplay between dietary components and intestinal responses in the context of allergy.

The involvement of the microbiome in the development and natural resolution of food allergies has been studied in humans, but limited evidence is currently available [81, 82]. As shown recently, 16S rRNA sequencing of faecal samples derived from children suffering from atopic dermatitis (AD) revealed a specific bacterial signature that could discriminate AD patients with additional food allergies from AD patients without food allergies [83]. Moreover, a specific microbial signature was recognized in food allergy-prone mice, and faecal transplantation to germ-free recipient mice could induce a similar food allergic phenotype as observed in the donors [84]. In this thesis, we showed a specific bacterial community structure in mice orally sensitized to the cow's milk protein whey compared to non-allergic control mice (chapter 7). Furthermore, oral supplementation with FOS in sensitized mice significantly shifted the bacterial community structure compared to sensitized mice receiving the control diet (chapter 7). Interestingly, OIT+FOS treatment induced a high level of similarity in bacterial community structure with non-allergic control mice. Our observation that faecal samples derived from OIT mice are enriched in members of the phylum Proteobacteria, raises the question whether OIT in cow's milk allergic mice might trigger microbial dysbiosis. Abundance of members of the Proteobacteria is associated with asthma and respiratory pathologies [85] and gastrointestinal inflammatory disorders [86]. A causal relation between abundance of facultative anaerobic Enterobacteriaceae family members (phylum Proteobacteria) and colitis

severity has been confirmed in mice: selective inhibition of specific bacterial respiratory pathways that are up-regulated during episodes of inflammation could prevent expansion of Enterobacteriaceae families and thereby symptoms of colitis [87]. The authors mentioned that 'microbiota-editing' was not effective during states of homeostasis [87]. Specifically, stimulating the growth of beneficial commensals via FOS supplementation during OIT to treat food allergies might prevent expansion of opportunistic dysbiotic bacterial strains and contributes to a healthy state of the gut.

Additional benefit of FOS supplementation during OIT was shown upon analysis of SCFA (chapter 6 and 7). Butyric acid and propionic acid concentrations were significantly increased in OIT+FOS mice compared to OIT mice (chapter 6 and 7). FOS supplementation in the absence of OIT did not significantly increase the availability of SCFA in any of the experiments, again suggesting a more complex relationship between dietary fiber fermentation and microbiota characteristics in the context of allergy. Although GPR-knock out studies should confirm the involvement of SCFA including butyrate in the allergy protective effect induced by OIT+FOS in cow's milk allergic mice, we suspect a major contribution of butyrate in supporting regulatory processes in the gut. In chapter 6 of this thesis, we showed that oral butyrate supplementation mimicked the supporting effect of FOS supplementation during OIT: butyrate enhanced OIT-induced desensitization of basophils and mast cells and regulatory T cell functionality. High fiber feeding in peanut allergic mice modulates gut microbiota composition and activity leading to increased levels of potent SCFA that can enhance oral tolerance induction and intestinal homeostasis [78]. Furthermore, increased fiber-derived SCFA can exert anti-inflammatory properties outside of the gastrointestinal tract and reduced allergic lung inflammation in mice [88]. Bacterial metabolites including SCFA influence immune precursor cells resident in bone marrow leading to alterations of the mucosal immune response in the airways of mice [88, 89]. The impact of the microbiota and their immunomodulatory metabolites on immune development and maturation gains more attention with respect to inflammatory disorders like asthma and food allergies [90, 91]. In chapter 7, we proposed a similar mechanism of action linking dietary fiber (FOS) and *in vitro* development of mast cells derived from bone marrow precursors. Expression of the mast cell receptors c-Kit (CD117) and FcεRI was lost in a substantial subset of the IL-3 and SCF-induced mast cells compared to the other groups after 6 weeks of culture. These findings need further confirmation; however, altered mast cell development and/or functionality might provide a potential long-term benefit of OIT+FOS as a treatment strategy for food allergy.

The relevance of SCFA analyses in the current experimental setting was further stressed by the observations derived from the topological data analysis in chapter 5: clinical outcomes of food allergy (e.g., acute allergic skin response, body temperature changes during anaphylaxis, symptom severity scores and mast cell degranulation-related mMCP-1 concentrations) showed the closest connection with SCFA levels measured in cecum content, after antigen-specific antibodies. This indicates that patterns in the SCFA data are strongly linked to patterns observed in clinical response data in the current pre-clinical model. Once advanced (non-invasive) methods become available to measure SCFA in humans, SCFA might be used as potential biomarkers to predict immunotherapy efficacy in food allergy trials.

Food for thought

The robust influence of OIT on antigen-specific IgE, IgG subtype and IgA levels as shown in serum samples of cow's milk allergic mice was in alignment with the current knowledge of antibody responses induced by OIT in food allergic patients [64]. Nevertheless, suboptimal clinical protection and less effective alteration of Th2-immunity were observed upon OIT. A possible explanation might be the dose of whey protein used (10 mg per daily oral gavage), since we aimed for an effective dose without reaching a maximal protective response. However, the microbiome analysis led to the observation that the Proteobacteria, a phylum associated with gut inflammation and dysbiosis [86], expanded in sensitized mice treated with OIT in the absence of the FOS intervention. In this project, we did not focus on parameters of (gut) inflammation (e.g., neutrophil infiltration, tissue damage, cytokines or signaling proteins) as a read-out for immunotherapy efficacy in allergic mice; however, targeting Th2-driven immune responses should result in reduced inflammation [73]. The current findings suggest exacerbation of gut inflammation in cow's milk allergic mice subjected to OIT. Simultaneous supplementation with FOS might prevent the inflammatory triggers induced by administration of allergens on a daily basis. More research is necessary to confirm this hypothesis; however, it might be worthwhile to study markers of gut inflammation and microbial dysbiosis in OIT-participants with unsuccessful clinical outcomes in future trials.

Future perspectives

With the current efficacy data in mind, future (pre-clinical) studies should focus on the potential improved safety of OIT via co-administration with dietary adjuvants. Concerns with regard to safety represent a major issue in the field of allergy and antigen-specific immunotherapy to date [15]. Furthermore, improving the efficacy of OIT via FOS interventions might allow lowering of the dose of the offending protein during treatment, and this will reduce the risk of adverse reactions in patients. Translation of the current findings from mice to humans should be performed with caution. The used pre-clinical food allergy model provides a valuable proof-of-concept for the combination strategy of OIT with FOS supplementation; however, it remains unknown to what extent bacterial community profiles or metabolic pathways in mice and humans are aligned. Non-digestible oligosaccharides including FOS are currently used as HMOS substitutes in infant formula and are regarded as safe food components [92, 93], which favors the use in food allergic patients during future OIT trials.

Overall conclusion - "Teaming up with the enemy"

In this thesis, we presented pre-clinical data showing the improved efficacy of a novel nutrition-based therapeutic strategy to treat food allergy in mice. Overall, the combination of OIT and supplementation with non-digestible oligosaccharides showed an improved allergy-suppressive effect compared to OIT. OIT+FOS induced the strongest reduction in acute allergic symptoms upon food challenge, and this was supported by alterations in antigen-specific antibody levels and shifted T cell and cytokine responses. Early desensitization effects in basophils and mast cells were accompanied by late effects including a reduction in the number of tissue-resident mast cells in the colon of treated mice. OIT+FOS induced microbiome alterations and increased concentrations of SCFA in cecum content. We sought to graphically summarize the highlights of the presented research in **Figure 1**, and propose the potential underlying mechanisms of action of OIT+FOS. The influence of dietary fiber and fermentation-derived SCFA on the innate and adaptive immune response, the intestinal epithelial lining and potentially also on bone marrow-resident immune precursors needs further exploration to prove a causal connection with the observed reduced allergic responses. Nevertheless, non-digestible oligosaccharides are shown to be promising candidates to support OIT efficacy in human food allergy patients.

