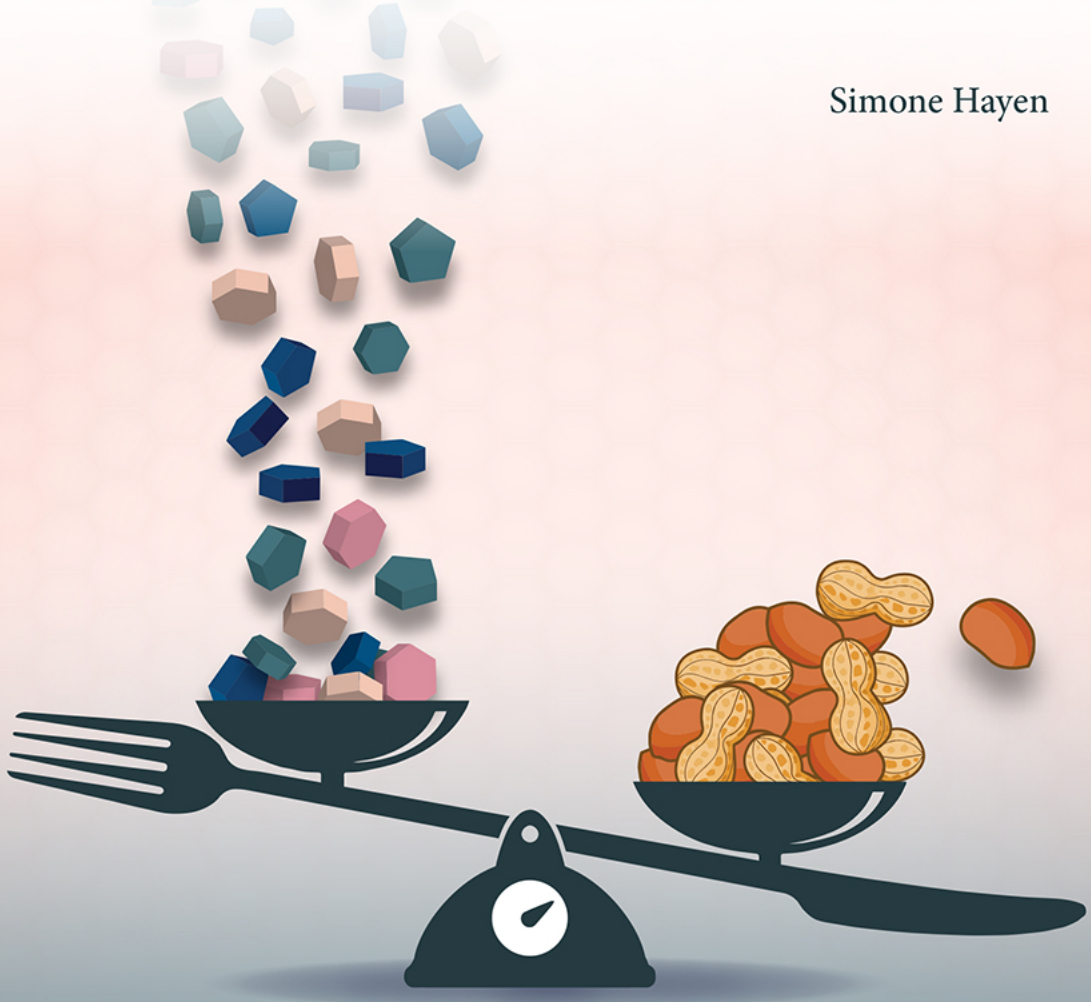
IMMUNOMODULATORY EFFECTS OF NON-DIGESTIBLE OLIGOSACCHARIDES IN PEANUT ALLERGY TREATING BY EATING

Simone Hayen



Immunomodulatory effects of non-digestible oligosaccharides in peanut allergy
Treating by eating

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Immunomodulatory effects of non-digestible oligosaccharides in peanut allergy

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TABLE OF CONTENTS

APPENDICES

CHAPTER 1

General introduction

Food allergy

The prevalence of food allergies is increasing worldwide, particularly but not only in developed countries[1-3] and has an impact on the quality of life of affected children or adults. According to several studies, genetic factors such as the human leukocyte antigen (HLA) locus play a role in the onset of allergies. However, the rate at which the prevalence of food allergies is increasing cannot be solely ascribed to genetic inheritance, and most likely is a combination of both environmental and genetic factors.[3] Food allergies can develop when immunological tolerance against the harmless food allergens is hampered. Major food allergens are peanut, tree nuts, milk, egg, wheat, soy and (shell)fish.[4] Among these food allergens, it is estimated that approximately 0.4-0.6% of the total population is affected by peanut allergy,[5,6] and in contrast to for example egg and cow's milk allergy that is mostly transient, peanut allergy is a highly persistent food allergy. Due to this persistent character and the frequently severe allergic symptoms,[5] this thesis will focus on diagnosis and hereditary background of peanut allergy, and investigates approaches to re-establish tolerance against peanut allergens. This introduction will give general background about food allergy and will indicate the scope of each chapter.

Allergic sensitization and elicitation phase

The development of peanut allergy can roughly be divided into two phases: the sensitization phase and the effector phase. Ingested peanut allergens are transported across the epithelium of the gastro-intestinal tract via M cells or intestinal epithelial cells (IECs)[7] and can be processed by antigen-presenting cells (APCs), such as dendritic cells (DCs). These DCs in turn can present these processed proteins via MHC class II to the T-cell receptor on T cells. Under the influence of certain cytokines, these T cells can develop into T helper 2 cells (Th2). Cytokines such as IL-4, IL-5 and IL-13 are secreted, and the release of IL-4 and IL-13 induces class switching in B cells, whereas IL-5 stimulates and recruits eosinophils.[8] The B cells, that have a B-cell receptor which also recognizes the allergen, subsequently start producing allergen-specific IgE. These specific IgEs can bind to the fragment crystallizable epsilon receptor 1 (FcεRI) that is expressed on among others basophils and mast cells. This whole process which finally results in the binding of specific IgEs to FcεRI is called the sensitization phase. Upon a second encounter with the specific peanut allergen, the allergen crosslinks the bound specific IgEs; this will induce the degranulation of the basophils or mast cells, and is called the effector phase, characterized by the allergic disease symptoms.[9,10] The degranulation of mast cells and basophils results in the release of several mediators such as histamines, which leads to clinical symptoms such as swelling of the oral mucosa, gastro-intestinal disturbances, wheezing, dermal urticaria or even (fatal) anaphylaxis. As most food allergies including peanut allergy are IgE-mediated, this thesis will focus on IgE-mediated peanut allergy.

I – Diagnosis of peanut allergy and hereditary factors

Peanut allergens

To diagnose peanut allergy, in most cases a skin prick test (SPT) is conducted, and in addition peanut-specific IgE antibodies can be detected in the blood.[11,12] However, the gold standard remains the use of a double-blind placebo-controlled food challenge (DBPCFC) where patients gradually receive increasing doses of peanut or placebo, while they are monitored on adverse clinical reactions. This DBPCFC has the disadvantage that it is labor intensive and burdensome for patients, sometimes resulting in severe reactions. Therefore, there is more interest towards the added diagnostic value of detection of specific IgE against components of peanut protein to predict peanut allergy, such as Ara h 2 and Ara h 6. **Chapter 2** elaborates more on these specific IgEs and their contribution in diagnosing peanut allergy.

Genetic background

While the exact origin of peanut allergy is not completely understood, there are indications that there is a certain link with the genetic background of the patients. The Human Leukocyte Antigen (HLA) system plays an important role in the presentation of allergens to T cells, and in this way the allergic cascade as described previously is activated. In **chapter 3**, the contribution of class I and II HLA molecules was described in a well-defined cohort of peanut-allergic patients, and is compared to the genetic background of a large control cohort of healthy stem cell donors.

II - Immunotherapeutic approaches

Currently, no lasting treatment options are available to induce long-lasting tolerance in peanut-allergic patients. Immunotherapeutic approaches aiming to restore tolerance are still under development. Major issues are efficacy and the frequent occurrence of sometimes severe allergic reactions. To further optimize these immunotherapeutic approaches, more interest is gained towards the use of specific adjuvants during immunotherapy, such as anti-IgE antibody omalizumab, Th1 inducing cytosine phosphate guanine (CpG) DNA or even dietary components. More examples of immunotherapeutic approaches and adjuvants are further discussed in the review as presented in **chapter 4**. The rationale behind the use of dietary adjunct therapy is that these components have immunomodulatory capacities, and may already skew the immune response away from the allergenic phenotype towards a more regulatory or T helper 1 (Th1) phenotype, and thus supports the immunotherapeutic approaches, rendering them more safe and efficacious.

III - Immune modulation by non-digestible oligosaccharides

One of the dietary adjunct strategies mentioned in **chapter 3** are non-digestible oligosaccharides (NDOs). These NDOs can be purified from sources such as inulin or prepared from lactose, and functionally resemble some of the characteristics of human milk oligosaccharides (HMOs) in breastmilk. These NDOs and HMOs can among others influence the gut microbiota[13,14] by enhancing the colonization of beneficial bacteria in the gut, such as lactobacilli and bifidobacteria[14]; a function that is labeled as a prebiotic function. A mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) is an example of a prebiotic NDO mixture, which has been studied extensively in different allergy prevention studies. In one of these studies, supplementation of a milk formula with scGOS/lcFOS was able to reduce the incidence of atopic dermatitis in children at risk.[15] In a different study, children that were breastfed or received a prebiotic-supplemented milk formula were eczema-free for longer periods than the placebo-group, indicating the potential of HMOs and NDOs.[16] These NDOs and HMOs thus have a beneficial effect on the intestinal homeostasis and promote the colonization and activation of so-called beneficial bacteria. As scGOS is synthetically prepared from cow's milk derived lactose, it may pose risks in people with a severe cow's milk allergy.[17] Therefore, a mixture of short- and long-chain fructo-oligosaccharides (scFOS/lcFOS) may be an alternative, since this mixture is derived from the vegetable source inulin (e.g. chicory, asparagus). Throughout this thesis, these two NDO mixtures are used, and their immunomodulatory capacities are assessed in an allergen-specific manner in different models, using cells of peanut-allergic patients. In this way, we investigated whether these NDOs might indeed be able to contribute to enhancing allergen-specific immunotherapeutic approaches by skewing the immune response away from the allergic phenotype.

Intestinal epithelial cell-PBMC co-culture

Chapter 5 focuses on the immunomodulatory capacities of scGOS/lcFOS and scFOS/lcFOS in an allergen-specific co-culture model between human intestinal epithelial cells (IECs) and peripheral blood mononuclear cells (PBMCs) of peanut-allergic patients. The gastrointestinal tract plays an important and pivotal role in the development of food allergies, since it is constantly discriminating between harmful and harmless antigens.[9,18] In this co-culture model the interaction between IECs and PBMCs can be studied in an allergen-specific environment.

Basophil degranulation

Previously, galectin-9, a soluble lectin, was found to be upregulated after whey-allergic mice received oral immunotherapy (OIT) in combination with scFOS/lcFOS.[19] Galectin-9 can among others be released by IECs and can inhibit the formation of the allergen-IgE complex

by binding to carbohydrate moieties of IgE.[20] Hereby mast cell or basophil degranulation can be reduced. Next to their function in the gut, NDOs also can become available systemically. Previous research indicated that HMOs and FOS, and most likely more oligosaccharide structures, were traced back in urine and the plasma compartment.[21-24] Therefore, **chapter 6** describes the direct effects of scGOS/lcFOS, scFOS/lcFOS and their indirect mediator galectin-9 using whole blood of peanut-allergic patients. In this model, blood was pre-incubated with NDOs or galectin-9, after which different cytokines and chemokines were measured in the plasma and basophil degranulation was assessed.

Autologous DC-T cell assay

The last experimental model focuses on the immunomodulatory effects of NDOs on DCs. Since DCs play an important role in antigen presentation, **chapter 7** investigates whether scFOS/lcFOS can affect antigen presentation of specific peanut epitopes to T cells. Hereto, monocyte-derived DCs (moDCs) were exposed to scFOS/lcFOS and peanut extract during maturation, followed by a DC-T cell co-culture with autologous T cells from the peanut-allergic patients. DCs would be an interesting target since these cells present antigens to T cells and determine the outcome of the consequent T cell response. Hence, if antigen presentation to T cells can be influenced, it can help to prevent sensitization or restore tolerance to the antigen of interest.

Finally, a summarizing discussion of the results obtained is provided in **chapter 8** and future perspectives on using oligosaccharides as adjunct therapy for allergen-specific immunotherapy are discussed.

Aims of this thesis

This thesis aimed to:

- Investigate the most important allergens of peanut and identify whether a genetic association can be found between HLA and peanut allergy
- Investigate the current strategies available to provide and improve treatment for peanut-allergy and to reduce side-effects
- Investigate the *in vitro* immunomodulatory properties of non-digestible oligosaccharides, and how these can support immunotherapeutic strategies.