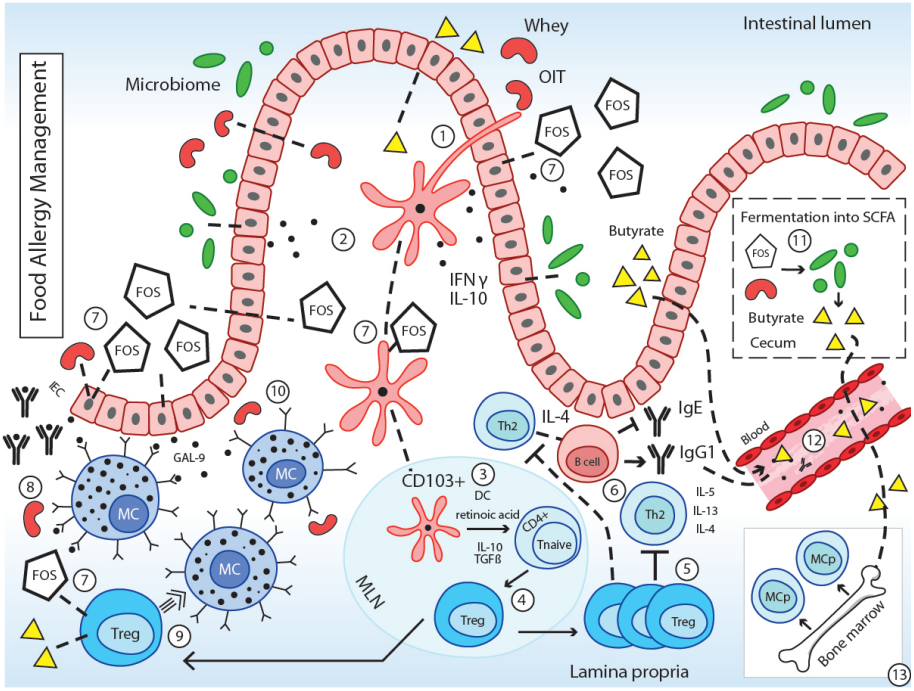


Figure 1. Potential underlying mechanisms of OIT+FOS-mediated suppression of food allergy. Summary of main findings derived from pre-clinical CMA studies as presented in this thesis. **(1)** Administration of whey proteins during OIT leads to uptake of protein by DC resident in the LP of the small intestine. **(2)** IEC-derived mediators, dietary and bacterial metabolites (retinoic acid and SCFA) and cytokines can shape the immune response, including the tolerogenic phenotype of CD103+ DC. **(3)** CD103+ DC present specific epitopes to naïve T cells in the MLN. **(4)** Under the influence of IL-10 and TGFβ, T cells differentiate into antigen-specific Tregs. **(5)** Expansion of Tregs and homing to the LP or peripheral tissues. **(6)** Suppression of Th2-mediated immunity and IL-4, IL-5 and IL-13 responses which is accompanied by stimulation of IgG producing B cells and reduced production of IgE. **(7)** Direct interaction of FOS with IEC and LP-resident immune cells, with increased release of IEC-derived galectin-9. **(8)** Galectin-9 forms complexes with IgE and interferes with mast cell activation. **(9)** Enhanced Treg suppression toward mast cells mediated by FOS and/or butyrate exposure. **(10)** OIT provides a 'high' protein load causing desensitization of mast cells and reduced expression of the FcεRI. **(11)** Fermentation of FOS by bacteria into SCFA including butyrate providing an energy source for colonocytes. **(12)** Uptake of fermentation-derived butyrate or orally administered butyrate into the systemic circulation. **(13)** Bacterial metabolites might epigenetically affect bone marrow resident mast cell precursors. OIT, oral immunotherapy; FOS, fructo-oligosaccharides; CMA, cow's milk allergy; DC, dendritic cell; LP, lamina propria; IEC, intestinal epithelial cells; SCFA, short-chain fatty acids; MLN, mesenteric lymph nodes; Tregs, regulatory T cells; Th2, T helper 2; GAL-9, galectin-9.

REFERENCES

1. Rona RJ, Keil T, Summers C, Gislason D, Zuidmeer L, Sodergren E, Sigurdardottir ST, Lindner T, Goldhahn K, Dahlstrom J, McBride D, and Madsen C. The prevalence of food allergy: A meta-analysis. J Allergy Clin Immunol 2007. 120(3): p. 638-46.
2. Osterballe M, Hansen TK, Mortz CG, Host A, and Bindslev-Jensen C. The prevalence of food hypersensitivity in an unselected population of children and adults. Pediatr Allergy Immunol 2005. 16(7): p. 567-73.
3. Longo G, Berti I, Burks AW, Krauss B, and Barbi E. Ige-mediated food allergy in children. Lancet 2013. 382(9905): p. 1656-64.
4. Arik Yilmaz E, Cavkaytar O, Buyuktiryaki B, Sekerel BE, Soyer O, and Sackesen C. Factors associated with the course of egg allergy in children. Ann Allergy Asthma Immunol 2015. 115(5): p. 434-438 e1.
5. Peters RL, Allen KJ, Dharmage SC, Koplin JJ, Dang T, Tilbrook KP, Lowe A, Tang ML, Gurrin LC, and HealthNuts S. Natural history of peanut allergy and predictors of resolution in the first 4 years of life: A population-based assessment. J Allergy Clin Immunol 2015. 135(5): p. 1257-66 e1-2.
6. Kono M, Akiyama M, Inoue Y, Nomura T, Hata A, Okamoto Y, Takeichi T, Muro Y, McLean WHI, Shimizu H, Sugiura K, Suzuki Y, and Shimojo N. Filaggrin gene mutations may influence the persistence of food allergies in japanese primary school children. Br J Dermatol 2018.
7. Neeland MR, Koplin JJ, Dang TD, Dharmage SC, Tang ML, Prescott SL, Saffery R, Martino DJ, and Allen KJ. Early life innate immune signatures of persistent food allergy. J Allergy Clin Immunol 2017. pii: S0091-6749(17)31754-2
8. Rachid R and Chatila TA. The role of the gut microbiota in food allergy. Curr Opin Pediatr 2016. 28(6): p. 748-753.
9. Bunyavanich S, Shen N, Grishin A, Wood R, Burks W, Dawson P, Jones SM, Leung DYM, Sampson H, Sicherer S, and Clemente JC. Early-life gut microbiome composition and milk allergy resolution. J Allergy Clin Immunol 2016. 138(4): p. 1122-1130.
10. Pajno GB, Fernandez-Rivas M, Arasi S, Roberts G, Akdis CA, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, Ebisawa M, Eigenmann P, Knol E, Nadeau KC, Poulsen LK, van Ree R, Santos AF, du Toit G, Dhami S, Nurmatov U, Boloh Y, Makela M, O'Mahony L, Papadopoulos N, Sackesen C, Agache I, Angier E, Halken S, Jutel M, Lau S, Pfaar O, Ryan D, Sturm G, Varga EM, van Wijk RG, Sheikh A, Muraro A, and Group EAIG. Eaaci guidelines on allergen immunotherapy: Ige-mediated food allergy. Allergy 2017. p. 28(8):728-745.
11. Nurmatov U, Dhami S, Arasi S, Pajno GB, Fernandez-Rivas M, Muraro A, Roberts G, Akdis C, Alvaro-Lozano M, Beyer K, Bindslev-Jensen C, Burks W, du Toit G, Ebisawa M, Eigenmann P, Knol E, Makela M, Nadeau KC,

O'Mahony L, Papadopoulos N, Poulsen LK, Sackesen C, Sampson H, Santos AF, van Ree R, Timmermans F, and Sheikh A. Allergen immunotherapy for ige-mediated food allergy: A systematic review and meta-analysis. Allergy 2017. 72(8): p. 1133-1147.

12. Andorf S, Manohar M, Dominguez T, Block W, Tupa D, Kshirsagar RA, Sampath V, Chinthrajah RS, and Nadeau KC. Feasibility of sustained response through long-term dosing in food allergy immunotherapy. Allergy Asthma Clin Immunol 2017. 13: p. 52.
13. Jones SM, Burks AW, Keet C, Vickery BP, Scurlock AM, Wood RA, Liu AH, Sicherer SH, Henning AK, Lindblad RW, Dawson P, Berin C, Fleischer DM, Leung DYM, Plaut M, Sampson HA, and Consortium of Food Allergy R. Long-term treatment with egg oral immunotherapy enhances sustained unresponsiveness that persists after cessation of therapy. J Allergy Clin Immunol 2016. 137(4): p. 1117-1127 e10.
14. Graham F, Tardio N, Paradis L, Des Roches A, and Begin P. Update on oral immunotherapy for egg allergy. Hum Vaccin Immunother 2017. 13(10): p. 2452-2461.
15. Vazquez-Ortiz M and Turner PJ. Improving the safety of oral immunotherapy for food allergy. Pediatr Allergy Immunol 2016. 27(2): p. 117-25.
16. Rachid R and Keet CA. Current status and unanswered questions for food allergy treatments. J Allergy Clin Immunol Pract 2017.
17. Burks AW, Sampson HA, Plaut M, Lack G, and Akdis CA. Treatment for food allergy. J Allergy Clin Immunol 2018. 141(1): p. 1-9.
18. Hayen SM, Kostadinova AI, Garssen J, Otten HG, and Willemsen LE. Novel immunotherapy approaches to food allergy. Curr Opin Allergy Clin Immunol 2014. 14(6): p. 549-56.
19. Begin P, Dominguez T, Wilson SP, Bacal L, Mehrotra A, Kausch B, Trela A, Tavassoli M, Hoyte E, O'Riordan G, Blakemore A, Seki S, Hamilton RG, and Nadeau KC. Phase 1 results of safety and tolerability in a rush oral immunotherapy protocol to multiple foods using omalizumab. Allergy Asthma Clin Immunol 2014. 10(1): p. 7.
20. Hol J, van Leer EH, Elink Schuurman BE, de Ruiter LF, Samsom JN, Hop W, Neijens HJ, de Jongste JC, Nieuwenhuis EE, Cow's Milk Allergy Modified by E, and Lactobacilli study g. The acquisition of tolerance toward cow's milk through probiotic supplementation: A randomized, controlled trial. J Allergy Clin Immunol 2008. 121(6): p. 1448-54.
21. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, and Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child 2006. 91(10): p. 814-9.
22. Senti G, Johansen P, Haug S, Bull C, Gottschaller C, Muller P, Pfister T, Maurer P, Bachmann MF, Graf N, and Kundig TM. Use of a-type cpg

oligodeoxynucleotides as an adjuvant in allergen-specific immunotherapy in humans: A phase i/iia clinical trial. Clin Exp Allergy 2009. 39(4): p. 562-70.

23. Tang ML, Ponsonby AL, Orsini F, Tey D, Robinson M, Su EL, Licciardi P, Burks W, and Donath S. Administration of a probiotic with peanut oral immunotherapy: A randomized trial. J Allergy Clin Immunol 2015. 135(3): p. 737-44 e8.
24. Flokstra-de Blok BM, DunnGalvin A, Vlieg-Boerstra BJ, Oude Elberink JN, Duiverman EJ, Hourihane JO, and Dubois AE. Development and validation of a self-administered food allergy quality of life questionnaire for children. Clin Exp Allergy 2009. 39(1): p. 127-37.
25. Kulmala P, Pelkonen AS, Kuitunen M, Paassilta M, Remes S, Schultz R, Dunder T, Turunen S, and Makela MJ. Wheat oral immunotherapy was moderately successful but was associated with very frequent adverse events in children aged 6-18 years. Acta Paediatr 2018.
26. Factor JM, Mendelson L, Lee J, Nouman G, and Lester MR. Effect of oral immunotherapy to peanut on food-specific quality of life. Ann Allergy Asthma Immunol 2012. 109(5): p. 348-352 e2.
27. Vazquez-Ortiz M, Alvaro M, Piquer M, Dominguez O, Giner MT, Lozano J, Jimenez-Feijoo R, and Plaza AM. Impact of oral immunotherapy on quality of life in egg-allergic children. Pediatr Allergy Immunol 2015. 26(3): p. 291-294.
28. Maeta A, Matsushima M, Muraki N, Asano M, Takaoka Y, Kameda M, and Takahashi K. Low-dose oral immunotherapy using low-egg-allergen cookies for severe egg-allergic children reduces allergy severity and affects allergen-specific antibodies in serum. Int Arch Allergy Immunol 2018.
29. Dantzer JA and Wood RA. The use of omalizumab in allergen immunotherapy. Clin Exp Allergy 2018.
30. Keet CA, Frischmeyer-Guerrerio PA, Thyagarajan A, Schroeder JT, Hamilton RG, Boden S, Steele P, Driggers S, Burks AW, and Wood RA. The safety and efficacy of sublingual and oral immunotherapy for milk allergy. J Allergy Clin Immunol 2012. 129(2): p. 448-55, 455 e1-5.
31. Yanagida N, Sato S, and Ebisawa M. Clinical aspects of oral immunotherapy for the treatment of allergies. Semin Immunol 2017. 30: p. 45-51.
32. Yanagida N, Okada Y, Sato S, and Ebisawa M. New approach for food allergy management using low-dose oral food challenges and low-dose oral immunotherapies. Allergol Int 2016. 65(2): p. 135-140.
33. Yanagida N, Sato S, Asaumi T, Nagakura K, Ogura K, and Ebisawa M. Safety and efficacy of low-dose oral immunotherapy for hen's egg allergy in children. Int Arch Allergy Immunol 2016. 171(3-4): p. 265-268.
34. Li XM, Srivastava K, Grishin A, Huang CK, Schofield B, Burks W, and Sampson

HA. Persistent protective effect of heat-killed escherichia coli producing "engineered," recombinant peanut proteins in a murine model of peanut allergy. J Allergy Clin Immunol 2003. 112(1): p. 159-67.

35. Wood RA, Sicherer SH, Burks AW, Grishin A, Henning AK, Lindblad R, Stablein D, and Sampson HA. A phase 1 study of heat/phenol-killed, e. Coli-encapsulated, recombinant modified peanut proteins ara h 1, ara h 2, and ara h 3 (emp-123) for the treatment of peanut allergy. Allergy 2013. 68(6): p. 803-8.
36. Canani RB, Nocerino R, Terrin G, Frediani T, Lucarelli S, Cosenza L, Passariello A, Leone L, Granata V, Di Costanzo M, Pezzella V, and Troncone R. Formula selection for management of children with cow's milk allergy influences the rate of acquisition of tolerance: A prospective multicenter study. Journal of Pediatrics 2013. 163(3): p. 771-+.
37. Canani RB, Nocerino R, Terrin G, Coruzzo A, Cosenza L, Leone L, and Troncone R. Effect of lactobacillus gg on tolerance acquisition in infants with cow's milk allergy: A randomized trial. Journal of Allergy and Clinical Immunology 2012. 129(2): p. 580-U482.
38. Berings M, Karaaslan C, Altunbulakli C, Gevaert P, Akdis M, Bachert C, and Akdis CA. Advances and highlights in allergen immunotherapy: On the way to sustained clinical and immunologic tolerance. J Allergy Clin Immunol 2017. 140(5): p. 1250-1267.
39. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, Shreffler WG, Steele P, Henry KA, Adair M, Francis JM, Durham S, Vickery BP, Zhong X, and Burks AW. Clinical efficacy and immune regulation with peanut oral immunotherapy. J Allergy Clin Immunol 2009. 124(2): p. 292-300, 300 e1-97.
40. Novak N, Mete N, Bussmann C, Maintz L, Bieber T, Akdis M, Zumkehr J, Jutel M, and Akdis C. Early suppression of basophil activation during allergen-specific immunotherapy by histamine receptor 2. J Allergy Clin Immunol 2012. 130(5): p. 1153-1158 e2.
41. Burton OT, Logsdon SL, Zhou JS, Medina-Tamayo J, Abdel-Gadir A, Noval Rivas M, Koleoglou KJ, Chatila TA, Schneider LC, Rachid R, Umetsu DT, and Oettgen HC. Oral immunotherapy induces igg antibodies that act through fcgammariib to suppress ige-mediated hypersensitivity. J Allergy Clin Immunol 2014. 134(6): p. 1310-1317 e6.
42. Burton OT, Tamayo JM, Stranks AJ, Koleoglou KJ, and Oettgen HC. Allergen-specific igg antibody signaling through fcgammariib promotes food tolerance. J Allergy Clin Immunol 2018. 141(1): p. 189-201 e3.
43. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, and Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory t-cell generation. Nature 2013. 504(7480): p. 451-5.
44. Smith PM, Howitt MR, Panikov N,

Michaud M, Gallini CA, Bohlooly YM, Glickman JN, and Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic treg cell homeostasis. Science 2013. 341(6145): p. 569-73.

45. Karaki S, Mitsui R, Hayashi H, Kato I, Sugiya H, Iwanaga T, Furness JB, and Kuwahara A. Short-chain fatty acid receptor, gpr43, is expressed by enteroendocrine cells and mucosal mast cells in rat intestine. Cell Tissue Res 2006. 324(3): p. 353-60.
46. Karaki S, Tazoe H, Hayashi H, Kashiwabara H, Tooyama K, Suzuki Y, and Kuwahara A. Expression of the short-chain fatty acid receptor, gpr43, in the human colon. J Mol Histol 2008. 39(2): p. 135-42.
47. Zhang H, Du M, Yang Q, and Zhu MJ. Butyrate suppresses murine mast cell proliferation and cytokine production through inhibiting histone deacetylase. J Nutr Biochem 2016. 27: p. 299-306.
48. Castillo-Courtade L, Han S, Lee S, Mian FM, Buck R, and Forsythe P. Attenuation of food allergy symptoms following treatment with human milk oligosaccharides in a mouse model. Allergy 2015. 70(9): p. 1091-102.
49. de Kivit S, Kraneveld AD, Knippels LM, van Kooyk Y, Garssen J, and Willemsen LE. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. J Innate Immun 2013. 5(6): p. 625-38.
50. Niki T, Tsutsui S, Hirose S, Aradono S, Sugimoto Y, Takeshita K, Nishi N, and Hirashima M. Galectin-9 is a high affinity ige-binding lectin with anti-allergic effect by blocking ige-antigen complex formation. Journal of Biological Chemistry 2009. 284(47): p. 32344-32352.
51. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BC, Knol J, Sprikkelman AB, van der Aa LB, Knippels LM, Garssen J, van Kooyk Y, and Willemsen LE. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 2012. 67(3): p. 343-52.
52. Akdis CA and Akdis M. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. World Allergy Organ J 2015. 8(1): p. 17.
53. Okayama Y and Kawakami T. Development, migration, and survival of mast cells. Immunol Res 2006. 34(2): p. 97-115.
54. Shamji MH and Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. J Allergy Clin Immunol 2017. 140(6): p. 1485-1498.
55. Shamji MH, Kappen JH, Akdis M, Jensen-Jarolim E, Knol EF, Kleine-Tebbe J, Bohle B, Chaker AM, Till SJ, Valenta R, Poulsen LK, Calderon MA, Demoly P, Pfaar O, Jacobsen L, Durham SR, and Schmidt-Weber CB. Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and

allergic asthma: An eaaci position paper. Allergy 2017. 72(8): p. 1156-1173.
56. Kulis M, Vickery BP, and Burks AW. Pioneering immunotherapy for food allergy: Clinical outcomes and modulation of the immune response. Immunol Res 2011. 49(1-3): p. 216-26.
57. van de Veen W, Wirz OF, Globinska A, and Akdis M. Novel mechanisms in immune tolerance to allergens during natural allergen exposure and allergen-specific immunotherapy. Curr Opin Immunol 2017. 48: p. 74-81.
58. Nagakura KI, Sato S, Yanagida N, Nishino M, Asaumi T, Ogura K, and Ebisawa M. Oral immunotherapy in japanese children with anaphylactic peanut allergy. Int Arch Allergy Immunol 2018.
59. Salmivesi S, Paassilta M, Huhtala H, Nieminen R, Moilanen E, and Korppi M. Changes in biomarkers during a six-month oral immunotherapy intervention for cow's milk allergy. Acta Paediatr 2016. 105(11): p. 1349-1354.
60. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, Jacobson MR, Kimber I, Till SJ, and Durham SR. Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. J Allergy Clin Immunol 2011. 127(2): p. 509-516 e1-5.
61. Santos AF, James LK, Bahnson HT, Shamji MH, Couto-Francisco NC, Islam S, Houghton S, Clark AT, Stephens A, Turcanu V, Durham SR, Gould HJ, and Lack G. Igg4 inhibits peanut-induced basophil and mast cell activation in peanut-tolerant children sensitized to peanut major allergens. J Allergy Clin Immunol 2015. 135(5): p. 1249-56.
62. Schouten B, van Esch BC, Hofman GA, van den Elsen LW, Willemsen LE, and Garssen J. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. Int Arch Allergy Immunol 2008. 147(2): p. 125-34.
63. Nozawa A, Okamoto Y, Moverare R, Borres MP, and Kurihara K. Monitoring ara h 1, 2 and 3-sige and sigg4 antibodies in peanut allergic children receiving oral rush immunotherapy. Pediatr Allergy Immunol 2014. 25(4): p. 323-8.
64. Wright BL, Kulis M, Orgel KA, Burks AW, Dawson P, Henning AK, Jones SM, Wood RA, Sicherer SH, Lindblad RW, Stablein D, Leung DY, Vickery BP, Sampson HA, and Consortium of Food Allergy R. Component-resolved analysis of iga, ige, and igg4 during egg oit identifies markers associated with sustained unresponsiveness. Allergy 2016. 71(11): p. 1552-1560.
65. Sampson HA, O'Mahony L, Burks AW, Plaut M, Lack G, and Akdis CA. Mechanisms of food allergy. J Allergy Clin Immunol 2018. 141(1): p. 11-19.
66. Sugimoto M, Kamemura N, Nagao M, Irahara M, Kagami S, Fujisawa T, and Kido H. Differential response in allergen-specific ige, iggs, and iga levels for predicting outcome of

oral immunotherapy. Pediatr Allergy Immunol 2016. 27(3): p. 276-82.