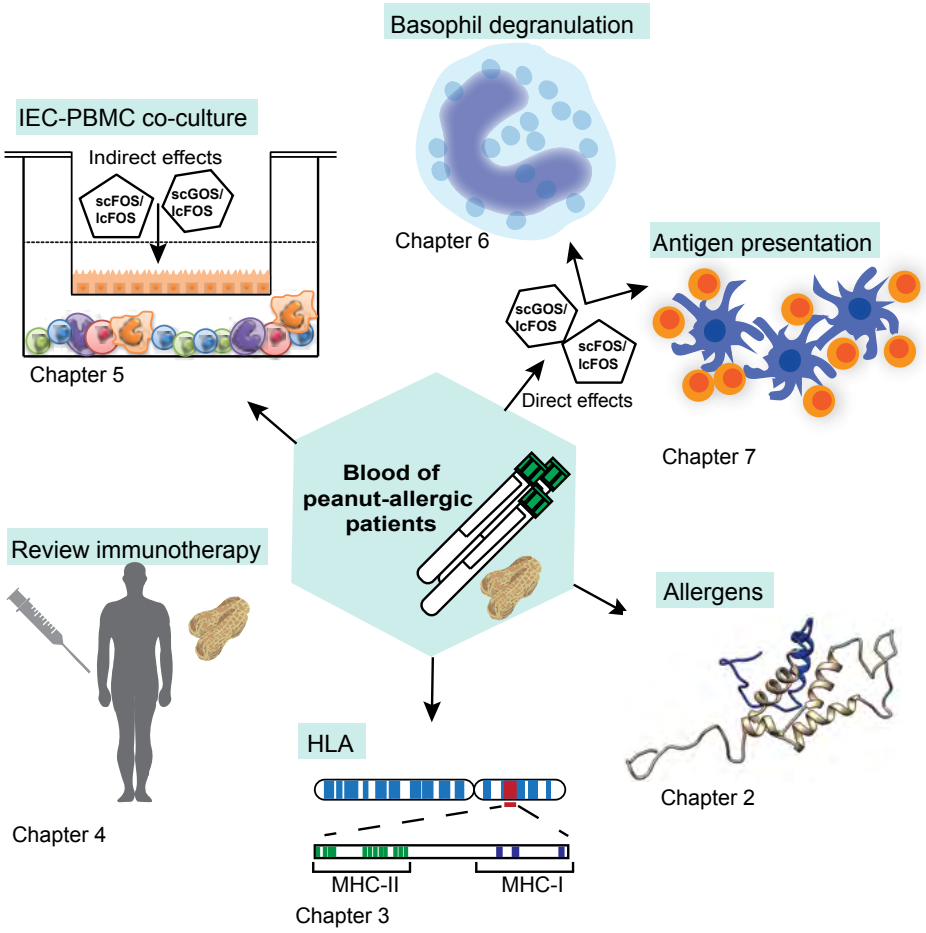

Figure 1. Outline of this thesis
Allergens involved in peanut allergy (chapter 2) and the hereditary influence of HLA (chapter 3) were investigated with blood of peanut-allergic patients. Chapter 4 elaborates on state of the art immunotherapeutic approaches. Blood of peanut-allergic patients was used to identify the immunomodulatory effects of non-digestible oligosaccharides (NDOs) in a co-culture model of intestinal epithelial cells (IECs) with PBMCs (chapter 5), basophil degranulation (chapter 6) or antigen presentation (chapter 7).

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PART 1
DIAGNOSIS OF PEANUT ALLERGY AND HEREDITARY FACTORS

CHAPTER 2

2S protein Ara h 7.0201 has unique epitopes compared to other Ara h 7 isoforms and is comparable to 2S proteins Ara h 2 and 6 in basophil degranulation capacity

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ABSTRACT

Background

Screening for specific IgE against 2S albumin proteins Ara h 2 and 6 has good positive predictive value in diagnosing peanut allergy. From the third 2S member Ara h 7, three isoforms have been identified. Their allergenicity has not been elucidated.

Objective

This study investigated the allergenicity of Ara h 7 isoforms compared to Ara h 2 and 6.

Methods

Sensitization of 15 DBPCFC confirmed peanut-allergic patients to recombinant Ara h 2.0201, 6.01 and isoforms of recombinant Ara h 7 was determined by IgE immunoblotting strips. A basophil activation test (BAT) was performed in nine patients to determine IgE-crosslinking capacities of the allergens. Sensitivity to the allergens was tested in five patients who were sensitized to at least one Ara h 7 isoform, by a concentration range in the BAT. 3D prediction models and sequence alignments were used to visualize differences between isoforms and to predict allergenic epitope regions.

Results

Sensitization to Ara h 7.0201 was most frequent (80%) and showed to be equally potent as Ara h 2.0201 and 6.01 in inducing basophil degranulation. Sensitization to Ara h 7.0201 together with Ara h 2.0201 and/or 6.01 was observed, indicating the presence of unique epitopes compared to the other two isoforms. Differences between the three Ara h 7 isoforms were observed in C-terminal cysteine residues, pepsin and trypsin cleavage sites and three single amino acid substitutions.

Conclusion & clinical relevance

The majority of peanut-allergic patients are sensitized to isoform Ara h 7.0201, which is functionally as active as Ara h 2.0201 and 6.01. Unique epitopes are most likely located in the C-terminus or an allergenic loop region which is a known allergenic epitope region for Ara h 2.0201 and 6.01. Due to its unique epitopes and allergenicity, it is an interesting candidate to improve the diagnostic accuracy for peanut allergy.

1 | INTRODUCTION

It is estimated that approximately 11.4%-13.1% of children (0-17 years) and 3.2%-5.1% of adults (>18 years) in European countries are sensitized against at least one food allergen, based on the detection of specific IgE in serum.[1] Most food allergies are IgE-mediated, and symptoms develop within minutes to a few hours after ingestion of the specific allergen. Among food allergies, peanut allergens are most frequent in eliciting a fatal food reaction, and it is estimated that 0.6% of the total population is affected by peanut allergy.[2] By determining specific IgE to recombinant or purified peanut proteins rather than crude peanut extract, component-resolved diagnostics (CRD) has proven to be a useful tool to improve diagnostic accuracy for peanut allergy.[3] However, the functionality of several potentially relevant allergens remains unknown, as their capacity to induce effector cell degranulation has never been tested in patient samples before.

To date, 17 peanut allergens (Ara h 1-17) are known and most of them have been sequenced and cloned.[3-6] Previous research indicated that screening for specific IgE against 2S albumins Ara h 2 and 6 to date is most effective in diagnosing peanut allergy, as the majority of peanut-allergic patients have specific IgE against these allergens.[6-9] Ara h 2 and Ara h 6 are both proteins belonging to the conglutin family, and Ara h 6 has approximately 53% sequence identity with Ara h 2.[5, 6]

Next to determining specific IgE against Ara h 2 and Ara h 6 for diagnosing peanut allergy, a third 2S protein Ara h 7 is currently gaining attention as predictor for peanut allergy.[10] Although Ara h 7 makes up only 0.5% of total peanut protein,[3] it is a storage protein and therefore considered an allergen with a potential strong diagnostic value.[11] In comparison, the abundance of Ara h 2 ranges between 5.9% and 9.3% of total peanut protein and for Ara h 6, this is 4% - 14%.[3, 12] By phage display technology, isoforms Ara h 7.0101 and Ara h 7.0201 were previously identified as allergens, but only Ara h 7.0201 was identified in peanut extract.[5] Ara h 7.0101 shares 42% sequence identity with Ara h 2 and 45% with Ara h 6, whereas Ara h 7.0201 shares 44% sequence identity with Ara h 2 and 52% with Ara h 6.[5, 13] Next to these two isoforms of Ara h 7, a third isoform labeled Ara h 7.0301 shares 70% sequence identity with Ara h 7, and was also identified in peanut extract.[5, 14] Previous research indicated the presence of unique epitopes in these Ara h 7 isoforms, and therefore, they may be relevant in peanut allergy diagnosis.[10]

As the functionality of Ara h 7 has not yet been studied extensively, the aim of this study was to identify whether the capacity of Ara h 7 to induce basophil degranulation using whole blood from peanut-allergic patients is similar to Ara h 2 and 6. In addition, the allergen recognition pattern of the three isoforms of Ara h 7 by peanut-allergic patients was studied. Furthermore, in relation to their functionality, the amino acid sequence and a 3D prediction protein model were used to predict epitopes or regions of Ara h 7 that are important in inducing basophil degranulation and allergenicity.

2 | MATERIALS & METHODS

2.1 | Study design and study population

Assessment of sensitization to 2S peanut allergens was performed with residual plasma of 15 peanut-allergic patients who visited the outpatient clinic of Dermatology/Allergology at the University Medical Center Utrecht in 2015-2017 for clinical research. Table 1 shows data on gender, age and historical data on SPT, subjective and objective doses determined by DBPCFC and Müller score.

Of these 15 patients, 9 random patients who were scheduled for visiting the UMC for clinical research were able to donate blood for the functional basophil activation test. Five random patients from the complete cohort who were sensitized to at least one Ara h 7 isoform were recruited for the concentration range BAT. Inclusion criteria consisted of a type I allergic reaction to peanut, confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC). Use of prednisone, other immunosuppressants or pregnancy were exclusion criteria. Informed consent was obtained of all patients prior to the study. The study was reviewed and approved by the Ethics Committee of the University Medical Center Utrecht (NL51606.041.15).

2.2 | Expression and purification of crude peanut extract and recombinant allergens

Crude peanut extract (CPE) was obtained by blending raw peanuts, followed by extraction at room temperature with Tris/NaCL buffer (20 mmol/L Tris, 150 mmol/L NaCL, pH 7.2). Supernatant was filtered twice and diluted in 1x PBS to the appropriate concentration. Recombinant peanut allergens Ara h 2.0201 (Acc.no. Q6PSU2), Ara h 6.01 (Acc.no Q647G9), Ara h 7.0101 (Acc.no. Q9SQH1), Ara h 7.0201 (Acc.no. B4XID4) and Ara h 7.0301 (Acc. No. Q647G8) were provided by EUROIMMUN and produced as described previously.[10, 15]

2.3 | Immunoblot

Immunoblots (Euroline, EUROIMMUN, Lübeck, Germany) were used to determine sensitization of 15 patients to isoforms of recombinant peanut proteins of Ara h 2.0201, Ara h 6.01, Ara h 7.0101, Ara h 7.0201 and Ara h 7.0301. Immunoblots and reagents were kindly provided by EUROIMMUN and experiments were performed according to the manufacturer's instructions. In short, the Euroline strips were incubated on a rocking shaker overnight at RT with 100 µL of 1:11 diluted patient plasma in universal buffer. Binding of antibodies was visualized with an enzyme-labelled anti-human IgE antibody in combination with substrate nitro blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate. The results were evaluated with EuroLineScan software. The intensity of the bands was measured and an intensity level of three or higher was considered positive (arbitrary units). Line blot intensity values of two and lower are considered negative.

TABLE 1 | Patient characteristics. Sex, age, Skin Prick Test (SPT), results of DBPCFC and Müller score per peanut-allergic subject.

Patient	Sex (M/F)	Age (years)	SPT peanut (mm)	Subjective ED (mg)	Objective ED (mg)	Müller score[a]
N01	F	41	3+	10	-	2
N02	M	37	3+	0.1	300	4
N03	M	45	4+	100		2
N04	F	50	4+	10	10	3
N05	F	35	4+	0.1	-	4
N06	F	27	4+	4	40	2
N07	M	42	5+	not known	300	3
N08	M	24	4+	100	>3,000	1
N09	F	24	3+	not known	>3,000	3
N10	F	18	4+	300	1,000	3
N11	F	32	4+	10	3,000	2
N12	M	27	5+	0.1	1,000	3
N13	M	25	3+	10	-	2
N14	F	26	4+	0.1	100	3
N15	F	34	4+	40	12,000	2

Skin prick test (mm) - a diameter of 3 mm (3+) was considered positive. Subjective and objective effective dose (ED) during DBPCFC indicated in mg. [a]Müller score 0: Symptoms oral cavity, 1: Symptoms of the skin and mucous membranes, 2: Gastro-intestinal symptoms, 3: Respiratory symptoms, 4: Cardiovascular symptoms.

2.4 | Basophil activation test

Whole heparinized blood was obtained from 9 out of 15 peanut-allergic patients and a BAT was performed. Blood samples were stimulated for 30 minutes with increasing concentrations or 1000 ng/mL of crude peanut extract (CPE), or separate recombinant peanut allergens in RPMI-1640 medium (Gibco, Life Technologies) supplemented with 1 ng/mL IL-3 (R&D Systems). Control samples for the basophil activation test were rVP40 (recombinant VP40, control protein) and buffer. Leukocytes were stained with an antibody cocktail of CD45-PO (Life Technologies), CD123-FITC (Biolegend), HLA-DR-PB (Biolegend), CD63-PE (Monosan), CD41-PE-Cy7 (Beckman coulter), CD203c (Biolegend). Basophils were defined as $CD45^+$ $CD203c^+$ $CD123^+$ and HLA-DR^- $CD41^-$, and degranulation was quantified by determining the surface expression of CD63. Results are expressed as percentage of CD63- bright basophils. A threshold above 5% degranulation was considered positive.

2.5 | 3D protein models and distance mapping

3D protein models were created with Protein Homology/analogY Recognition Engine (PHYRE).[16] Further graphics and analyses such as distance mapping were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.[17]

2.6 | Data analysis and statistics

Correlation between percentage degranulation of basophils and intensity of the Euroline strips was determined with Spearman's correlation coefficient, as the data were not normally distributed. GraphPad Prism 7 (GraphPad Software, USA) was used for statistical testing and visualizing data.

3 | RESULTS

3.1 | Peanut-allergic patients can be sensitized to Ara h 2.0201, Ara h 6.01 and all isoforms of Ara h 7

In 15 peanut-allergic patients, sensitization to Ara h 2.0201, 6.01 and the three isoforms of Ara h 7 was established by means of the immunoblot strips (Figure 1A). Sensitization to Ara h 2.0201 was most abundant; 14 out of 15 patients were sensitized to this allergen, followed by sensitization to Ara h 6.01 and 7.0201, which were both recognized by 12 patients (80%). Two patients were monosensitized to Ara h 2.0201 (N01, N09), while also cosensitization for multiple allergen occurred. 40% of the patients were sensitized to all allergens, while one patient (N15) recognized all allergens except Ara h 2.0201 (Figure 1B). Ara h 7.0201 sensitization in combination with Ara h 2.0201 and/or Ara h 6.01 was found for three patients (N03, N04, N11), while sensitization to Ara h 7.0101 or Ara h 7.0301 was never observed in the absence of Ara h 7.0201.

3.2 | All Ara h 7 isoforms can induce basophil degranulation, and their line blot intensity levels correlates with basophil degranulation

Basophil degranulation experiments were performed for nine peanut-allergic patients, with the optimal allergen concentration of 1000 ng/mL (Figure 2A). All allergens were able to induce basophil degranulation. Of the three Ara h 7 isoforms, Ara h 7.0201 was most effective, as it induced basophil degranulation in six patients, followed by Ara h 7.0301 which induced basophil degranulation in two patients. Ara h 7.0101 was able to induce degranulation in only one patient. Next to basophil degranulation, the correlation between the intensity levels of the Euroline strips, which are directly related to levels of specific IgE, and the percentage of basophil degranulation was determined (Figure 2B). A significant correlation ($P < 0.05$), r = 0.8-0.9 was observed between the lineblot intensity and basophil degranulation upon Ara h 2.0201, 6.01 or 7.0201 exposure (Figure 2B).

3.3 | Isoforms Ara h 7.0201 and Ara h 7.0301 can induce basophil degranulation at least as well as Ara h 2.0201 and 6.01.

To compare the ability to induce basophil degranulation at low allergen concentrations, a concentration range of allergens was used in the BAT in whole blood of five patients who were sensitized against at least one isoform of Ara h 7. Patient N06 was sensitized to Ara

A

		Line blot intensity (Arbitrary Units)				
Patient	CAP peanut (kU/L)	Ara h 2.0201	Ara h 6.01	Ara h 7.0101	Ara h 7.0201	Ara h 7.0301
N01	1.7	4	2	2	2	2
N02	44	67	71	5	3	0
N03	1.8	3	11	0	7	0
N04	12	4	0	2	5	1
N05	85	18	31	10	34	5
N06	12.8	3	62	8	3	1
N07	42.7	57	58	26	74	25
N08	1.9	4	5	1	2	0
N09	1	3	2	2	2	0
N10	>100	167	81	56	115	106
N11	n.d.	12	26	1	5	1
N12	66	74	96	6	106	12
N13	11.2	29	4	3	40	4
N14	9.7	50	61	8	54	4
N15	1.55	2	17	8	12	3
		14/15 (93%)	12/15 (80%)	9/15 (60%)	12/15 (80%)	7/15 (47%)

B

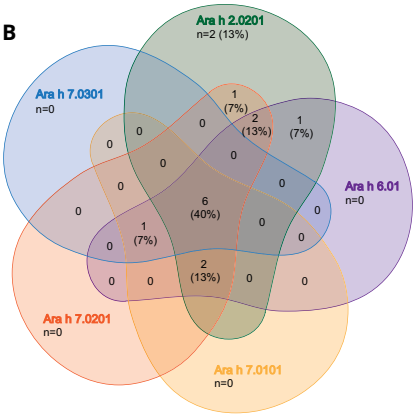

FIGURE 1 | Co-sensitization to peanut allergens of 15 peanut-allergic patients
Specific IgE (kU/L) against peanut determined by ImmunoCAP are indicated per patient in the left column. Sensitization to allergens Ara h 2.0201, 6.01, 7.0101, 7.0201 and 7.0301 by lineblot are shown as intensity values (arbitrary units) with intensity values > 3 considered positive **(A)**. Venn diagram illustrating co-sensitization to peanut allergens Ara h 2.0201, 6.01, 7.0101, 7.0201 and 7.0301 based on intensity level Euroline strips (n = 15) **(B)**.

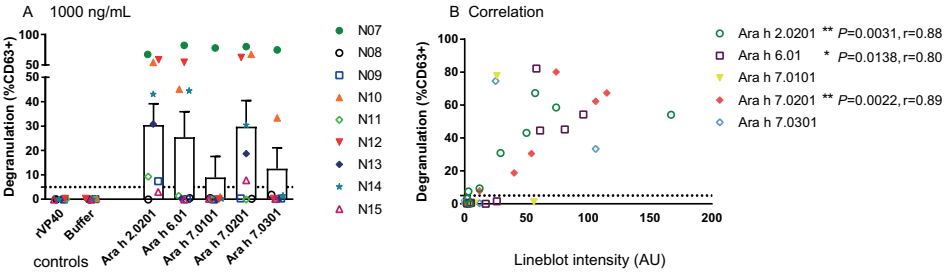

FIGURE 2 | Functionality Ara h 7 isoforms in basophil activation test
BAT assay in peanut-allergic patients at an allergen concentration of 1000 ng/mL **(A)**. Degranulation is indicated as percentage of $CD63^+$ cells (n = 9). Spearman correlation between degranulation in the BAT assay versus the intensity levels of the lineblot strips (n = 9) **(B)**, * $P < 0.05$, ** $P < 0.01$.

h 2.0201, 6.01, 7.0101 and 7.0201. However, only basophil degranulation was detected upon exposure to CPE and Ara h 6.01 (Figure 3A), which is probably related to relatively low intensity levels of sensitization for Ara h 2.0201 and 7. N10 and N14 showed already high basophil degranulation upon low concentrations (0.3 ng/mL) of isoforms Ara h 7.0201 and Ara h 7.0301 (Figure 3C, E), while basophils of other patients degranulated around 10 ng/mL of Ara h 2, 6 and 7. In the other two patients (N07, N12) the maximal degranulation upon exposure to the Ara h 7 isoforms was comparable to Ara h 2.0201 and 6.01. This indicates that isoform Ara h 7.0201 is at least as effective as recombinant Ara h 2.0201 and Ara h 6.01 in terms of inducing basophil degranulation.

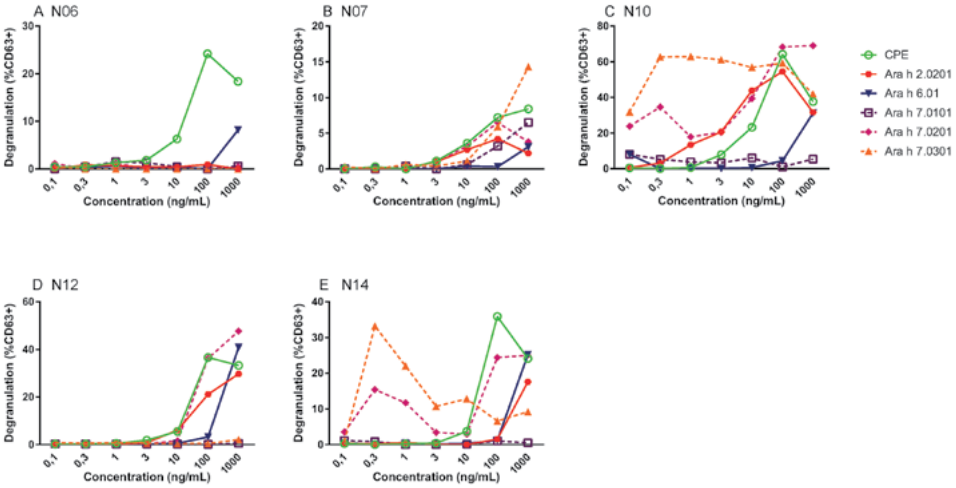

FIGURE 3 | Degranulation of basophils after exposure to different allergen concentration
Basophil activation of five peanut-allergic patients in a BAT assay with an allergen concentration range **(A-E)** (CPE, Ara h 2.0201, 6.01, 7.0101, 7.0201 and 7.0301).

3.4 | Sequential differences in sequence alignment of Ara h 7 isoforms compared to Ara h 2.0201 and 6.01

A sequence alignment between Ara h 2.0201, 6.01 and the three Ara h 7 isoforms was performed, to explain the differences in the efficacy of Ara h 7.0201 to induce basophil degranulation in more patients than the other two Ara h 7 isoforms (Figure 4A). Known linear epitopes of Ara h 2 and 6 recognized by allergic patients are highlighted in color.[18] Of the three isoforms, Ara h 7.0201 showed most sequence similarity with Ara h 2.0201 and 6.01 in the C-terminal regions that are known to be allergenic linear epitopes in Ara h 2 and 6 (orange underlined sequence).[18] Similar to the conserved cysteine pattern of at least eight conglutins of Ara h 2.0201 and Ara h 6.01, Ara h 7.0201 is the only isoform containing eight cysteine residues (underlined C-residues), whereas Ara h 7.0101 and Ara h 7.0301 only contain six cysteine residues. Cysteine residues play an important role in the folding and stability of proteins.[11] Furthermore, Ara h 7.0201 differed in three amino acid positions from both other isoforms (Figure 4B, arrows). These differences influence polarity, hydrophobicity, charge and trypsin cleavage sites (blue residues). In addition, more differences in trypsin (blue) and pepsin (red) cleavage sites were observed in the C-terminus (highlighted end) between the three different isoforms, which plays an important role in the enzymatic digestion and thus can influence stability and allergenicity of proteins (Figure 4B).

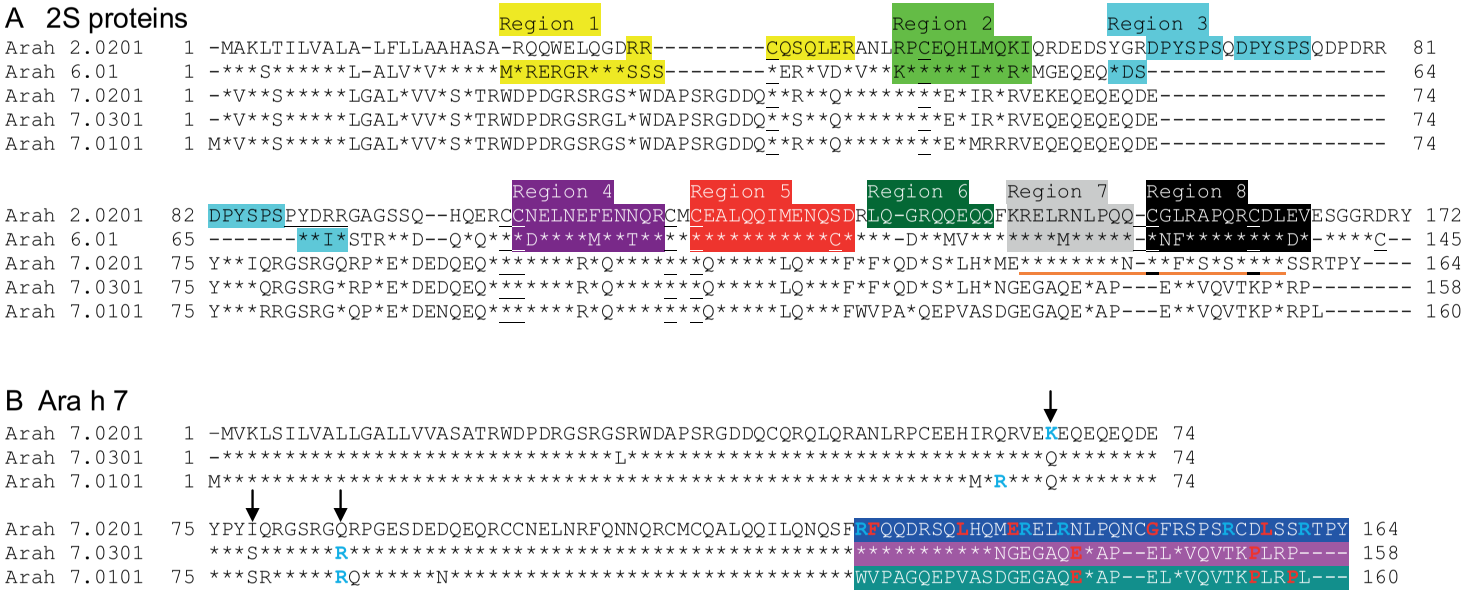

FIGURE 4 | Sequence alignment of Ara h 2.0201, 6.01, 7.0201, 7.0101 and 7.0301
Known allergenic epitopes of Ara h 2.0201 and 6.01 are color highlighted and explained below. Cysteine residues are underlined. Stars indicate similarities compared to Ara h 2.0201. C-terminal similarity of Ara h 7.0201 compared to Ara h 2.0201 and 6.01 is underlined in orange **(A)**. Sequence alignment of Ara h 7.0201, 7.0101 and 7.0301. Similarities compared to Ara h 7.0201 are indicated with stars. Arrows indicate differences in amino acid sequence of Ara h 7.0201 to both other isoforms. Blue residues indicate trypsin cleavage sites and red residues pepsin cleavage sites. The C-terminus is highlighted at the end **(B)**.

3.5 | 3D structural differences between Ara h 7 isoforms related to known allergenic epitope sites of Ara h 2.0201 and 6.01

In contrast to Ara h 2 and 6, of which crystal structures have been described,[2, 19] no crystal structure is available for the isoforms of Ara h 7 and isoforms Ara h 2.0201 and Ara h 6.01. In addition to the sequence alignment, predictive protein 3D models of all isoforms were therefore created with PHYRE and UCSF Chimera (Figure 5). Known allergenic linear epitopes of Ara h 2.0201 and 6.01 are highlighted in the same colors as displayed in Figure 4 (Figure 5A, B).[18] Figure 5C-E shows the predicted 3D models of the Ara h 7 isoforms. The sequence alignment indicated that most differences were located in the C-terminus. In the 3D models, a main structural difference with Ara h 7.0101 is observed in this C-terminus (turquoise), and some smaller differences are observed between Ara h 7.0201 and Ara h 7.0301 in this region (pink vs blue). The three amino acids that differ between these three isoforms (light blue) are all located in a loop which is a known epitope region for Ara h 2.0201 and 6.01 (region 3). As these changes in amino acids can influence hydrophobicity, polarity and charge, an amino acid distance analysis was performed (UCSF Chimera, Figure S1). Indeed, mainly in the loop region (green circle), differences in distance between amino acid residues were observed.

4 | DISCUSSION

Ara h 2 and Ara h 6 have proven to be two of the most informative peanut-allergens in the diagnosis of peanut allergy, as most patients have specific IgE against one or both allergens.[7] In addition, the current study shows that 80% of the 15 peanut-allergic patients studied were sensitized to one or multiple isoforms of a third recombinant 2S albumin member Ara h 7, mostly in combination with recombinant Ara h 2.0201 or Ara h 6.01. This is probably explained by the sequence identity between these three isoforms and Ara h 2.0201 and 6.01.[2, 5, 6, 8, 9] Ara h 7.0201 showed the highest sensitization frequency among peanut-allergic patients (80%), which was comparable to sensitization to Ara h 2.0201 and 6.01 (93 and 80%, respectively).