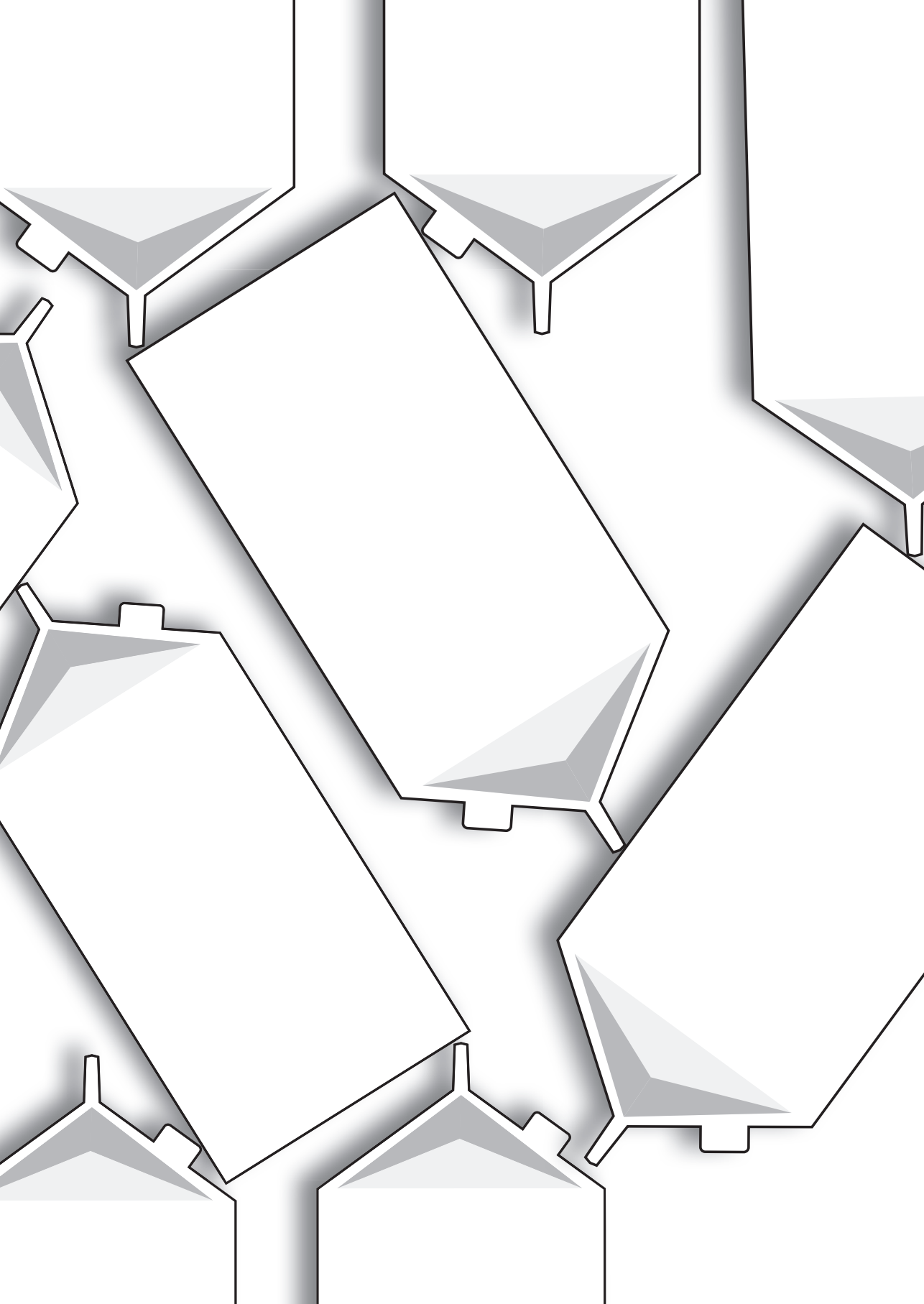
67. Janzi M, Kull I, Sjoberg R, Wan J, Melen E, Bayat N, Ostblom E, Pan-Hammarstrom Q, Nilsson P, and Hammarstrom L. Selective iga deficiency in early life: Association to infections and allergic diseases during childhood. Clin Immunol 2009. 133(1): p. 78-85.
68. Annunziato F, Romagnani C, and Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. J Allergy Clin Immunol 2015. 135(3): p. 626-35.
69. Berin MC and Shreffler WG. Mechanisms underlying induction of tolerance to foods. Immunol Allergy Clin North Am 2016. 36(1): p. 87-102.
70. Radulovic S, Jacobson MR, Durham SR, and Nouri-Aria KT. Grass pollen immunotherapy induces foxp3-expressing cd4+ cd25+ cells in the nasal mucosa. J Allergy Clin Immunol 2008. 121(6): p. 1467-72, 1472 e1.
71. Perezabad L, Reche M, Valbuena T, Lopez-Fandino R, Molina E, and Lopez-Exposito I. Oral food desensitization in children with ige-mediated cow's milk allergy: Immunological changes underlying desensitization. Allergy Asthma Immunol Res 2017. 9(1): p. 35-42.
72. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, Ruckert B, Akdis CA, and Akdis M. Igg4 production is confined to human il-10-producing regulatory b cells that suppress antigen-specific immune responses. J Allergy Clin Immunol 2013. 131(4): p. 1204-12.
73. Renz H, Allen KJ, Sicherer SH, Sampson HA, Lack G, Beyer K, and Oettgen HC. Food allergy. Nat Rev Dis Primers 2018. 4: p. 17098.
74. Polukort SH, Rovatti J, Carlson L, Thompson C, Ser-Dolansky J, Kinney SR, Schneider SS, and Mathias CB. Il-10 enhances ige-mediated mast cell responses and is essential for the development of experimental food allergy in il-10-deficient mice. J Immunol 2016. 196(12): p. 4865-76.
75. Akdis CA and Akdis M. Advances in allergen immunotherapy: Aiming for complete tolerance to allergens. Sci Transl Med 2015. 7(280): p. 280ps6.
76. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, and Powrie F. A functionally specialized population of mucosal cd103+ dcs induces foxp3+ regulatory t cells via a tgf-beta and retinoic acid-dependent mechanism. J Exp Med 2007. 204(8): p. 1757-64.
77. Goverse G, Molenaar R, Macia L, Tan J, Erkelens MN, Konijn T, Knippenberg M, Cook EC, Hanekamp D, Veldhoen M, Hartog A, Roeselers G, Mackay CR, and Mebius RE. Diet-derived short chain fatty acids stimulate intestinal epithelial cells to induce mucosal tolerogenic dendritic cells. J Immunol 2017. 198(5): p. 2172-2181.
78. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, Macia L, and Mackay CR. Dietary fiber and bacterial scfa enhance

oral tolerance and protect against food allergy through diverse cellular pathways. Cell Rep 2016. 15(12): p. 2809-24.

79. Daien CI, Pinget GV, Tan JK, and Macia L. Detrimental impact of microbiota-accessible carbohydrate-deprived diet on gut and immune homeostasis: An overview. Front Immunol 2017. 8: p. 548.
80. Zehra S, Khambati I, Vierhout M, Mian MF, Buck R, and Forsythe P. Human milk oligosaccharides attenuate antigen-antibody complex induced chemokine release from human intestinal epithelial cell lines. J Food Sci 2018.
81. Huang YJ, Marsland BJ, Bunyavanich S, O'Mahony L, Leung DY, Muraro A, and Fleisher TA. The microbiome in allergic disease: Current understanding and future opportunities-2017 practall document of the american academy of allergy, asthma & immunology and the european academy of allergy and clinical immunology. J Allergy Clin Immunol 2017. 139(4): p. 1099-1110.
82. Lynch SV and Boushey HA. The microbiome and development of allergic disease. Curr Opin Allergy Clin Immunol 2016. 16(2): p. 165-71.
83. Fieten KB, Totte JEE, Levin E, Reyman M, Meijer Y, Knulst A, Schuren F, and Pasmans S. Fecal microbiome and food allergy in pediatric atopic dermatitis: A cross-sectional pilot study. Int Arch Allergy Immunol 2018. 175(1-2): p. 77-84.
84. Rivas MN, Burton OT, Wise P, Zhang YQ, Hobson SA, Lloret MG, Chehoud C, Kuczynski J, DeSantis T, Warrington J, Hyde ER, Petrosino JF, Gerber GK, Bry L, Oettgen HC, Mazmanian SK, and Chatila TA. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. Journal of Allergy and Clinical Immunology 2013. 131(1): p. 201-212.
85. Huang YJ, Nariya S, Harris JM, Lynch SV, Choy DF, Arron JR, and Boushey H. The airway microbiome in patients with severe asthma: Associations with disease features and severity. J Allergy Clin Immunol 2015. 136(4): p. 874-84.
86. Shin NR, Whon TW, and Bae JW. Proteobacteria: Microbial signature of dysbiosis in gut microbiota. Trends Biotechnol 2015. 33(9): p. 496-503.
87. Zhu W, Winter MG, Byndloss MX, Spiga L, Duerkop BA, Hughes ER, Buttner L, de Lima Romao E, Behrendt CL, Lopez CA, Sifuentes-Dominguez L, Huff-Hardy K, Wilson RP, Gillis CC, Tukel C, Koh AY, Burstein E, Hooper LV, Baumler AJ, and Winter SE. Precision editing of the gut microbiota ameliorates colitis. Nature 2018. 553(7687): p. 208-211.
88. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, and Marsland BJ. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 2014. 20(2): p. 159-66.
89. Fonseca W, Lucey K, Jang S, Fujimura KE, Rasky A, Ting HA, Petersen J,

Johnson CC, Boushey HA, Zoratti E, Ownby DR, Levine AM, Bobbit KR, Lynch SV, and Lukacs NW. Lactobacillus johnsonii supplementation attenuates respiratory viral infection via metabolic reprogramming and immune cell modulation. Mucosal Immunol 2017. 10(6): p. 1569-1580.

90. Lloyd CM and Marsland BJ. Lung homeostasis: Influence of age, microbes, and the immune system. Immunity 2017. 46(4): p. 549-561.
91. McKenzie C, Tan J, Macia L, and Mackay CR. The nutrition-gut microbiome-physiology axis and allergic diseases. Immunol Rev 2017. 278(1): p. 277-295.
92. Akkerman R, Faas MM, and de Vos P. Non-digestible carbohydrates in infant formula as substitution for human milk oligosaccharide functions: Effects on microbiota and gut maturation. Crit Rev Food Sci Nutr 2018: p. 1-12.
93. Burks AW, Harthoorn LF, Van Ampting MT, Oude Nijhuis MM, Langford JE, Wopereis H, Goldberg SB, Ong PY, Essink BJ, Scott RB, and Harvey BM. Synbiotics-supplemented amino acid-based formula supports adequate growth in cow's milk allergic infants. Pediatr Allergy Immunol 2015. 26(4):p.316-22.