To the best of our knowledge, this is the first time that the functionality of recombinant Ara h 7 isoforms was tested, rather than only determining specific IgE binding in patient samples. Although the BAT assay can be a variable assay, grouped results indicate that overall Ara h 7.0201 was able to induce basophil degranulation comparable to Ara h 2.0201 and 6.01. In two independent patients, Ara h 7.0201 and Ara h 7.0301 were able to induce basophil degranulation at relatively low concentrations of allergen compared to CPE and Ara h 2.0201 and 6.01, suggesting that these specific Ara h 7 epitopes can be recognized by sensitized individuals and increase efficacy in stimulating basophil degranulation. While this could not be directly related to sensitization levels of the line blot strips, it indicates

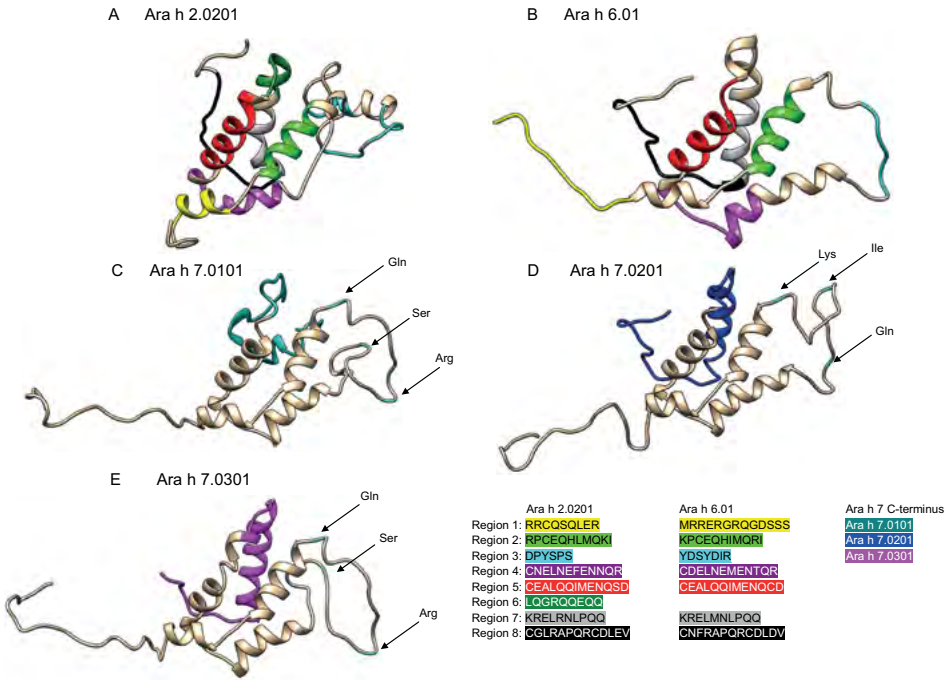

FIGURE 5 | 3D protein prediction models of Ara h 2.0201, 6.01 and 7
3D model of Ara h 2.0201 and 6.01, known allergenic epitopes are indicated in color according to the color-scheme of Figure 4 **(A-B)**. 3D prediction models of the three Ara h 7 isoforms. Turquoise (Ara h 7.0101), blue (Ara h 7.0201) or pink (Ara h 7.0301) indicates the C-terminus, light blue residues indicated with arrows indicate the main differences between Ara h 7.0201 and the other two isoforms in loop region 3 **(C-E)**.

that some patients can react to low concentrations of Ara h 7. Although Ara h 7 represents only 0.5% of peanut protein content, in contrast to 4%–14% for Ara h 2 and 6,[3, 12] this allergen has the potency to induce responses at low concentrations. Sensitization to isoform Ara h 7.0101 was observed in 60% of peanut-allergic patients, although biologic activity was observed in only one patient. Ara h 7.0101 was identified with phage display technology, but could not be retrieved in peanut extract,[5] which is most likely the explanation for this reduced biologic activity. Cross-reactivity between the three isoforms may explain the observed sensitization for this isoform.

A limitation of this study is that currently there is no native Ara h 7 available. All experiments were performed with recombinant proteins. Native Ara h 2 and 6 have been shown to induce basophil degranulation at lower concentrations of allergen than those of the recombinant proteins used in this study.[9] Nevertheless, the recombinant Ara h 7 proved to be able to induce basophil degranulation in some patients at already low concentration, indicating that it might even be more reactive in crude peanut extract.

A limitation of the BAT assay is that the response of patients to specific allergens can be significantly variable and not always follows the typical bell-shaped dose-response curve[20] as for instance, is observed in patient N10 and N14. The BAT tests performed in this study were only performed once for each patient. Combining the obtained results, Ara h 7.0201 appears the most promising Ara h 7 isoform in optimizing peanut allergy diagnosis, as it possesses a similar sensitization profile and efficacy in basophil degranulation as recombinant Ara h 2.0201 and 6.01. Ara h 7.0201 contains more unique epitopes than the other two Ara h 7 isoforms, as patients are sensitized more often to this isoform, and they can be sensitized to this particular isoform combined with only Ara h 2.0201 and Ara h 6.01 (N03, N04 and N11). Sensitization to either of the other two isoforms in combination with Ara h 2.0201 and/or 6.01 was not observed.

Although Ara h 7.0201 contains cross-reactive epitopes with Ara h 2.0201 and 6.01, a previous study indicated that monosensitization against Ara h 7.0201 was observed in 2 out of 15 patients.[10] This suggests that Ara h 7.0201 indeed contains one or more epitopes not present on the other Ara h 7 isoforms or Ara h 2.0201 and 6.01. The similarity of Ara h 7.0201 in sensitization and basophil degranulation with Ara h 2.0201 and 6.01 is most likely related to the C-terminus of Ara h 7.0201, as it fits into the conserved cysteine conglutin family pattern of at least eight cysteine residues, in contrast to the other two isoforms.[5] These C-terminal cysteine residues are important for protein stability and determine the IgE binding of allergens.[21] By 3D protein modeling, the three main differences in amino acid sequence of the Ara h 7 isoforms were visualized in a loop region that is a known allergenic epitope for Ara h 2.0201 and 6.01. Due to these amino acid substitutions, small changes in distance between amino acids occur, which could contribute to enhanced exposure to an epitope. Combining these two findings, it is expected that the unique epitopes of Ara h 7.0201 are located in either the C-terminus or in this loop region 3. Therefore, differences in enzymatic digestion by pepsin and trypsin may influence the allergenicity of Ara h 7 isoforms.

Taken together previous data[10] and the data presented in this paper, we hypothesize that determining specific IgE for Ara h 7.0201 can be of additional value in peanut allergy diagnosis. Ara h 7.0201 contains unique epitopes and is functionally as active as Ara h 2.0201 and Ara h 6.01 in inducing basophil degranulation. In addition, in some patients Ara h 7 can already provoke basophil degranulation at low concentrations. Due to cross-reactivity between Ara h 2.0201, 6.01 and 7, the latter one could have a potential strong diagnostic value.

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SUPPLEMENTAL FIGURE

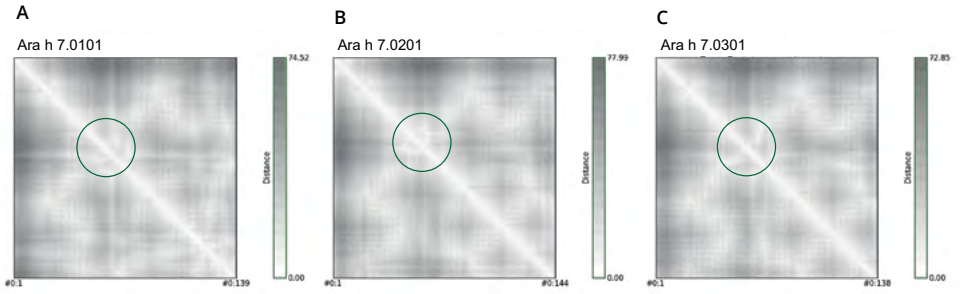

FIGURE S1: Residue distance map
Distance maps of Ara h 7.0101, 0201 and 0301. The distance of each amino acid is related to every other amino acid. The distances between the residues are indicated according to the color scale (Å), where white indicates that amino acid residues are in close proximity **(A-C)**.

CHAPTER 3

No association found between high-resolution HLA-B or HLA-DQB1 alleles and peanut allergy in a West-European cohort

Submitted for publication

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ABSTRACT

Background

Peanut allergy is a food allergy which exists worldwide. The gold standard for establishing a diagnosis remains the double-blind placebo-controlled food challenge (DBPCFC), which is time-consuming and sometimes can lead to severe allergic reactions. The exact reason for the development of food allergies in general is not yet understood, however genetic associations have been made. Previous Genome-Wide Association Studies (GWAS) have found that single nucleotide polymorphisms (SNPs) in HLA-B and HLA-DQB1 are associated with peanut allergy and general allergic sensitization.

Goal

The aim of this study was to analyze the association between HLA-B or HLA-DQB1 and peanut-allergy in a West-European Caucasian population compared to a large control cohort of stem cell donors.

Methods and Results

For 86 well-defined peanut-allergic patients (62% DBPCFC, 36% convincing history and sensitization, 2% Ara h 2 >1.75 kU/L), high-resolution HLA-typing was performed. Univariate χ^2 analysis indicated a decreased frequency in allele DQB1*03:01g in peanut-allergic patients, which coincided with increased frequencies of DQB1*02:01g, 04:02 and 06:04. In addition, increased frequencies for B*40:01g and B*51:01g were found, while frequencies of B*44:02g decreased. However, after Bonferroni correction only a trend was observed for an increased frequency of HLA-DQB1*06:04.

Conclusion

No significant associations between high-resolution HLA-B and HLA-DQB1 typing results and peanut allergy were observed in this study. High-resolution HLA typing is recommended for future research when investigating relations between HLA and well characterized diseases. In addition, cohorts should be large enough to have sufficient statistical power to overcome the correction needed for the multiple comparisons.

1 | INTRODUCTION

Worldwide, peanut allergy is one of the most common food allergies, which may cause severe and even fatal allergic reactions.[1] Current diagnostics include a skin prick test and the detection of peanut-specific IgE antibodies. In combination with an appropriate history of the patient, a clinical diagnosis can be made for most patients. The gold standard is a double-blind placebo-controlled food challenge (DBPCFC), which is expensive, time consuming and burdensome. The detection of IgE antibodies against specific peanut allergens, such as Ara h 2 and 6, are good predictors for peanut-allergy. However, it is known that also non-allergic individuals may have circulating IgE molecules against the same components of peanut. Therefore, exclusion of peanut allergy is not possible based on measurement of specific IgE alone.

Although the underlying mechanism of the development of food allergies is not completely understood, research has been performed to discover the link between peanut allergy and genetics.[2-4] The Human Leukocyte Antigen (HLA) system plays an important role in the onset of allergy by presenting allergens via class II molecules to T cells, subsequently activating allergen-specific B cells resulting in class-switching and maturation into IgE producing plasma cells. As specific HLA class II molecules may be able to present peptides of peanut allergens, the HLA class II background of peanut-allergic patients might influence the immune response to peanut allergens. Vice versa, a specific HLA class II background of individuals might be related to inability to generate an immune response to major peanut allergens rendering them unable to get peanut allergy. This would resemble the situation in celiac disease in which HLA-DQ2/DQ8 testing is currently employed to rule out the presence of celiac disease, which saves patients from going through small bowel biopsy when they are DQ2/DQ8 negative.[5] Previous studies have shown mixed results in the association of HLA class II with peanut allergy.[6-8] A study that reported no associations with HLA, was performed on a relatively small cohort, and patients were genotyped at low resolution.[7] Another study performed on a Canadian Caucasian cohort with children has pointed towards an association between HLA-DQB1*02 and DQB1*06:03P and peanut allergy.[8] Also a Genome-Wide Association Study (GWAS) indicated that a single nucleotide polymorphism (SNP) between the HLA-DQB1 and HLA-DQA2 genes was related to peanut allergy[4] and another GWAS study found an association between asthma and HLA-DQB1[9]. In addition to HLA class II, general associations with allergic sensitization have been found for HLA-B as well.[10] However, no data is yet available on the relation between HLA-B and sensitization for peanut or peanut allergy.

In this study we hypothesized that peanut allergy requires the presence of specific HLA-B or HLA-DQ molecules. To examine the contribution of HLA-B and HLA-DQ, a well-defined West-European cohort of patients with a persistent peanut-allergy was high-resolution typed for HLA-B and HLA-DQB1. As reference cohort, an existing cohort of stem cell donors was used, which was described earlier.[11]

2 | MATERIALS & METHODS

2.1 | Study population

106 West-European patients with a suspected peanut allergy were included in this study. The final cohort of peanut-allergic patients (n = 86) was identified by their medical records by means of either a positive DBPCFC (n = 53, 62%), sensitization with a convincing medical history after ingestion of peanut (n = 31 (36%)). When these data were not available, a value of Ara h 2 > 1.75 kU/L was used (n = 2, (2%)), which correlates with 100% positive predictive value for diagnosing peanut allergy.[12] Left over peripheral blood mononuclear cells (PBMCs) of patients was obtained from previous studies. All patients gave informed consent before their material was used in this study.

2.2 | High resolution HLA typing

For 86 patients with an established peanut allergy, genomic DNA was isolated from PBMC samples with MagnaPure (Roche) according to the manufacturer's protocol. These samples were used for high resolution typing for HLA class I and II (HLA-B and HLA-DQB1) using Next Generation Sequencing by MiSEQ with protocols, reagents, NGSengine version 2.9 software, and IMGT/HLA version 3.31, all used according to the manufacturer's protocol (GenDX, Utrecht, the Netherlands). Some HLA-DQB1 and HLA-B alleles in the control or peanut-allergic cohort were not expressed, or expressed at low abundance (< 5%), and were therefore grouped in statistic calculations. The other HLA-DQB1 and HLA-B alleles with an occurrence > 5% were grouped according to the paper of Maiers *et al* .[11]

2.3 | Statistical analysis

Antigens with a frequency distribution > 5% in the control or allergic group were analyzed for association with susceptibility for peanut allergy by using a χ^2 test. Because nine HLA types were considered in the analysis, a Bonferroni correction factor of 9 was applied. Therefore a *P*-value of 0.005 was considered significant, and 0.01 was considered a trend. The correspondence between specific Ara h 2 levels and HLA-DQB1 or HLA-B alleles were investigated with one-way ANOVA.

3 | RESULTS

Demographic data of the peanut-allergic cohort is displayed In Table 1. The ratio between males and females was 0.78:1 and the average age of the peanut-allergic patients was 30. Furthermore, the majority of patients (62%) underwent a DBPCFC, where 96% reported objective symptoms. Patients diagnosed with peanut-allergy based on convincing history and sensitization (36%) or specific IgE against Ara h 2 > 1.75 kU/L (2%), reported in 76%

TABLE 1 | Demographic data peanut-allergic cohort

	Peanut-allergic patients (n = 86)	
Sex ratio, M:F	0.78:1	
Age Male	26 (18-31)	
Age Female	30 (21-38)	
Age Total	28 (20-38)	
	DBPCFC (n = 53)	**History/Ara h 2 (n = 33)**
Subjective symptoms	2 (4%)	4 (12%)
Objective symptoms	51 (96%)	25 (76%)
Unknown	0 (0%)	4 (12%)
sIgE peanut (kU/L)	18 (3.14-93)[a]	4.05 (1.75-33.7)
sIgE Ara h 1 (kU/L)	1 (0-17.05) [b]	0.02 (0-4.31)
sIgE Ara h 2 (kU/L)	3.39 (0.54-21.20) [c]	2.32 (0.12-16.6)
sIgE Ara h 3 (kU/L)	0.26 (0.06-7.72) [d]	0.11 (0.03-0.55)
sIgE Ara h 8 (kU/L)	2.11 (0.13-6.41) [d]	6.66 (4.51-16.25)
sIgE Ara h 9 (kU/L)	0.06 (0.03-0.09) [e]	0.06 (0.04-0.14)

86 peanut-allergic patients were included in this study. Age is indicated as median (Q1,Q3). Symptoms are indicated as number of subjects (% population), while sIgE is indicated as median (Q1,Q3). Patients were either characterized based on DBPCFC (left column) or based on convincing history and sIgE or Ara h 2 > 1.75 kU/L (right column).
[a]2 unknown, [b]29 unknown, [c]22 unknown, [d]30 unknown, [e]31 unknown

objective symptoms. In the majority of patients specific IgE (sIgE) against Ara h 1, 2, 3, 8 and 9 was measured by ImmunoCAP ISAC. The average sIgE concentration with a range is shown in Table 1.

To assess whether HLA-B or HLA-DQB1 alleles were related to peanut allergy, their frequency in allergic patients was compared to a previously reported cohort of > 10.000 healthy controls[11] (Table 2). This analysis indicated that there were eight HLA-DQB1-alleles with a frequency > 5% in either the control or peanut-allergic cohort, which were used for further analysis. The other HLA-DQB1 alleles were grouped together. Univariate analysis shows that the frequency of DQB1*03:01g was decreased, which coincided with an increase in DQB1*02:01g, 04:02 and 06:04. However, after Bonferroni correction for multiple comparisons, only a trend was observed for the increased frequency of DQB1*06:04 ($P = 0.01$).

For the relation between HLA-B and peanut allergy, a similar approach was used as described for HLA-DQB1 (Table 3). Univariate analysis shows increased frequencies for B*40:01g and B*51:01g, while frequencies of B*44:02g decreased. However, no significant differences were found after Bonferroni correction for multiple comparisons.

Since Ara h 2 is a good predictor for peanut allergy, we investigated whether certain HLA-DQB1 or HLA-B alleles correspond to levels of specific IgE against Ara h 2. No significant differences were found neither for HLA-B nor HLA-DQB1 (data not shown). Based on the differences in frequency distribution displayed in Table 2, patients were divided into two

TABLE 2 | Frequency distribution HLA-DQB1 in the control and peanut-allergic cohort

HLA-DQB1	Frequency control cohort	Frequency Peanut allergy	*P* value
02:01g	0.237	0.297	0.04
03:01g	0.187	0.116	0.02
03:02	0.095	0.081	0.54
04:02	0.025	0.052	0.04
05:01	0.122	0.093	0.23
06:02	0.141	0.140	0.91
06:03	0.061	0.087	0.24
06:04	0.033	0.070	0.01
Other	0.102	0.063	0.09

Frequencies of alleles >5% in either the control or allergic cohort were analyzed using a $\chi 2$ test. After Bonferroni correction (nine comparisons) a *P* value < 0.005 was considered significant.

TABLE 3 | Frequency distribution HLA-B in the control and peanut-allergic cohort

HLA-B	Frequency Control cohort	Frequency Peanut Allergy	*P* value
07:02g	0.140	0.116	0.37
08:01g	0.125	0.140	0.57
13:02	0.026	0.052	0.06
15:01g	0.067	0.070	0.87
35:01g	0.057	0.029	0.15
40:01g	0.056	0.099	0.02
44:02g	0.090	0.047	0.05
44:03	0.050	0.052	0.97
51:01g	0.045	0.081	0.04
Other	0.320	0.331	0.75

Frequency of alleles > 5% in either the control or allergic cohort were analyzed using a $\chi 2$ test. After Bonferroni correction (nine comparisons), a *P* value < 0.005 was considered significant.

groups: one group consisting of patients with HLA-DQB1*02:01g, 04:02 and 06:04, but not 03:01g and one group with patients expressing HLA-DQB1*03:01g, but not the other three HLA-DQB1 alleles. Also in these two groups, no significant correlation was observed with regard to Ara h 2 or peanut-specific IgE levels (data not shown). For HLA-B, this grouping could not be performed, since specific Ara h 2 levels were only known for two patients expressing HLA-B*44:02g.

4 | DISCUSSION

In the development of peanut allergy, antigens are presented to the immune system by HLA class II molecules. Previous research indicated that of the three MHC-II molecules, HLA-DQB1 might play an important role in the development of peanut allergy, since a SNP was

located in the region where HLA-DQB1 is expressed.[13] In addition to class II, an association between HLA-B and allergic sensitization was described previously,[10] however no association studies between HLA-B and peanut allergy have been performed. In this study, high-resolution HLA-B and HLA-DQB1 expression was investigated in a cohort of West-European peanut-allergic patients (based on positive DBPCFC, medical history and sensitization or specific IgE against Ara h 2), and was compared with a large cohort of Caucasian controls. In a univariate analysis, a decreased frequency of DQB1*03:01g was observed, which coincided with increased frequencies of DQB1*02:01g, 04:02 and 06:04. However, after correction for multiple comparison, only a trend in increased frequency of DQB1*06:04 was identified. To the best of our knowledge, this is the first study to investigate a possible relation between HLA-B and peanut allergy. For HLA-B, significant increased frequencies for HLA-B*40:01g and 51:01g were observed, while frequencies of HLA-B*44:02g were found to decrease when using a univariate analysis. These significant changes in HLA-B frequencies were lost after Bonferroni correction for multiple comparisons. Since this might be related to the size of the cohort, associations between peanut-allergy and HLA-B and HLA-DQB1 cannot be excluded.

The increased frequency of DQB1*02:01g after univariate analysis is in contrast to a previously reported study, where DQB1*02 frequencies (which also encompasses all DQB1*02 alleles) were decreased in a peanut-allergic cohort compared to a non-allergic cohort[8]. This difference might be related to the descent of the cohort (Canadian versus European, although both are Caucasian), or the fact that our cohort existed mostly of adults instead of children (15%) and is smaller. Although the average age of the peanut-allergic cohort previously described was 11 years, from literature it is known that approximately 20% of children at a young age can outgrow peanut allergy.[14] In a smaller study with 48 peanut-allergic individuals, an association with HLA-DQB1*04 was found, although this was typed at low-resolution.[6] This is in accordance with the univariate analysis in our study for HLA-DQB1*04:02. Another study that did not observe any associations with HLA class II and peanut allergy, was based on low-resolution typing, which is in line with our data.[7] When looking into our high-resolution allele typing, we can appreciate that a small change in allele frequency at high resolution level is easily lost when these alleles are pooled into one single allele as is the case for low-resolution typing.

The limitations of this study are that it is not known what the incidence of peanut allergy is in the control cohort. This control cohort represents a normal Caucasian population, so approximately 1% of this cohort might experience peanut allergy.[15] It is not expected that this will influence the associations observed, as the control cohort was rather large. A second limitation is that most subjects have multiple allergies, instead of only being allergic to peanuts and this is an important drawback in performing these kinds of studies.

In this study, no association between HLA-B or HLA-DQB1 and peanut allergy was found after correction for multiple comparisons. This is a common drawback in examining

associations between diseases and HLA,[16] and can only be overcome by analyzing data of a larger number of individuals. Since it is not known which allergen is most important in the onset of peanut allergy, and which HLA subtype therefore most likely is associated, no sample size calculation could be made before conducting this study. It might therefore be possible that our cohort was too small to overcome this correction for multiple comparisons. In addition, next to MHC class I or II genes several other factors are important in the development of peanut allergy. Previously, risk factors were found in other innate and adaptive immune pathways, such as CD14, filaggrin and IL-9[17-19] and also DNA methylation might partly mediate the genetic risk for food allergy.[13] Although increased risks have been found among family members with allergies, MHC class I or II genes might not be the most important factor in the development of peanut allergy.

In conclusion, this study did not find any significant multivariate associations between HLA-B, HLA-DQB1 and peanut allergy after multivariate analysis in a West-European cohort of peanut-allergic patients that were high-resolution typed. Studies that are investigating associations between HLA and allergy, autoimmune disorders or related diseases will benefit from performing high-resolution typing, as associations within one allele can easily be missed with low-resolution typing. In addition, due to correction for multiple comparisons, one should take into account that the cohorts are large enough when searching for associations between HLA and disease.

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PART II
IMMUNOTHERAPEUTIC APPROACHES

CHAPTER 4

Novel immunotherapy approaches to food allergy

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ABSTRACT

Purpose of review

Despite reaching high percentages of desensitization using allergen-specific immunotherapy (SIT) in patients with food allergy, recent studies suggest only a low number of patients to reach persistent clinical tolerance. This review describes current developments in strategies to improve safety and long term efficacy of SIT.

Recent findings

Modified allergens or tolerogenic peptides, ultimately optimized for human leukocyte antigen background of the patient, are explored for tolerance induction, whereas anti-IgE antibody (omalizumab) may be used to facilitate SIT safety. Adjunct therapies to enhance efficacy may make use of Th1 polarizing agents, for example CpG-oligodeoxynucleotides combined with modified allergen packaged in nanoparticles. Preclinical studies showed insulin-like growth factor-2, intravenous immunoglobulin, Tregitopes or allergen encased oligomannose-coated liposomes capable of inducing regulatory T cells, recognized for their importance in clinical tolerance induction. Dietary intervention strategies utilizing herbal formula 2, VSL3#, nondigestible short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) plus *Bifidobacterium breve* M-16V or n-3 long chain polyunsaturated fatty acids may facilitate safety and/or a favourable milieu for tolerance induction.

Summary

Combining SIT using (adapted) allergens or tolerogenic peptides with adjunct therapy may be essential to improve safety and/or efficacy. Beyond using targeted approaches, specific dietary components may be explored to reduce side effects and support clinical tolerance induction by SIT.

1 | INTRODUCTION

The rising prevalence of allergic diseases relates to the increase in noncommunicable diseases, and food allergy may be seen as an early onset noncommunicable disease.[1] Food allergy is one of the first manifestations of atopic constitution and affects 6% of children and 3-4% of adults in westernized countries. Cow's milk and hen's egg are the main contributors to early childhood allergy and are outgrown in 80% of patients, by contrast, peanut allergy is resolving in less than 20%. Intake of the culprit food results in local (oropharyngeal/gastrointestinal) and/or systemic (atopic dermatitis, asthma) symptoms, ranging from mild (itching) to extremely severe (anaphylactic shock). Food allergen specific immunotherapy (SIT) is being explored but safety and long-term efficacy issues currently question its applicability for general clinical practice.[2, 3] This review provides an update on clinical trials published over the last year using SIT in IgE mediated cow's milk, hen's egg and peanut allergy. Furthermore novel developments in allergen modification and adjunct therapies to improve safety and (long-term) efficacy are discussed.

1.1 | Mechanism of allergy and immunotherapy

Food allergy results from a defect in the adaptive immune response upon presentation of food proteins by antigen presenting cells to T cells resulting in Th2 polarization and IgE-producing plasma cells rather than tolerance.[3] Allergen specific-IgE binds fragment crystallizable epsilon receptor 1 (FcεRI) on mast cells and basophils (sensitization) and allergen reencounter cross-links membrane-bound IgE triggering degranulation, resulting in allergic symptoms.[3] Immunotherapy involves desensitization ultimately by reducing antigen-specific IgE and internalization of membrane-bound allergen-specific IgE,[4] while increasing IgG4. Immunotherapy also aims at restoring tolerance by supporting Th1 and regulatory T cells (Tregs) ($CD4^+CD25^{high}FoxP3^+$, Tr1 or T_H3 cells). Tregs suppress Th2 prone immune responses via secretion of IL-10 and/or TGF-β.[5-7] Indeed, allergen induced Tregs were recently found to associate with reestablishment and maintenance of clinical tolerance using SIT.[6]

1.2 | Allergen-specific immunotherapy for food allergy

SIT for food allergy utilizes conventional or rush (within days) allergen dose-escalation protocols followed by daily home maintenance with the tolerated threshold dose. Dose-escalation aims at desensitizing, increasing the threshold dose for clinical reactivity to the culprit food. Maintenance aims at reaching (persistent) clinical tolerance (no clinical symptoms for the threshold dose after ceasing therapy for at least four weeks).[2, 3] For food allergy oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) are most often studied.[2, 3] OIT lowers the relative risk ratio for reactions to 0.19 as a result of desensitization.[8] However, only in a few studies, clinical tolerance after ceasing therapy was determined and

results are disappointing.[6, 9] Apart from OIT and SLIT, also subcutaneous immunotherapy (SCIT) was shown effective in for example, peanut allergy, but coincided with a high frequency of severe side-effects.[3] Novel approaches include epicutaneous immunotherapy (EPIT).

1.2.1 | Oral immunotherapy

Several recent studies [6, 10-13] for peanut allergy showed desensitization in patients undergoing OIT. Anagnostou *et al.*[10] found 62% desensitization allowing an eliciting dose threshold increase of 25 times compared to the placebo group, but 20% of OIT patients experienced respiratory difficulties and in one case epinephrine was administered. These adverse effects are not uncommon for OIT and pose a serious drawback (Table 1). Immunological changes required to reach a state of clinical tolerance rather than desensitization are largely unknown. Recently, OIT in peanut-allergic patients increased the frequency of highly suppressive antigen-induced forkhead box P3 (FoxP3) expressing Tregs in tolerant participants. Only 13% of all participants in the OIT group remained unresponsive after six months of peanut avoidance, but persistent hypomethylation of the FoxP3 locus was associated with maintaining clinical tolerance.[6] Keet *et al.* [9] also found that persistent clinical tolerance was only reached in 31% of patients undergoing OIT in cow's milk allergy. It was suggested that the duration of OIT plays a role in sustained unresponsiveness.[14] OIT is a common method for inducing desensitization in hen's egg allergic children and raw egg extract desensitized 80-90%.[15, 16] Current recruiting clinical trials are implementing baked egg or egg protein to desensitize patients.[17]

1.2.2 | Sublingual immunotherapy

SLIT is extensively investigated as potential therapy for food allergies.[12, 18] Fleischer *et al.*[18] showed desensitization in 70% of participants in a peanut SLIT trial compared to placebo controls. Adverse effects mostly included mild oral-pharyngeal symptoms. Another recent study found OIT to a greater extent to induce immunological changes, such as lowering the peanut IgE over IgG4 ratio, than SLIT. Also dose eliciting thresholds were higher and more stable in OIT than SLIT treated patients.[12] Although the efficacy of SLIT is lower compared to OIT, its safety profile could be beneficial for further clinical trials.[12, 19]

1.2.3 | Epicutaneous immunotherapy

EPIT can induce effector responses via Langerhans cells, which are activated by a skin-patch containing allergenic proteins.[19] Current Phase I/II trials for peanut allergy are ongoing; so far results regarding safety seem promising.[19] An EPIT trial in peanut-sensitized mice demonstrated the contribution of Tregs to desensitization, which maintained their suppressive capacity after treatment was completed.[20]