APPENDICES

NEDERLANDSE SAMENVATTING

DANKWOORD

ABOUT THE AUTHOR

LIST OF PUBLICATIONS

Nederlandse Samenvatting

Voedselallergieën komen voor bij 6-8% van de kinderen en 1-2% van de volwassenen in de Westerse wereld. De laatste jaren neemt het aantal kinderen, jongeren en volwassenen dat lijdt aan een voedselallergie toe. De verminderde blootstelling aan (pathogene) micro-organismen op jonge leeftijd als het immuunsysteem nog niet optimaal is ontwikkeld en de afname van de hoeveelheid niet-verteerbare vezels in het dagelijks dieet, zorgen voor een verandering in de samenstelling en activiteit van bacteriën in de darmen en voor een overgevoelig immuunsysteem (**hoofdstuk 1**). De 'standaard' reactie van het immuunsysteem na opname van een voedingseiwit is gebaseerd op tolerantie, wat betekent dat het immuunsysteem het eiwit verwerkt en erkent als een veilig eiwit. In het geval van een voedselallergie, is het mechanisme van tolerantie verstoord en ontstaat er een overgevoeligheidsreactie. Preventieve maatregelen die kunnen leiden tot verbetering van tolerantie en vermindering van allergieën in kinderen zijn beschreven in **hoofdstuk 2** van dit proefschrift.

Opname van een voedingseiwit kan plaatsvinden via de huid of via het verteringsstelsel en kan leiden tot een T helper 2 cel (Th2)-gemedieerde immunologische respons en de productie van specifiek IgE door B cellen. Specifiek IgE kan binden aan de IgE-receptor (FcεRI) op basofielen in de circulatie en mestcellen in de mucosale weefsels, een proces genaamd sensibilisatie. Bij een tweede blootstelling reageert het immuunsysteem via het IgE mechanisme en zorgt voor degranulatie van basofielen en mestcellen. De vrijgekomen pro-inflammatoire cytokines zorgen voor klinische symptomen zoals vasodilatatie, oedeemvorming, respiratoire problemen, huiduitslag, misselijkheid, diarree, constipatie en zelfs systemische anafylaxis. Het vermijden van voedingseiwitten, genaamd allergenen, waartegen een individu een acute allergische reactie kan ontwikkelen, is op dit moment de enige beschikbare behandelmethode voor allergische patiënten. Daarnaast wordt gebruik gemaakt van adrenaline injecties om ernstige acute allergische symptomen zoals anafylaxis te onderdrukken. De dagelijkse beperkingen met betrekking tot het dieet gaan gepaard met sociale problemen en suboptimale voedselinname bij (jonge) kinderen. Daarnaast heeft de angst voor een onverwachte allergische reactie aanzienlijk effect op de kwaliteit van leven van individuen met een voedselallergie.

De noodzaak om een effectieve en veilige therapie te ontwikkelen wordt benadrukt door het toegenomen aantal patiënten met een voedselallergie en de beperkte behandelmethoden die momenteel beschikbaar zijn. De toepassing van allergeen-specifieke immunotherapie wordt momenteel onderzocht in patiënten die lijden aan voedselallergieën. Het hoofddoel van deze behandelmethode is het induceren

of herstellen van orale tolerantie tegen voedingsallergenen, zodat de patiënt het allergeen weer kan (her)introduceren in het dagelijkse dieet. Met behulp van een (lage) dagelijkse dosering van het allergeen wordt ervoor gezorgd dat de drempelwaarde voor degranulatie van basofielen en mestcellen wordt verhoogd (desensibilisatie). Vervolgens wordt de dosering opgeschroefd zodat de Th2-gemedieerde immuunrespons naar een regulatoire immuunrespons kan worden gestuurd. Hoge dosering met het allergeen activeert regulatoire T cellen en B cellen en zorgt voor de productie van allergeen-specifiek IgG4 ten koste van IgE. Allergeen-specifiek IgG4 kan voorkomen dat het allergeen bindt aan IgE en de activatie van mestcellen en basofielen onderdrukken. Na een langdurige behandeling wordt de patiënt blootgesteld aan een (hoge) testdosering met het allergeen, om te meten of de allergische respons en de daarbij behorende klinische symptomen zijn afgenomen. Verschillende toedieningsroutes worden momenteel onderzocht in immunotherapie studies (o.a. orale, sublinguale, epidermale en subcutane toediening) en worden vergeleken op basis van effectiviteit en veiligheid. Ondanks veelbelovende resultaten, vooral na orale immunotherapie, wordt klinische toepassing op grote schaal voorkomen door het optreden van (ernstige) allergische bijwerkingen tijdens behandeling en het terugkeren van de allergische status nadat de behandeling is gestopt.

Het gebruik van voedingssupplementen met immunogene werking of met een positief effect op de samenstelling en de activiteit van bacteriën in de darmen (**hoofdstuk 2**: probiotica, prebiotica, synbiotica, meervoudig onverzadigde vetzuren en anti-oxidanten), lijkt een veelbelovende methode om de effectiviteit en veiligheid van immunotherapie te verbeteren. Het is bewezen dat de bacteriën in de darmen invloed hebben op de inductie van orale tolerantie en/of het ontstaan van voedselallergieën. Een verstoorde balans in de hoeveelheid en het soort bacteriën in de darmen beïnvloedt het immuunsysteem en wordt geassocieerd met ontstekingsziekten en allergieën. Niet-verteerbare vezels (koolhydraten) zijn mogelijke kandidaten om de groei van darmbacteriën en het immuunsysteem te stimuleren tijdens immunotherapie. In eerdere studies in zowel mensen als muizen is bewezen dat plantaardige niet-verteerbare vezels allergische symptomen kunnen verminderen. Daarnaast stimuleren de niet-verteerbare vezels de ontwikkeling van het immuunsysteem via de groei van gunstige darmbacteriën en gaan ze directe interacties aan met epitheel- en immuuncellen in de darmen. De werkzaamheid van plantaardige niet-verteerbare vezels komt overeen met de activiteit van vezels die aanwezig zijn in moedermelk en de ontwikkeling van de darmflora en het immuunsysteem van de pasgeborene kunnen bevorderen. Niet-verteerbare vezels blijven intact tijdens de spijsvertering en bereiken de darm in hun oorspronkelijke

vorm. Darmbacteriën fermenteren de vezels en produceren daarbij korte-keten vetzuren zoals butyraat, acetaat en propionaat. Korte-keten vetzuren hebben bewezen anti-inflammatoire effecten, zowel in de darm als in perifere weefsels via opname in de systemische circulatie. Korte-keten vetzuren zijn een energiebron voor darmcellen en kunnen direct communiceren met epitheelcellen en immuuncellen via specifieke receptoren. Orale toediening van korte-keten vetzuren leidde tot vermindering van allergische symptomen in muismodellen voor allergisch astma en pinda-allergie en dit effect was vergelijkbaar met het effect geobserveerd na toediening van de niet-verteerbare vezels. De exacte bijdrage van niet-verteerbare vezels, de samenstelling en activiteit van darmbacteriën en/of korte-keten vetzuren in het verminderen van de allergische respons is tot dusver nog niet bekend.

In dit proefschrift is onderzocht of de effectiviteit van orale immunotherapie kan worden verbeterd door gelijktijdig de inname van niet-verteerbare vezels te verhogen met behulp van een dieet. Hierbij is gebruik gemaakt van een muismodel voor koemelkallergie, waarin gezonde muizen werden gesensibiliseerd tegen het koemelkeiwit wei en vervolgens werden behandeld met orale immunotherapie en/of een dieet met toegevoegde niet-verteerbare vezels. In **hoofdstuk 3** is het model voor de behandeling van koemelkallergie geoptimaliseerd en zijn allergische muizen blootgesteld aan zowel orale als subcutane immunotherapie in oplopende doseringen. Na behandeling ondergingen de muizen specifieke provocaties met (hoge) doseringen wei. De mate van anafylaxis en de daarbij behorende symptomen (acute zwelling van de huid na injectie en daling van de lichaamstemperatuur) na behandeling werden gebruikt om de effectiviteit van orale en subcutane immunotherapie te bepalen. Dit hoofdstuk laat zien dat zowel orale als subcutane immunotherapie de acute allergische reactie na provocatie kan verminderen in een muismodel voor koemelkallergie. Deze vermindering ging gepaard met verlaagde allergeen-specifieke IgE en verhoogde allergeen-specifieke IgG1 en IgG2a niveaus. Deze bevindingen waren in overeenstemming met effecten van orale en subcutane immunotherapie in patiënten die last hebben van respiratoire allergieën en/of voedselallergieën. Vervolgens is het koemelkallergie model met de in **hoofdstuk 3** bepaalde (sub)optimale dosering orale immunotherapie gebruikt om het ondersteunende effect van niet-verteerbare vezels en korte-keten vetzuren verder te onderzoeken.

De resultaten in **hoofdstuk 4** lieten zien dat de combinatie van orale immunotherapie en een dieetinterventie met niet-verteerbare vezels de allergische reactie na provocatie sterk kon verminderen. Orale immunotherapie zonder dieetinterventie liet suboptimale bescherming tegen acute allergische symptomen zien. Daarnaast werd

de toename in wei-specifiek IgE, geïnduceerd door provocatie met het allergeen, voorkomen in allergische muizen die behandeld waren met de combinatietherapie. De niveaus wei-specifiek IgG1 en IgA in serum namen juist toe. Zowel toename in IgG1 als IgA is geassocieerd met bescherming tegen allergische symptomen. Halverwege het immunotherapie protocol zijn subgroepen muizen opgeofferd om de aanwezige immuuncellen te typeren met flow cytometrie. De resultaten toonden aan dat het percentage regulatoire T cellen aanwezig in de mesenterische lymfeknopen significant toegenomen was in dieren behandeld met de combinatietherapie, in tegenstelling tot dieren die enkel orale immunotherapie ondergingen. Het overbrengen van immuuncellen uit de milt van donor muizen naar naïeve ontvanger muizen, liet zien dat deze regulatoire T cellen een belangrijke rol spelen in de bescherming tegen allergische symptomen na provocatie. De combinatietherapie liet het percentage Th2 cellen in de milt dalen en het percentage Th1 cellen stijgen en onderdrukte de daar bijhorende cytokineproductie. Deze bevindingen waren in overeenstemming met resultaten uit therapie studies in mensen.