1.3 | Allergen adaptation to improve safety and/or efficacy

Adaptations of food allergens to reduce allergenicity, while retaining their capacity to induce desensitization or tolerance induction, can improve safety and efficacy of SIT. Allergenic proteins in cow's milk and hen's egg comprise of conformational heat labile (e.g. β-lactalbumin, ovalbumin (OVA)) as well as sequential heat stable (e.g. α_{s1}-casein, ovomucoid (OVM)) IgE binding epitopes.[21] Hence heating will not completely reduce the allergenic risk, and in a recent study[22] it was shown that 64% of egg-allergic children could tolerate baked egg. The heating process may influence tolerogenic properties of the allergenic protein as was tested in mice expressing T cell receptors (TCRs) specific for dominant T cell epitope $OVA_{323-339}$. Unheated egg white, 80 or 121°C heated egg white enhanced the allergenicity or immunogenicity, whereas 100°C heated egg white showed reduced allergenicity while maintaining tolerogenic capacities.[23] Indeed in OVM sensitized mice desensitization via OIT was equally effective using the native or heated (30' 100°C) OVM and introduction of baked egg in the diet of egg-allergic patients resulted in desensitization for raw egg in 50% of cases.[24, 25]

1.4 | Peptide immunotherapy

As an alternative for (adapted) allergen treatment, an increasing amount of encouraging preclinical data is published on peptide SIT for food allergy.[26] OIT using OVM immunodominant T cell epitope-containing peptides reduced the frequency of clinical signs and allergen-specific IgE in OVM-allergic mice, while increasing Treg frequency in the circulation.[27] T cell epitopes as candidates for peanut allergy SIT are selected on the basis of proliferation of $CD4^+$ T cell lines generated from peanut allergic patients, on basophil activation assays identifying the non-IgE cross linking peptides and human leukocyte antigen (HLA)-genotyping.[28, 29] A recent genome-wide association study[30] in 5,789 allergen sensitized individuals versus 10,076 controls showed, amongst others, that sensitization is associated with HLA-B and –DQB1. T cell responses towards allergens are initiated by allergen internalization and degradation followed by peptide presentation in the context of HLA molecules. Cognate T cell help is required for isotype class switching and induction of antigen-specific memory B cells and plasma cells.[31] Specific HLA molecules exhibit distinct peptide specificities thus the responsiveness of individuals to epitopes within allergens can theoretically be predicted per individual, grouped according to their major histocompatibility complex (MHC) background. By using NETMHCII algorithms, a number of Ara h 2 peptides binding to MHC class-II were identified and proven to be functional as similar peptides were shown to induce T cell proliferation.[32] In a direct approach to identify Ara h 1 specific peptides, T cell stimulation with 20-mer overlapping peptides from different individuals resulted in 145 Ara h 1-specific T cell clones. HLA restriction analysis showed HLA-DQ and –DR as presenting molecules for different peptides defining apparently without a background preference for specific (e.g. DRB1*03:01)

TABLE 1 | Summary of recent clinical trials for OIT, SLIT and EPIT

Reference	Allergen/ Therapy	Number of patients	Desensitization/ Tolerance	Immunological changes	Safety
Anagnostou 2014	Peanut; OIT	85 (age 7-16) Placebo:46 OIT:39	Desensitization; Full: 62%; Partial:22% Increased median threshold 25-fold	Not investigated	Epinephrine given to one subject. 19% β2-agonist
Chin 2013	Peanut; OIT/SLIT	50 (age 2-11) OIT:23 SLIT:27	OIT three times more likely to pass DBPCFC at 12 months. Variable and lower eliciting dose thresholds by SLIT	OIT larger changes in peanut IgE and IgG4 levels than SLIT	4 doses epinephrine for 2 patients in OIT
Fleischer 2013	Peanut; SLIT	40 (age 12-37) Placebo: 20 SLIT: 20	Desensitization; SLIT: 70% ;Placebo: 15%	Increase allergen-specific IgG4 for SLIT patients. No difference responders and non-responders.	Dose-related oral-pharyngeal symptoms One dose epinephrine
Syed 2014	Peanut; OIT	43 (age 4-55) OIT: 23 ; Abstaining peanut: 20	Desensitization; OIT:87%; Lasting tolerance: 13%	Immune tolerant patients higher antigen-induced Tregs. IgG4 no predictor of lasting tolerance	Not assessed
Vickery 2014	Peanut; OIT	39 (age 1-16)	Sustained unresponsiveness: 31% (one month after OIT)	Sustained unresponsiveness correlated with lower baseline IgE levels. No role for IgG4	15% withdrawal allergic side effects
Dello Iacono 2013	Hen's egg; OIT	20 (5- 11 years) OIT: 10 Placebo: 10	Desensitization; Partial: 90 % (median 20 mL)	Increase threshold not correlated with level serum-specific IgE	Adverse effects in all patients, dose related. 66% grade III, 9% grade IV
Meglio 2013	Hen's egg; OIT	20 (age ≥4 years)	Desensitization; Full: 80% (25 mL); Partial:10%	IgG4 levels for ovalbumin increased significantly	30% reached full dose symptom free.
Keet 2013	Cow's milk; OIT	32 Follow-up from two studies	Follow-up study, 31% full serving with minimal or no symptoms	Baseline IgE important for outcome	19% at least one anaphylaxis, 9% at least one dose of epinephrine
Vázquez-Ortiz 2013	Cow's milk; OIT	81 (age 5-18)	Desensitization;Full: 72% (200 mL) ; Partial:21%	Baseline IgE important for outcome	14 patients persistent reactions 6 adverse effects

alleles.[29] Prickett *et al.*[29]designed seven short peptides containing ten core T cell epitopes of Ara h 1 and three peptides containing five core T cell epitopes of Ara h 2.[28] It is suggested that a potential peptide immunotherapy with the combined Ara h 1 and Ara h 2 peptides targets a HLA-diverse patient population; however, preclinical and clinical studies still need to examine the therapeutic potential of those peanut peptides. In cow's milk allergy, T cell epitopes have been identified for both beta-lactoglobulin[33] and alfa-lactalbumin.[34] Oral delivery of two specific beta-lactoglobulin T cell epitope peptides prior to whey sensitization reduced the development of the whey induced acute allergic skin response in mice.[33] Innovative approaches for peptide immunotherapy aim at enhancing the tolerogenic properties, for example, by adding sugar moieties suggested to induce Tregs by activating C-type lectin receptor specific ICAM-3 grabbing non-integrin-related 1 (SIGNR1) on tolerogenic dendritic cells (DC).[35, 36]

1.5 | Mucosal vaccines

Approaches using allergen delivery to mucosal surfaces via nanoparticles or bacterial vectors are under development. Recently EMP-123, a rectally administered suspension of killed *Eschericia.coli* expressing encapsulated modified recombinant Ara h 1, Ara h 2, Ara h 3 and shown effective in mice, induced severe acute side effects including two anaphylactic reactions in five out of ten peanut-allergic patients.[37] An alternative for the use of *E.coli* as a vector may be the *Lactococcus lactis.*[38] Prophylactic oral delivery of *Lactococcus lactis* engineered to secrete Ara h 2 or have cell wall anchored Ara h 2 reduced peanut specific IgE and Th2 polarization in peanut sensitized mice.[38]

1.6 | Adjunct treatment added to SIT to enhance desensitization

Humanized anti-IgE antibody Omalizumab binds free IgE and dissociates IgE from the surface of mast cells, consequently reducing FcεRI expression.[39, 40] Combining SLIT or OIT with Omalizumab seems promising since in a pilot study in high-risk peanut-allergic patients rapid oral desensitization was reached with only mild adverse effects, while monotherapy with Omalizumab resulted in high rates of severe side effects in patients with peanut-allergy.[2, 39, 40] Designed ankyrin repeat proteins (DARPin) also prevent binding of free IgE and DARPin bi53_79 completely dissociated IgE from FcεRI and blocked cutaneous anaphylaxis in mice expressing human FcεRIα chain better than Omalizumab.[39] Hence, beyond anti-IgE antibodies, in the future DARPin may be added to SIT as adjunct therapy to improve safety (Figure 1).

1.6.1 | Th1 inducing CpG-oligodeoxynucleotides

Toll-like receptor 9 agonists, for example, bacterial cytosine phosphate guanine (CpG) DNA rich in nonmethylated CG motifs or synthetic CpG oligodeoxynucleotides (ODN), induce Th1 polarization. Oral delivery of digestion-protected CpG ODN treated allergic symptoms

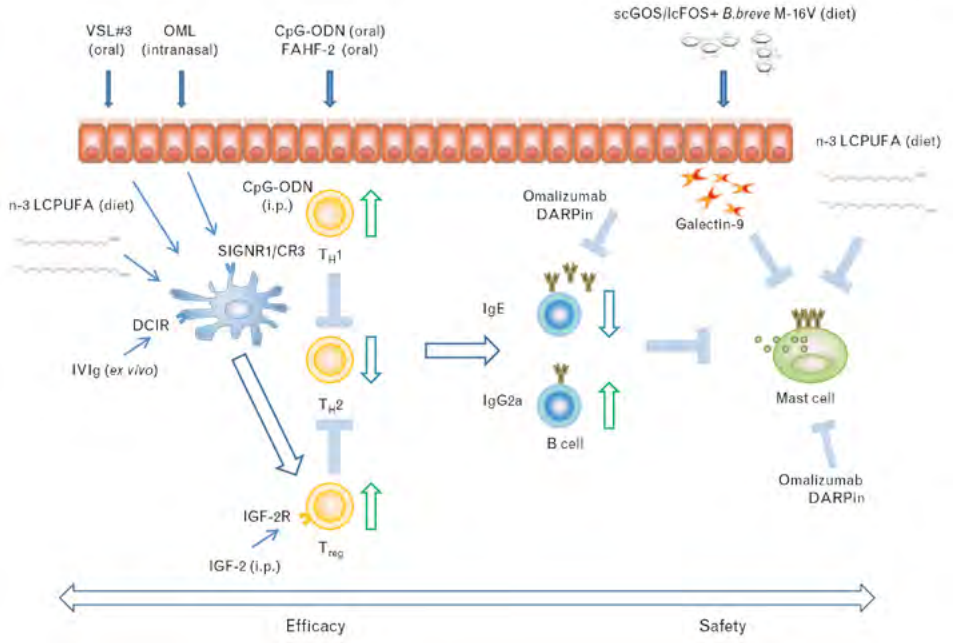

FIGURE 1 | Future possibilities for adjunct therapy to be combined with SIT using adapted allergens or tolerogenic peptides in food allergy (preclinical data)
CpG ODN (oral or i.v; treatment) enhanced systemic Th1 (IFN-γ), reduce Th2 (IL-13, IL-5) and decreased in peanut IgE and/or increased IgG2a while suppressing mast cell degranulation and allergic symptoms in mice.[41, 42] Similar findings were observed for FAHF-2,[50] and VSL3# (a cocktail of 4 different lactobacilli spp., 3 bifidobacteria spp. and *Streptococcus thermophilus*).[51] IGF-2 enhanced antigen specific Tregs by binding IGF-2R on Tregs and suppressed Th2 (IL-4, IL-13, IL-5), OVA specific IgE, mast cell degranulation and allergic symptoms, when injected during oral OVA sensitization of mice.[7] IVIg binds DCIR hereby inducing tolerogenic DCs that instruct Tregs and prevent OVA induced airway hyperresponsiveness.[46] OVA encased oligomannose-coated liposomes instruct tolerogenic DC via SIGNR1 and CR3 and intranasal treatment suppressed food-allergic symptoms in OVA-sensitized mice.[48] Omalizumab and DARPin bind IgE and induce internalization of FcεRI contributing to desensitization.[39] Dietary scGOS/lcFOS and Bifidobacterium breve M-16 V increase galectin-9, and reduce allergic symptoms when provided during whey sensitization.[52] Dietary n-3 LCPUFAs enhanced the frequency of tolerogenic DCs and Tregs and prevented allergic sensitization and symptoms in mice.[53] CpG ODN, CpG oligodeoxynucleotides; CR3, complement receptor 3; DARPin, designed ankyrin repeat proteins; DC, dendritic cells; IGF-2, insulin-like growth factor-2; LCPUFAs, long-chain polyunsaturated fatty acids; OVA, ovalbumin; SIGNR1, specific ICAM-3 grabbing nonintegrin-related 1; SIT, specific immunotherapy; scGOS/lcFOS, short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides; Tregs, regulatory T cells.

in peanut sensitized mice,[41] type B and C CpG-ODN (i.p.) proved to be most effective.[42] In a phase I/IIa trial with house dust mite allergic asthma patients QbG10 (bacteriophage Qbeta-derived virus –like particle with CpG-motif G10 inside) containing type A CpG as adjuvant in allergen specific subcutaneous immunotherapy, effectively relieved symptoms lasting for 38 weeks after treatment.[43] QbG10 without allergen treatment also reduced asthmatic symptoms and improved the forced expiration volume up until 12 weeks after steroid withdrawal[44]. Combined injection of Ara h 2 with CpG ODN packaged in

biodegradable protamine (proticles) suppressed generation of Ara h 2 specific IgE in BALB/c mice,[45] indicating nanoparticles as a novel way of combined delivery of allergens and adjuvant in SIT.