In **hoofdstuk 5** van dit proefschrift werd de data uit de koemelkallergie experimenten gebruikt om een model te ontwerpen (Bayesiaans netwerk) dat het effect van sensibilisatie, orale immunotherapie en/of een dieetinterventie met niet-verteerbare vezels op de belangrijkste (klinische) allergische parameters kan voorspellen. Daarnaast liet een topologisch netwerk gebaseerd op alle gemeten parameters in de koemelkallergie experimenten zien dat er een belangrijk verband bestaat tussen de mate van allergische symptomen en de aanwezigheid van korte-keten vetzuren in muizen. Deze innovatieve methoden kunnen de identificatie van nieuwe (voorspellende) biomarkers bevorderen en het onderzoek naar het optimaliseren van immunotherapie ondersteunen in toekomstige studies.

De bevinding dat er een sterk verband is tussen allergische symptomen en de aanwezigheid van korte-keten vetzuren in koemelkallergische muizen, versterkte de theorie dat korte-keten vetzuren een rol spelen in de beschermende effecten van orale immunotherapie en niet-verteerbare vezels. Om dit experimenteel te onderzoeken, werden er in **hoofdstuk 6**, naast de dieetinterventie met niet-verteerbare vezels, groepen muizen geïncludeerd waar oraal butyraat werd toegediend tijdens immunotherapie. De uitkomsten na provocatie met wei lieten zien dat orale toediening van butyraat in koemelkallergische muizen net zo effectief de mate van allergische symptomen kan verminderen als het dieet met niet-verteerbare vezels. Muizen die behandeld waren met orale immunotherapie en butyraat lieten een verlaagde activiteit van basofielen in de circulatie en mestcellen in de mucosale weefsels na provocatie zien. De concentratie butyraat in de darmen van allergische

muizen was alleen significant verhoogd na orale immunotherapie en de interventie met niet-verteerbare vezels. Deze bevinding gaf aan dat de gunstige effecten op de samenstelling en/of de activiteit van de darmbacteriën alleen worden gestimuleerd door de aanwezigheid van niet-verteerbare vezels en dat dit mogelijk een voordeel kan zijn ten opzichte van het gebruik van butyraat tijdens therapie.

Fecesmonsters van koemelkallergische muizen zijn geanalyseerd om de rol van darmbacteriën tijdens bescherming tegen de allergische respons na orale immunotherapie en de dieetinterventie met niet-verteerbare vezels verder te onderzoeken. De data in **hoofdstuk 7** van dit proefschrift laten zien dat er significante veranderingen in de aanwezigheid van specifieke bacteriestammen volgen na de dieetinterventie met niet-verteerbare vezels en/of orale immunotherapie. Een toename van bacteriën die horen binnen het geslacht *Allobaculum* (producenten van korte-keten vetzuren) was gemeten in fecesmonsters van allergische muizen die het dieet verrijkt met niet-verteerbare vezels ontvingen. Allergische muizen die alleen blootgesteld waren aan orale immunotherapie lieten een toename zien in de aanwezigheid van bacteriën die behoren tot de stam Proteobacteriën. Een toename in Proteobacteriën wordt geassocieerd met verstoring van de balans in aanwezige darmbacteriën en is te zien bij ontstekingsziekten zoals colitis en allergisch astma. Naar aanleiding van deze resultaten werd geconcludeerd dat er een mogelijk verband is tussen de suboptimale klinische bescherming gemeten na orale immunotherapie in koemelkallergische muizen en de verstoorde samenstelling van de darmflora. Het gelijktijdig toedienen van niet-verteerbare vezels zou deze verstoring tegen kunnen gaan via de groei van korte-keten vetzuur-producerende bacteriën. Behandeling met de combinatie van orale immunotherapie en de dieetinterventie met niet-verteerbare vezels leidde tot de sterkste bescherming tegen acute allergische symptomen en liet daarnaast een bacterieprofiel zien dat een grote mate van overeenkomst had met het bacterieprofiel in niet-allergische (gezonde) controle muizen.

Om in de toekomst de huidige behandelingsstrategie met orale immunotherapie en niet-verteerbare vezels te vertalen naar studies in mensen, werd de rol van de belangrijkste (voorspellende) allergische parameter allergeen-specifiek IgE in **hoofdstuk 8** geëvalueerd met behulp van *in vitro* experimenten. Serum van koemelkallergische muizen was afgenomen tijdens en na immunotherapie en na provocatie om wei-specifieke IgE niveaus te bepalen. Het serum werd vervolgens gebruikt om gekweekte basofielen en mestcellen te sensibiliseren met wei-specifiek IgE en te activeren met wei. Er werd een positieve correlatie gevonden tussen wei-specifieke IgE niveaus in serum en de mate van *in vitro* degranulatie van basofielen. De mate van degranulatie *in vitro* was eveneens geassocieerd met de mate van mestcel

degranulatie *in vivo* na provocatie. Deze observatie bevestigde dat vermindering van allergische symptomen zoals mestcel degranulatie na orale immunotherapie en een dieetinterventie met niet-verteerbare vezels in koemelkallergische muizen (tussentijds) geëvalueerd kan worden aan de hand van wei-specifieke IgE niveaus en functionele *in vitro* testen.

Samenvattend hebben de studies in dit proefschrift laten zien dat de effectiviteit van orale immunotherapie kan worden verbeterd door een dieetinterventie met niet-verteerbare vezels in een muismodel voor koemelkallergie. De combinatietherapie liet de meest effectieve vermindering van allergische symptomen zien na provocaties met (hoge) doseringen allergeen. In overeenstemming met de effecten van orale immunotherapie in voedselallergie studies in mensen, werd er in het koemelkallergie model een toename waargenomen van wei-specifieke IgE, IgG1 en IgA niveaus tijdens immunotherapie. De combinatie van orale immunotherapie en de dieetinterventie met niet-verteerbare vezels kon de stijging in wei-specifiek IgE na een allergeen provocatie voorkomen. De stijging in wei-specifiek IgG1 en IgA was afhankelijk van orale immunotherapie en leek niet beïnvloed door het dieet. De combinatietherapie kon de aanwezige T cel populaties in de milt en mesenterische lymfeknopen beïnvloeden: het percentage allergie-geassocieerde Th2 cellen daalde en het percentage Th1 cellen en regulatoire T cellen steeg. Zowel Th1 cellen als regulatoire T cellen worden geassocieerd met tolerantie. Analyses van korte-keten vetzuren in de darm en de samenstelling van darmbacteriën in fecesmonsters lieten significante veranderingen zien na orale immunotherapie en/of de dieetinterventie met niet-verteerbare vezels. Ondanks het feit dat mechanistische studies nodig zijn om de rol van korte-keten vetzuren in de vermindering van allergische symptomen na de combinatietherapie te bevestigen, geven de resultaten in dit proefschrift weer dat veranderingen in de samenstelling en activiteit van de darmbacteriën belangrijke factoren kunnen zijn die de effectiviteit van orale immunotherapie kunnen bevorderen. In **Figuur 1** (**hoofdstuk 9**) van dit proefschrift, zijn de belangrijkste experimentele resultaten grafisch weergegeven en worden onderliggende mechanismen van orale immunotherapie en een dieetinterventie met niet-verteerbare vezels geschetst die mogelijk leiden tot bescherming tegen koemelkallergie. Concluderend, een dieetinterventie met niet-verteerbare vezels is een veelbelovende strategie om de effectiviteit van orale immunotherapie in voedselallergie studies in mensen te verbeteren.

Dankwoord

Ongelofelijk maar waar, het einde is in zicht en bij deze begin ik aan het meest gelezen hoofdstuk van mijn proefschift: het dankwoord! De afgelopen vier jaar zijn voorbij gevlogen en ik ben trots op het eindresultaat, maar dit had ik zonder de hulp van de volgende bijzondere mensen nooit voor elkaar gekregen:

Mijn promotor Johan, lieve Johan, je onuitputtelijke enthousiasme en positieve instelling hebben me altijd erg geholpen om het goede van de dingen te blijven zien. Je liet me erg vrij in de keuzes die voor het project gemaakt moesten worden en ondanks je drukke agenda was je altijd op de hoogte van de laatste ontwikkelingen. Dank voor het vertrouwen dat je altijd in me hebt en voor je hulp bij het maken van de juiste carrière keuzes. Daarnaast grote dank voor het feit dat ik altijd alles tegen je mocht zeggen, zelfs toen ik je een hyena vond.

Mijn copromotor Betty, lieve Betty, dank voor je geduld met me en voor alles wat je me hebt geleerd vanaf de eerste dag dat ik aan de slag ging in jouw team. Je hulp tijdens de experimenten was onmisbaar en daarnaast gaf je me de ruimte om mijn eigen weg te gaan. Dank voor je interesse en betrokkenheid tijdens het project, maar vooral ook in mij als persoon. Vooral tijdens de grootste uitdaging van mijn promotietraject (de inspecteur), noem het maar zwartste bladzijde, stonden we samen sterk en is alles goed gekomen.

Mijn copromotor Léon, lieve Léon, dank voor het feit dat jij altijd de stabiele, nuchtere factor in ons team was. Ik kon blind op je bouwen en je zette me ook af en toe met beide benen terug op de grond als ik dat nodig had. Je eerlijkheid en steun waren erg belangrijk en ik wil je bedanken voor de zelfstandigheid die je van me verwachtte. De dansvloer bleef nooit lang leeg op een feestje waar wij allebei waren, ik hoop dat er nog velen zullen volgen!