1.6.2 | Regulatory T-cell inducing strategies

Apart from enhancing Th1 polarization, allergen-specific Tregs may improve SIT efficacy (Figure 1). Recently the insulin-like growth factor-2 (IGF-2) receptor was found to be expressed on $CD4^+$ $CD25^+$ $FoxP3^+CD127^-$ Treg in mouse and human.[7] IGF-2 induced proliferation of functional Tregs upon concurrent TCR activation while preventing oral OVA sensitization in mice.[7] Intravenous immunoglobulins (IVIg) (polyclonal IgG) provide another way of enhancing Treg numbers. The sialic acid fraction of IVIg instructs tolerogenic dendritic cells (DCs) via binding of the dendritic cell immunoreceptor. *In vivo* treatment with sialic acid IVIg (i.p.), as well as adoptive transfer of OVA primed *ex vivo* sialic acid IVIg exposed DCs, prevented OVA induced airway hyperresponsiveness in mice while enhancing pulmonary Treg frequency.[46] Furthermore, HLA class II restricted peptides, selected based on Treg cell epitope sequences (Tregitopes) on the IgG Fc and F(ab)2 regions, suppress immune responses to co-administered antigens by stimulating the expansion of Tregs.[47] Combined allergen with adjuvant exposure by intranasal treatment with OVA encased oligomannose-coated liposomes was also found to expand Treg and suppressive $CD8^+$ T cells, while reducing OVA specific IgE and allergic symptoms in mice by targeting SIGNR1 and complement receptor 3 (CR3) on DCs.[48] Biologic agents such as *Lactobacillus rhamnoses* (Lcr35) may also install tolerogenic DCs. Adoptive transfer of Lcr35 treated DCs or feeding Lcr35 prior to sensitization, lowered the allergic airway response in OVA allergic mice, while enhancing Tregs in the MLN.[49]

1.6.3 | Dietary intervention

Dietary components favoring SIT may involve herbal formula-2 (FAHF-2), probiotic mixture VSL3#, non-digestible oligosaccharides or n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) (Figure 1). FAHF-2, a traditional Chinese medicine cocktail of nine herbs, of which active components include alkaloids berberine, palmatine and jatrorrhizine, successfully treated peanut-allergy in mice, without using allergen. Beyond systemic Th1 polarization FAHF-2 suppressed mast cell FcεRI expression and reduced mast cell and basophil numbers. FAHF-2 is currently being tested in a phase II trial in peanut-allergic patients.[50] Beyond using herbs, also treatment with VSL3#, enhanced Tregs while suppressing Th2 and peanut-allergic symptoms in mice.[51] A 9:1 mixture of short-chain galacto- and long-chain fructo-oligosaccharides (scGOS/lcFOS) reduces the incidence of atopic dermatitis in infants at risk.[54] *In vitro* scGOS/lcFOS combined with bacterial CpG DNA enhanced release of soluble type lectin galectin-9 (known to bind IgE) by human intestinal epithelial cells and instructed Treg and Th1 polarization.[55] Dietary intervention with scGOS/lcFOS reduced allergic symptoms in whey sensitized mice in correlation with

increased serum galectin-9 levels, most effectively when combined with *Bifidobacterium breve* M-16V.[52] Furthermore, n-3 LCPUFA, found in oily fish, prevented allergic sensitization in mice by increasing the frequency of tolerogenic DCs and Tregs and suppressing the allergic effector response.[53, 56] Studies are warranted using these dietary components as adjunct therapy in SIT.

2 | CONCLUSION

Improvement of SIT regarding persistence of clinical tolerance while reducing risk of treatment related side effects is needed. Safety of SIT can be enhanced by using modified allergens. Heat treatment retains desensitizing properties of allergens, while reducing allergenicity, but is not tolerated by all patients. Small peptides derived from immunodominant T cell epitopes of allergens, ultimately matching the HLA background of the patient, are now being tested for their tolerogenic properties. Beyond allergen modification, adjunct therapies are developed. Combining Omalizumab with OIT may improve safety, and future adjunct therapies for safety may involve DARPin, which bind IgE and effectively dissociate IgE from FcεRI. SIT efficacy can be enhanced by instructing Th1 polarization and combined exposure of CpG ODN with modified allergens, for example, packaged in nanoparticles, could facilitate this. Preclinical studies have shown IGF-2, sialic acid IVI9, Tregitopes or allergen encased oligomannose-coated liposomes capable of inducing allergen-specific Tregs, which may be key for clinical tolerance induction and maintenance. Dietary FAHF-2 induces Th1 polarization and suppresses effector responses, and is now tested in a phase II clinical trial in peanut-allergic patients. VSL3# induces Treg, and TGF-β depending Th2 suppression while lowering peanut-allergic symptoms in mice. Furthermore, dietary scGOS/lcFOS plus *Bifidobacterium breve M-16V* enhanced serum galectin-9 levels correlating with reduced allergic symptoms in mice and may be explored as SIT adjunct therapy. Furthermore, adequate n-3 LCPUFA status may facilitate a favorable milieu for tolerance induction. Combining SIT using (adapted) allergens or tolerogenic peptides with adjunct therapy may be essential to further improve safety and/or efficacy.

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PART III
IMMUNE MODULATION BY NON-DIGESTIBLE OLIGOSACCHARIDES

CHAPTER 5

Exposure of intestinal epithelial cells to short- and long chain fructo-oligosaccharides and CpG ODN enhances peanut-specific Th1 polarization

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ABSTRACT

Background

Non-digestible oligosaccharides promote colonization of beneficial gut bacteria and have direct immunomodulatory effects. Apical exposure of intestinal epithelial cells (IECs) to short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) in a transwell co-culture model enhanced the CpG-induced (TLR-9 ligand) Th1-phenotype and regulatory IL-10 response of underlying peripheral mononuclear cells (PBMCs) of healthy donors. scGOS is derived from lactose and may pose risks in severe cow's milk allergic patients, and scFOS/lcFOS may be an alternative. The goal of this study was to determine the immunomodulatory effects of scGOS/lcFOS and scFOS/lcFOS in an allergen-specific transwell co-culture model using PBMCs from peanut-allergic patients.

Methods

IECs cultured on transwell filters were apically exposed to CpG, either or not in combination with oligosaccharides. These IECs were co-cultured with basolateral PBMCs of peanut-allergic patients that were either activated with aCD3/28 or peanut-extract. Basolateral cytokine production and T cell polarization were measured and the contribution of galectin-9 and the dectin-1 receptor in immune modulation were assessed.

Results

IECs exposed to CpG increased IFN-γ, IL-10 and galectin-9 production by aCD3/28-stimulated PBMCs, whereas IL-13 decreased. Both scGOS/lcFOS and scFOS/lcFOS further enhanced IFN-γ and IL-10, while suppressing IL-13 and TNF-α. In the peanut-specific model, only scFOS/lcFOS further increased IFN-γ and IL-10 production, coinciding with enhanced Th1-frequency. Expression of CRTH2 reduced after CpG exposure, and was further reduced by scFOS/lcFOS. Galectin-9 inhibitor TIM-3-Fc abrogated the additional effect of scFOS/lcFOS on peanut-specific IFN-γ production, while neutralization of the dectin-1 receptor was not effective.

Conclusion

Epithelial exposure to scFOS/lcFOS enhanced the CpG induced Th1 and regulatory IL-10 response in a peanut-specific co-culture model. These effects suggest scFOS/lcFOS as candidate for dietary adjunct in allergen-specific immunotherapy.

1 | INTRODUCTION

Over the past decades, the prevalence of food allergies has increased in Western countries.[1,2] Harmless food proteins are recognized as being immunogenic by the immune cells of food-allergic patients, resulting in allergic sensitization. In sensitized individuals, these allergens can provoke a variety of symptoms when ingested, ranging from itching and swelling in the mouth to anaphylaxis. Next to eliminating these food proteins from the diet, there are currently no therapies available for treating food allergies that induce sustained oral tolerance. Several studies were able to induce desensitization in patients undergoing oral immunotherapy (OIT), hereby increasing the eliciting dose (ED).[3-5] However, inducing sustained non-responsiveness or tolerance remains difficult and is often combined with severe side effects.[1,4] Combining OIT with additional immunomodulatory agents, such as prebiotics as dietary adjuvant, may enhance safety and efficacy of immunotherapy and support clinical tolerance induction.[6]

The gastrointestinal (GI) tract plays an important role in the development of food allergies, and is constantly discriminating between harmful and harmless antigens.[7,8] A monolayer of intestinal epithelial cells (IECs) separates the intestinal contents from the underlying immune compartment and forms a barrier, hereby keeping away harmful bacteria or antigenic proteins. They can interact with innate and adaptive immune cells via the release of immune mediators such as galectin-9, or via cell-cell contact.[9,10] Under inflammatory conditions these IECs express pathogen recognition receptors, such as toll-like receptors (TLRs). These TLRs can recognize bacterial fragments from the gut microbiota or invading pathogens.

TLR-2 and TLR-9 have been described as important TLRs in recognition of certain probiotic strains.[11] Ligation of TLR-9 by bacterial DNA rich in unmethylated CpG islands maintained intestinal homeostasis, and oral administration of a synthetic TLR-9 agonist was effective in both prevention and treatment of peanut allergy in mice by redirection of the immune response towards a T helper 1 (Th1) phenotype.[12] *In vitro,* IECs apically exposed to synthetic CpG oligodeoxynucleotides (ODN) enhanced IFN-γ and IL-10 production by PBMCs in the basolateral compartment, while decreasing IL-13.[13] Therefore, targeting specific TLRs on IECs may be of interest in modulating immune responses.[13]

Previous research showed that dietary intervention with specific mixtures of non-digestible oligosaccharides (prebiotics) and/or beneficial bacteria (probiotics) may help to prevent infants from developing allergic diseases.[14-16] A prebiotic mixture containing short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) was able to reduce the incidence of atopic dermatitis in children at risk.[15] The functioning of these prebiotics is not fully elucidated, although it is known that they can improve intestinal tolerance and promote colonization of beneficial microbiota. Indeed, children receiving such a prebiotic mixture of scGOS/lcFOS, showed an increased presence of *Bifidobacteria*

and *Lactobacilli* in the gut.[17] Also, the addition of scFOS or inulin to the diet increased *Bifidobacteria* counts.[18-21] Beyond their effect on the microbiome, these prebiotics may suppress mast cell and basophil degranulation by enhancing galectin-9 levels among others secreted by IECs.[22] Furthermore, they may induce polarization of Th1 and regulatory T cells (Tregs) when combined with CpG ODN.[10,22,23]

Previously, in a transwell co-culture model using IECs and activated PBMCs, prebiotic mixture scGOS/lcFOS indeed enhanced galectin-9 levels secreted by IECs. Apical TLR-9 ligation of IECs in the presence of scGOS/lcFOS supported the production of IFN-γ and IL-10 by PBMCs, while IL-13 production was reduced.[10] Since scGOS is produced from cow's milk derived lactose, it may pose risks in people with severe cow's milk allergy.[24] A synbiotic mixture of scFOS/lcFOS with *Bifidobacterium breve* was also able to reduce allergic manifestations in a murine model.[25] This study will compare these two mixtures and their immunomodulatory effects.

Next to galectin-9, which was shown to contribute to these immunomodulatory effects, dectin-1 may play a role in the binding of these oligosaccharides. Dectin-1 is a C-type lectin receptor that is present on human IECs and the human IEC line HT-29. It can bind carbohydrates such as β-glucans, and may therefore be a possible candidate receptor for the oligosaccharides.[26,27] Dectin-1 is expressed at high levels at entry sites for pathogens such as the intestine, therefore, it may play an important role in immune surveillance.[28]

The aim of this study was to investigate the immunomodulatory effects and mechanism of action of the two prebiotic mixtures scGOS/lcFOS and scFOS/lcFOS in a transwell co-culture model simulating the cross-talk between IECs and activated PBMCs. IECs were exposed to scGOS/lcFOS or scFOS/lcFOS in combination with CpG ODN, and co-cultured with PBMCs of peanut-allergic patients, either stimulated in an aspecific (aCD3/28) or peanut-specific manner.

2 | MATERIALS & METHODS

2.1 | Study population

Fifteen peanut-allergic patients were recruited from the outpatient clinic of Dermatology/Allergology at the University Medical Center Utrecht. This number was calculated based on previous experiments with healthy donors. Demographic data, severity of symptoms (Skin Prick Test (SPT) and Müller score)) and the ED as established by double-blind placebo-controlled food challenge (DBPCFC) are displayed in Table 1. Inclusion criteria consisted of a type I allergic reaction to peanut, confirmed by a positive DBPCFC. Exclusion criteria were pregnancy or the continuous use of systemic immunosuppressants, such as prednisone. All patients gave written informed consent before enrollment in the study. Five patients that responded best to the peanut-extract were asked for a second visit for additional studies. The study was reviewed and approved by the Ethics Committee of the University Medical Center Utrecht (NL51606.041.15).

Table 1 | Patient characteristics

Patient	Age (years)	Sex (M/F)	Müller score[a]	SPT peanut (mm)	Subjective ED (mg)	Objective ED (mg)	CAP peanut (kU/L)
N01	41	F	2	3+	10	-	1.7
N02	37	M	4	3+	0.1	300	44
N03	45	M	2	4+	100	-	1.8
N04	50	F	3	4+	10	10	12
N05	35	F	4	4+	0.1	-	85
N06*	27	F	2	4+	4	40	12.8
N07*	42	M	3	5+	Not known	300	42.7
N08	24	M	1	4+	100	>3,000	1.9
N09	24	F	3	3+	Not known	>3,000	1
N10*	18	F	3	4+	300	1,000	>100
N11	32	F	2	4+	10	3,000	No data
N12*	27	M	3	5+	0.1	1,000	66
N13	25	M	1	3+	10	-	11.2
N14*	26	F	2	4+	0.1	100	9.7
N15	34	F	2	4+	40	12,000	1.55

Age, sex, Müller score, Skin Prick Test (SPT), results of DBPCFC and specific IgE per peanut-allergic subject. *: Subjects that visited a second time. [a]Müller score 0: Symptoms oral cavity, 1: Symptoms of the skins and mucous membranes. 2: Gastro-intestinal symptoms, 3: Respiratory symptoms, 4: Cardiovascular symptoms. Skin prick test (mm), diameter of 3 mm (3+) is considered positive. All patients underwent a double-blind placebo-controlled food-challenge (DBPCFC), subjective and objective effective doses are displayed.

2.2 | PBMC isolation

100 mL blood of peanut-allergic patients was withdrawn in heparin tubes. Blood was diluted 1:1 with 1x PBS (Sigma-Aldrich Chemie BV, the Netherlands), followed by isolation of PBMCs using a Ficoll-Paque PLUS (GE Healthcare Life Sciences, Sweden) density gradient centrifugation (2400rpm, 20 min). PBMCs were resuspended in RPMI 1640 (Gibco, Life Technologies, the Netherlands) with 2.5% pooled human AB serum and penicillin/streptomycin (100x, Gibco, Life Technologies).

2.3 | Culture of intestinal epithelial cells HT-29

Undifferentiated human colon adenocarcinoma HT-29 cells (ATCC, HTB-38; passages 144-149), were cultured in 75 cm^2 culture flasks (Greiner Bio-One B.V., the Netherlands) in McCoy's 5A medium (Gibco, Life Technologies, the Netherlands) supplemented with 10% heat-inactivated FCS (Gibco, Life Technologies, the Netherlands) and penicillin/streptomycin (100x, Gibco, Life Technologies). These cells are a representative model for crypt epithelium and can respond to bacterial stimuli.[29] In the absence of an activating agent for the underlying immune cells, the HT-29 cells have very low background levels of cytokine that are being produced.[13]

HT-29 cells were kept in an incubator at 37 °C and 5% CO_2. Cells were passaged once a week and medium was refreshed every 3-4 days. Previous studies have shown that HT-29

in a similar manner as polarized T84 cells contribute to the immunomodulatory effects of CpG ODN in presence or absence of oligosaccharides, and can be used as a model to mimic the cross-talk between IECs and underlying immune cells.[10] Therefore these cells were chosen for the current studies.