De PhD-coördinator Paul H., lieve Paul, ik wil je bedanken voor je support en voor je interesse tijdens de afgelopen vier jaar. Je was de eerste die ik een hand gaf en je bent me altijd blijven volgen. Bedankt voor de gezellige tijd tijdens de PhD committee van Drug Innovation, alle uitjes en borrels (waar jij vaak het langst bleef en het fanatiekst en het onhandigst ging opruimen). Je bent de stille kracht van de afdeling en daarvoor ben ik je zeer dankbaar.

Mijn paranimf Suzanne, lieve Suus, waar moet ik beginnen?! Je bent mijn steun en toeverlaat geweest tijdens het grootste deel van mijn promotie en het doet pijn in

mijn hart dat ik je ga verlaten. Tijdens mijn experimenten was jij nog meer gedreven dan ik en bleef je tot het bittere einde helpen. Je week niet van mijn zijde toen ik een paar uur op de grond heb gelegen in het DDW en met de charmante evac-chair het gebouw verliet. We hebben prachtige reisjes gemaakt, skiën in Spanje en Zwisterland, de EAACI congressen, het was een feest! Work hard, play hard en vooral hebben we elkaar altijd gemotiveerd om het beste van onszelf te laten zien. Ik weet dat ik je vreselijk heb afgeleid tijdens het werk, dat ik vaak mijn filter niet aan had staan en dat ik overal wat van vond, maar het leek jou niet te deren. Waarvoor dank! Je bent een topper en ik wens je alle succes bij het afronden van je eigen project. Ik geloof in je en ik weet zeker dat je ook deze laatste periode je schouders eronder blijft zetten. Mijn paranimf Laura, lieve Lau, wij hebben van begin tot eind lief en leed gedeeld in het NUTRALL consortium. We zijn begonnen als twee kneuzen en hebben het geschopt tot doctor (bijna dan haha)! De dierexperimenten waren een gigantische klus en ik wil je bedanken voor je hulp en support. De NUTRALL meetings waren nooit zo goed vol te houden geweest zonder jou, vooral omdat je het voor mij allemaal goed wist te relativeren en we konden blijven lachen. We hebben samen genoten van het skiën in Italië en hebben een prachtige reis door Sri Lanka gemaakt. Lau, je bent een keiharde werker en ik waardeer je nuchtere instelling enorm! Ik weet zeker dat ook jouw verdediging niet lang op zich laat wachten, houd vol meid, zeker nu de laatste loodjes het zwaarst wegen.

Mijn collega's uit het NUTRALL consortium: Simone, Joost, Raymond, Linette, Henny, Marianne, Jolanda, Tim en Lars, dank voor de prettige samenwerking en voor jullie input tijdens het project. Ik heb erg veel geleerd van de discussies en het feit dat iedereen vanuit zijn eigen achtergrond naar een vraag of resultaat kijkt. Speciale dank voor de partners van TNO, ik ben erg blij dat we een stap hebben kunnen zetten in het gebruik van netwerk analyses om meer uit onze data-sets te halen. Lieve Simone, ik vond het erg fijn om met je samen te werken binnen ons project en ik wil je alle geluk wensen bij het afronden van jouw stuk van het NUTRALL project.

De mestcel-man Bart, lieve Bart, ik wil je enorm bedanken voor je hulp en inzet tijdens de in vitro experimenten die we hebben uitgevoerd. Jouw liefde voor het labwerk deel ik helaas niet, maar samen met jou in de flowkast werken was toch altijd wel een feestje! We hebben gezellig op de racefiets gezeten tijdens de Tourtocht en de Giro in Utrecht en zelfs een reisje naar Praag mogen maken voor het EMBRN congres. We konden elkaar af en toe in de weg zitten, maar hoe harder jij roept "uit het oog, uit het hart", hoe meer ik weet dat je me gaat missen. Ik ga jou in ieder geval zeker missen, onwijs bedankt voor de gezellige tijd en ik wil je heel veel geluk wensen met je twee kleine kanjers.

De opper mestcel-man Frank, dank voor je hulp en nuttige input in het onderzoek en voor je kritische blik op de laatste twee manuscripten. Ik vond het erg leuk om tijdelijk deel uit te maken van het mestcel-team, bekroond met de gezellige BBQ bij jou in de achtertuin! Ook wil ik Daniëlle bedanken voor je inzet en de fijne samenwerking tijdens je stage bij mij op het mestcel project.

Mijn (ex)bureau-genoten, lieve Mara, Kirsten, Atanaska, Saskia B., Kim en Jitske, vooral jullie gezelligheid ga ik enorm missen! Jullie waren stuk voor stuk altijd bereid om te helpen tijdens de secties, tijdens het organiseren van borrels en ook jullie interesse en zorgzaamheid heb ik erg gewaardeerd. Lieve Mara, ik wil jou nog even extra bedanken voor je hulp tijdens de experimenten en voor alle taken die je voor me wilde overnemen. Je bent een onmisbare en fijne collega! Lieve Kirst, bedankt voor je niet-aflatende interesse en voor de geweldige tijd die we samen hebben gehad! Zet hem op de komende tijd, blijf in jezelf geloven, want je bent echt een topper! Lieve Nas, dank voor je steun en het feit dat je altijd wilde meedenken! Zelfs als je het bere-druk had, stond je voor me klaar om te helpen. Lieve Sas en Kim, dank voor het lachen en de gezellige klets-momenten, ik heb veel van jullie kunnen leren en waardeer jullie enorm! Lieve Jits, de nuchtere feestganger uit het Oosten, dank voor je gezelligheid en voor je heerlijke kijk op de dingen "gewoon afstoempen dat proefschrift". Heel veel succes bij je nieuwe uitdaging in Nijmegen!

Lieve Paul J, het is eigenlijk een klein wonder dat ik mijn proefschrift op tijd heb kunnen inleveren met jou als vaste bron van afleiding. Zelfs in het schrijfhokje waar ik me vier maanden heb opgesloten wist je me te vinden om me even op te vrolijken of gewoon om extreem vervelend te doen. Ook al heb ik je menig maal achter het behang willen plakken, wil ik je bedanken voor de gezellige tijd, heel veel succes met je project!

My dearest Chinese friends, dear YingXin, Ling and Yulong, it has been a pleasure having you around! I would like to thank you for your interest and friendship, I truly admire your openness and your ability to adapt to the typical Dutch culture. Especially Wingyan, you are a special one, thanks for the nice talks.

Mijn lieve collega's en ex-collega's die wilden helpen tijdens het experimentele werk: Gemma, Gerard, Suzan T., Astrid, Veronica, Caroline en Saskia O. Lieve allemaal, ontzettend bedankt voor jullie hulp en inzet, zonder jullie had ik het tijdens de secties niet gered! Dank dat jullie van die fijne collega's voor me zijn geweest. Lieve Marianne, Manon en Laura van het IRAS, dank voor jullie hulp, het was erg fijn dat ik ook op jullie kon bouwen in tijden van nood.

Mijn (ex)collega's van de Pharmacology group in het DDW: Martje, Sandra, Soheil, Mojtaba, Katja, Anne Metje, Arash, Paula, Peyman, Daphne, beide Marjoleins, Lucianne, Aletta, Gert, Lidija, Roos, Hamed, Adele, Charlotte, Meng, Yang, Silvia, Manoe, Judith, Lei, Susan V., Koen, Monika, Ingrid, Thea, Amer, Milos, Michèle, Puqiao, Rolf, Pieter, Liesbeth, Jing, Jelle, Brenda, Negisa and many others, thanks for the great ambiance that you help to create in our department! It was a pleasure getting to know you all and I wish you all the best for the future. Extra dank voor Daphne vanwege de hilarische lunch-momenten waarin we de laatste aflevering van Dreamschool konden analyseren. Dear Sarah, thanks for the great times when you visited our group for several months. It was a pleasure having you around!

Mijn collega's van de Immunology group van Nutricia Research: Desiree, Tjalling, Prescilla, Bea, Selma, Nicole, Laura B., Reinilde, Anita, Lieke, Karen, Laura M., Joost, Nienke en Jeroen. Dank voor de warme ontvangst die ik altijd kreeg als ik langskwam bij jullie en voor de interesse tijdens mijn PhD project. Ik denk dat de goede basis is gelegd tijdens mijn afstudeerstage bij jullie in de groep. Speciale dank aan Desiree, vanwege de fijne samenwerking en je behulpzaamheid. Ik wil je heel veel succes wensen bij je nieuwe uitdaging bij UIPS. Lieve Jeroen, dank voor je interesse en je humor en voor de goede start die je mij binnen de alliantie hebt gegeven. Lieve Prescilla, ik wil je heel erg bedanken voor je hulp tijdens mijn banenzoektocht, zo waardevol!

Bij deze zou ik ook graag van de gelegenheid gebruik willen maken om alle fijne mensen om me heen nog een keer extra te vertellen hoe enorm blij ik ben dat jullie er zijn. Jullie interesse en support, met name tijdens de laatste loodjes van het promotietraject, hebben me zeker geholpen.

Mijn lieve senaatjes, ik ben er trots op dat we nog zo'n hechte band hebben. Dank voor het lachen, de gezellige etentjes en voor de spontane ideeën die ons zelfs in een auto-rally in Limburg doen belanden. Lieve Em, bedankt voor de fijne gesprekken en de mooie vriendschap.

Mijn lieve schoolvriendinnen, ondanks dat we allemaal ons eigen pad volgen, blijven we bij elkaar. Jullie zijn stuk voor stuk kanjers waar ik heel blij mee ben.
Mijn gezellige hockeyteam bij Kampong, lieve teamies, dank voor de fijne afleiding en voor de kans om promotie-frustraties eruit te slaan op het veld. Op naar de winst en nog meer gezelligheid!

Mijn lieve clubgenootjes, ik ben heel erg blij met jullie en wil jullie bedanken voor de waardevolle vriendschappen en oneindige gezelligheid! Speciale dank aan Charly en Linnie voor jullie vriendschap en de beste Tomorrowland feestjes ever! Lieve Lous, dank voor je luisterende oor en je vermogen om de juiste vragen te stellen. Daarnaast verdient de hele wintersport crew een speciale benoeming vanwege de fanatische week waarin alle promotie-stress eruit geskied en gedanst kon worden (volgend jaar weer?).

Mijn lieve oud-huisgenootjes uit Wageningen, dank voor jullie steun en interesse en voor de fijne weekendjes weg! Tussen alle drukte door is er bij jullie altijd ruimte om je verhaal te doen, heel waardevol!

Lieve familie, in het bijzonder Liesbeth en Rene, ik wil jullie enorm bedanken voor de hulp en inzichten die jullie me gaven ter voorbereiding op de stappen na het promotie-traject. Fijn om op je familie terug te kunnen vallen. Lieve Patries, Duncan en Mika en lieve nichtjes Marissa en Lenneke, dank voor jullie gezelligheid en interesse, fijn om jullie dichtbij te hebben! Lieve oma Trees, ik ben er trots op dat je deze mijlpaal nog mee mag maken, je blijft altijd mijn grootste fan en ik bewonder je doorzettingsvermogen enorm.

En dan the wolfpack! Lieve Fien, je bent een ontzettend waardevolle vriendin, een luisterend oor en een steun en toeverlaat. Bedankt dat je er altijd voor me bent en dat je me zo goed kunt lezen. Ik waardeer je eerlijkheid en je enthousiasme voor de kleine dingen om je heen. Lieve As, je talent om altijd het goede in mensen te zien waardeer ik enorm. Waar ik soms wat bot uit de hoek kan komen, weet jij altijd het juiste te zeggen. Je bent een rots in de branding en hebt oneindig geduld en interesse in de mensen om je heen. Lieve meiden, dank voor de ontspanning tijdens het sporten (dank ook aan Amber en Rens!) en tijdens de etentjes bij de Spaghetteria, maar vooral dank voor deze fijne vriendschap.

Homies! Lieve Richel, ex-huisgenoot aan de ASW, vanaf onze eerste dag in Wageningen zijn onze wegen onafscheidelijk. Ik zou je enthousiasme, je interesse in iedereen en je onuitputtelijke energie voor geen goud willen missen! Dank voor je support tijdens mijn promotie-traject, maar vooral dank voor je positieve kijk op het leven en voor het feit dat je me af en toe wakker kan schudden. Je bent er één uit duizenden en ik hoop dat we nog lang samen het licht uit doen! Lieve Lin, huisgenoot aan de ASW, maar vooral buddy for life! Je hebt het hele promotie-traject meegemaakt vanaf de voorste rij, mijn god wat ben ik blij dat het erop zit. Dank voor je interesse en je zakelijke blik tijdens moeilijke situaties. Dank voor de vele keren dat je al gekookt

had en ik tot laat kon doorschrijven aan mijn thesis. Ik waardeer jou en je nuchtere blik enorm en ben blij dat onze directe manier van communiceren altijd goed heeft gewerkt.

Lieve Maaike en Alex, lieve zus en broer, dank voor jullie onvoorwaardelijke liefde en voor de interesse en steun. Ik heb me als kleine zus altijd veilig gevoeld bij jullie en wil jullie bedanken voor jullie open blik. Vooral toen ik alles anders ging doen dan we thuis gewend waren. Lieve Boris en Mariëlle, ik ben enorm blij dat jullie erbij gekomen zijn en dat jullie de typische gang van zaken bij ons thuis hebben weten te waarderen. Lieve Finn, Dex, Kai en Bente, jullie zijn nu nog kleine draakjes, maar ooit ga ik jullie vertellen hoe ik op een dag doctor werd. Ik ben nu al mega trots op jullie en ik zal er altijd voor jullie zijn.

Lieve ouders, papa en mama, ik bedank jullie als allerlaatst maar ook het allermeest, omdat jullie er altijd voor me zijn en omdat jullie nergens voor terugdeinzen. Met jullie als thuisfront, kunnen wij de wereld aan. Dank voor de onvoorwaardelijke liefde, maar vooral voor het vertrouwen dat ik altijd heb gevoeld. Wat de toekomst ook zal brengen, ik weet dat jullie achter me staan. Van hier tot aan de maan…en terug.

Het zit erop, vier jaar hard werken en zoveel mogelijk leren, het was een intense tijd en ik had het voor geen goud willen missen. Wat heb ik genoten van alle gezelligheid en uitdagingen, deze herinneringen nemen ze me nooit meer af. Iedereen die hier op wat voor manier dan ook aan heeft bijgedragen (en ik wellicht niet genoemd heb): BEDANKT! Zo, en nu aan het bier en hup die dansvloer op!

About the author

Marlotte Marianne Vonk was born on the 25th of May 1989 in Gouda, the Netherlands. From 2001 to 2007, she attended the Erasmus College in Zoetermeer, resulting in an atheneum diploma in Nature and Health. Afterwards, she enrolled in the Bachelor program Nutrition and Health at Wageningen University in Wageningen, the Netherlands. Marlotte included a minor in Immunology in the educational program and performed her literature thesis under the supervision of Dr. Ruth Adriaansen at the Department of Cell Biology and Immunology, which was led by Prof. dr. Huub Savelkoul. During her studies in Wageningen, she decided to join the full-time board of the student association W.S.V. Ceres for one year (2010-2011) to gain more operational experience. Afterwards, she successfully attained her Bachelor diploma (2011) and pursued her interest in medical sciences in the Master program Biomedical Sciences from 2012-2014 at the Radboud UMC in Nijmegen, the Netherlands. In 2013, she moved to Stockholm, Sweden, to conduct a 6-month internship in the Malaria Research Group led by Prof. dr. Anders Björkman at the Department of Medicine of the Karolinska Institutet. Marlotte conducted her second 6-month internship at the Immunology Platform led by Prof. dr. Johan Garssen at Danone Nutricia Research, Utrecht, the Netherlands. Under the supervision of Dr. Jeroen van Bergenhenegouwen, she studied the immunomodulatory effects of a synbiotic concept, consisting of non-digestible oligosaccharides and CpG DNA, in an *in vitro* co-culture system. After graduation, Marlotte conducted her PhD project at the Department of Pharmacology of the Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands under the supervision of Prof. dr. Johan Garssen and the daily supervision of Dr. Betty van Esch and Dr. Léon Knippels (2014-2018). The PhD project was part of the NUTRALL research consortium in which partners from industry (Danone Nutritica Research), academia (Institute for Risk Assessment Sciences and UMCU) and the government (TNO) collaborated. During her PhD trajectory, Marlotte was trained in the PhD program Drug Innovation of the Graduate School of Life Sciences. She received two travel grants from the European Academy of Allergy and Clinical Immunology (EAACI) to attend the annual congress in Barcelona (2015) and Helsinki (2017). During her PhD, she won a poster award at the EAACI annual congress in Barcelona (2015), at the UIPS bi-annual symposium in Utrecht (2016, 2nd place) and at the EAACI Winterschool in Switserland (2018).

List of publications

Jolanda H.M. van Bilsen, Lars Verschuren, Laura Wagenaar, Marlotte M. Vonk, Betty C.A.M. van Esch, Léon M.J. Knippels, Johan Garssen, Joost J. Smit, Raymond H.H. Pieters and Tim J. van den Broek. *A network-based approach for identifying suitable biomarkers for oral immunotherapy of food allergy.* 2018. (To be submitted)

Marlotte M. Vonk, Bart R.J. Blokhuis, Mara A.P. Diks, Laura Wagenaar, Joost J. Smit, Raymond H.H. Pieters, Johan Garssen, Léon M.J. Knippels and Betty C.A.M. van Esch. *Butyrate enhances desensitization induced by oral immunotherapy in cow's milk allergic mice*. 2018. (Submitted for publication)

Marlotte M. Vonk, Phillip A. Engen, Ankur Naqib, Stefan J. Green, Ali Keshavarzian, Bart R.J. Blokhuis, Frank A. Redegeld, Johan Garssen, Léon M.J. Knippels and Betty C.A.M. van Esch. *Altered microbial community structure and metabolism in cow's milk allergic mice treated with oral immunotherapy and fructo-oligosaccharides.* 2018. (Submitted for publication)

Laura Wagenaar, Manon van Roest, Laura Kruijsen, Marlotte M. Vonk, Betty C.A.M. van Esch, Léon M.J. Knippels, Johan Garssen, Raymond H.H. Pieters and Joost J. Smit. *Improvement of safety of subcutaneous immunotherapy for peanut allergy with the non-digestible oligosaccharides scFOS/lcFOS.* 2018. (Submitted for publication)

Laura Wagenaar, Marianne W.H.C. Bol-Schoenmakers, Giulio Giustarini, Marlotte M. Vonk, Betty C.A.M. Van Esch, Léon M.J. Knippels, Johan Garssen, Joost J. Smit and Raymond H.H. Pieters. *Dietary supplementation with non-digestible oligosaccharides reduces allergic symptoms and supports low dose oral immunotherapy in a peanut allergy mouse model.* 2018. (Submitted for publication)

Marlotte M. Vonk, Mara A.P. Diks, Laura Wagenaar, Joost J. Smit, Raymond H.H. Pieters, Johan Garssen, Betty C.A.M. van Esch and Léon M.J. Knippels. *Improved efficacy of oral immunotherapy using non-digestible oligosaccharides in a murine cow's milk allergy model: a potential role for Foxp3+ regulatory T cells*. 2017. Frontiers in Immunology, 8:1230, doi: 10.3389/fimmu.2017.01230

Marlotte M. Vonk*, Laura Wagenaar*, Raymond H.H. Pieters, Léon M.J. Knippels, Linette E.M. Willemsen, Joost J. Smit, Betty C.A.M. van Esch and Johan Garssen. *The efficacy of oral and subcutaneous antigen-specific immunotherapy in murine cow's milk- and peanut allergy models*. 2017. Clinical and Translational Allergy, 7:35, doi: 10.1186/s13601-017-0170-y

Marlotte M. Vonk*, Atanaska I. Kostadinova*, Matthias V. Kopp, Betty C.A.M van Esch, Linette E.M. Willemsen, Léon M.J. Knippels and Johan Garssen. *Dietary interventions in infancy.* 2016. Allergy, Immunity and Tolerance in Early Childhood: The First Steps of the Atopic March, Academic Press, Elsevier, 261-284, ISBN: 978-0-12-420226-9

*These authors contributed equally to this work