2.3 | IEC transwell co-culture model

One week prior to the experiment, HT-29 cells were seeded four times diluted in transwell inserts (12 well plates, 0.4 μm polyester membrane, Corning, USA). After reaching confluence, IECs were apically exposed to 2.5 μM of CpG ODN (M362 ODN type C, Invitrogen) either or not combined with 0.5% w/v (5 mg/mL) of a 9:1 mixture of scGOS (Vivinal GOS syrup 45% pure, Borculo Domo, the Netherlands) and lcFOS (Raftiline HP, Orafti) or a 0.5% w/v 9:1 mixture of scFOS (Raftilose P95, Orafti) and lcFOS. In the basolateral compartment, 3 x 10^6 PBMCs from peanut-allergic patients were either stimulated for 24 hours with anti-CD3 (PeliCluster CD3, CLB-T3/4.E, 1XE) and anti-CD28 antibodies (PeliCluster CD28, CLB-CD28/1, 15E8, both 1:10.000, Sanquin, the Netherlands) or 6 days with 50 μg/mL crude peanut extract (CPE) (Figure 1). PE was made by blending peanuts, followed by extraction with Tris/NaCl buffer (20 mM Tris, 150 mM NaCl, pH 7.2) at room temperature. After extraction, supernatant was filtered twice and diluted to the desired concentration in 1xPBS. Incubation times for the peanut-specific and aspecific model were based on previous experiences.[10,30] Due to limitation of patient material, both the aspecific and peanut-specific model could be performed once per patient.

Part of the medium was refreshed every 2-3 days. After 24 hours or 6 days, culture supernatants from the basolateral compartment were stored at -20°C until cytokine measurement. After 24 hours of co-culture with aCD3/28 stimulated PBMCs, the IECs were washed and the insert was transferred to a new plate with fresh medium without PBMCs for another 24 hours, to determine galectin-9 production by IECs. In the peanut-specific model, galectin-9 was measured directly in the basolateral compartment after six days of culture.

To study the involvement of galectin-9 in immune modulation, 1 μg/mL TIM-3-Fc fusion protein (Bio-Techne, USA) was added to the basolateral compartment of the peanut-specific model, to neutralize galectin-9. Additionally, the role of dectin-1 as a candidate receptor for the oligosaccharides was investigated in the peanut-specific model, by means of a neutralizing antibody applied in the apical compartment (3 μg/mL, Bio-Techne, USA).[27,31,32]

2.5 | Flow cytometric analysis

After 24 hours (aCD3/28) or 6 days (CPE), lymphocytes were collected from the basolateral compartment. Cells were stained with a panel of antibodies (CD3, CXCR3, CRTH2, CD25 (all Biolegend), CD127, FoxP3, CD4 (all eBioscience) and CXCR5 (BD Biosciences) after which T cell polarization of Th1 ($CD3^+CD4^+CXCR3^+$), Th2 ($CD3^+CD4^+CRTH2^+$), Tfh

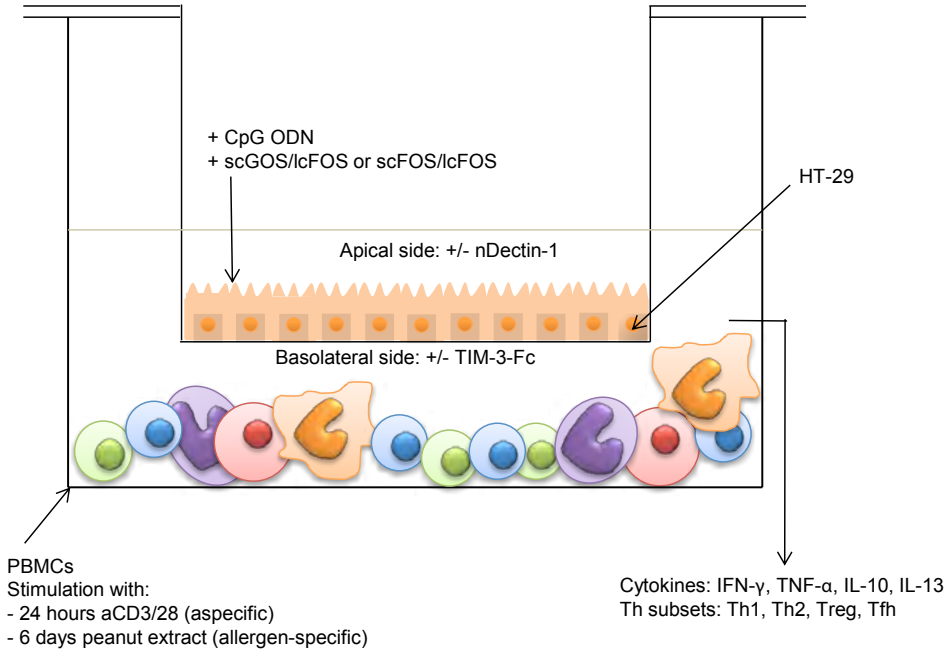

FIGURE 1 | Intestinal epithelial cells (IEC) transwell co-culture model
HT-29 cells (IECs) cultured in transwells were apically exposed to synthetic CpG oligodeoxynucleotides (ODN) in the presence or absence of either short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) or scFOS/lcFOS. Simultaneously, 3×10^6 peripheral mononuclear cells (PBMCs) in the basolateral compartment were either stimulated aspecifically for 24 hours with aCD3/CD28, or for 6 days with peanut extract. Basolateral cytokine production and T cell polarization were measured. After 24 hours, the insert of the aspecific model was transferred to a new plate with fresh medium, to measure production of galectin-9 by IECs. The contribution of dectin-1 and galectin-9 in the peanut-specific model was investigated by either neutralizing dectin-1 with an antibody on the apical side, or by neutralizing galectin-9 with TIM-3-Fc on the basolateral side.

(follicular T helper) ($CD3^+CD4^+CXCR5^+$) and Treg ($CD3^+CD4^+CD25^{high}CD127^-FoxP3^+$) was determined. FoxP3 staining was performed according to the manufacturer's protocol (FoxP3 Transcription Factor Staining Buffer Set, Thermofisher, USA).

2.6 | Cytokine production of PBMCs in the basolateral compartment

In the basolateral supernatants, IFN-γ, TNF-α, IL-10, IL-13 and IL-4 were measured by means of ELISA, according to the manufacturer's protocol (Ready-Set-Go, eBioscience). IL-4 was below the ELISA detection limit for both the aspecific and the allergen-specific co-culture supernatants. IL-13 and TNF-α production in the peanut-specific co-culture model was below the ELISA detection limit. In co-cultures using blood samples of four patients, PBMCs were restimulated with phorbol 12-Myristate 13-Acetate (PMA, 10ng/mL, Sigma-Aldrich, The Netherlands) and ionomycin (1μg/mL, Sigma-Aldrich, The

Netherlands) for 24 hours which did yield detectable levels of IL-13. Galectin-9 production was analyzed using human-galectin-9 polyclonal and biotinylated polyclonal antibodies (BioTechne). Data was analyzed by 4-parametric curve fitting using Microplate Manager software.

2.7 | Statistical analysis

Data are expressed as mean ± SEM. The statistical significance of the data was analyzed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Normally distributed data were analyzed using a paired Student's t-test or one-way repeated measures ANOVA followed by Bonferroni *post hoc* analysis. Not normally distributed data were first transformed (square-root or LOG) before analysis. Data were considered significant at $P < 0.05$.

3 | RESULTS

3.1 | Enhanced production of IL-10 and IFN-γ by aspecific or peanut-specific activated PBMCs upon combined exposure of IECs to CpG ODN and oligosaccharides

For this study, PBMCs of 15 peanut-allergic patients (six male and nine female; age 18-50; Müller 1-4) were studied in an IEC transwell co-culture model, and the immunomodulatory effects of two prebiotic mixtures were assessed. Hereto, PBMCs of these peanut-allergic patients were either stimulated aspecifically with aCD3/28 or peanut-specific by using a crude peanut extract. These PBMCs were co-cultured with IECs that were apically exposed to prebiotic mixtures in the presence of CpG ODN (TLR-9 ligand).

The peanut-allergic patients showed similar responses in this aspecific model as healthy donors (Figure 2). Apical exposure of IECs to oligosaccharides alone did not affect cytokine concentrations in the basolateral compartment, but modified CpG ODN-induced immune responses in the aspecific co-culture model (Figure 2). To better appreciate these effects, the subsequent data of the CpG exposed IECs co-cultured with PBMCs of peanut-allergic patients are represented as ratios compared to the intrinsic medium control (Figure 3). Exposure of IECs to CpG ODN resulted in increased basolateral IFN-γ and IL-10 release by PBMCs of both healthy and allergic donors in the aspecific co-culture model (Figure 2A, B, E, F and 3A-B). Both scGOS/lcFOS and scFOS/lcFOS further significantly enhanced this CpG induced increase in IFN-γ and IL-10 in the aspecific co-culture model. In addition, IL-13 production was decreased by CpG ODN, and was further significantly decreased in the presence of the oligosaccharides in peanut-allergic patients (Figure 2C, G and 3C). Combined exposure of IECs to CpG ODN and scGOS/lcFOS or scFOS/lcFOS also resulted in a significant decrease in TNF-α, while CpG alone did not (Figure 2D, H and 3D).

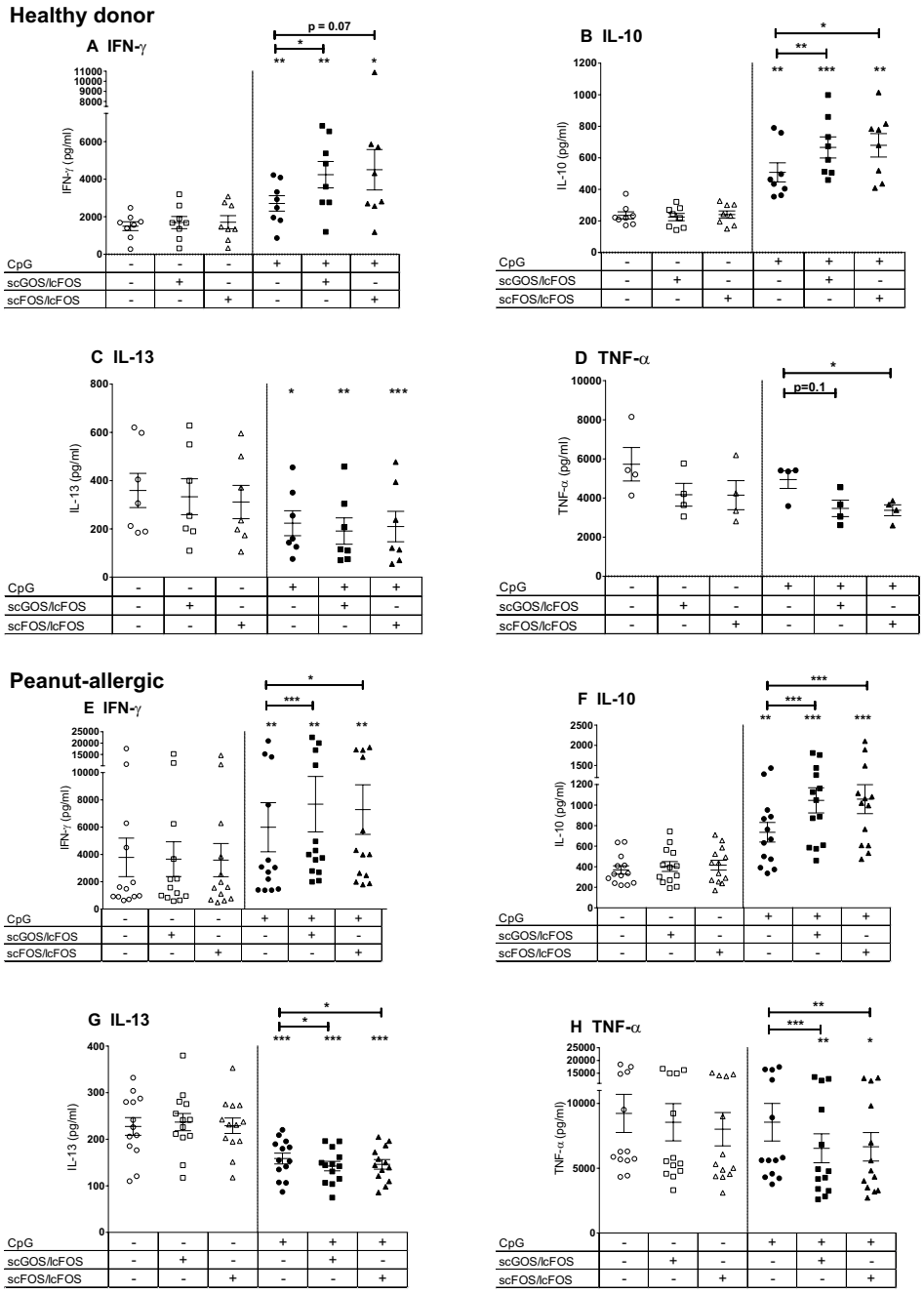

FIGURE 2 | Comparison aspecific co-culture model between healthy donors and peanut-allergic donors Apical exposure of intestinal epithelial cells (IECs) to non-digestible oligosaccharides in absence of CpG ODN did not affect cytokine concentrations in the basolateral compartment **(A–H)**. >>>

Legend Figure 2 Continued
CpG exposure increased IFN-γ and IL-10 production **(A, B)** in healthy donors, which was further increased by the combined exposure to CpG ODN and oligosaccharides. In addition, IL-13 was decreased by CpG exposure alone **(C)** and TNF-α production decreased in the combined presence of oligosaccharides and CpG **(D)**. Peanut-allergic donors showed similar results in terms of these response patterns upon exposure of IECs to CpG, oligosaccharides, or the combination of CpG and oligosaccharides **(E–H)**.

In previous studies, in absence of epithelial cells the CpG ODN did enhance IL-10 and reduced IL-13 secretion by activated PBMCs, but was unable to further enhance IFN-γ production compared to the control sample. Only in the presence of HT-29 cells CpG ODN increased IFN-γ production of underlying immune cells and additional exposure to oligosaccharides further increased this.[10,13]

In the peanut-specific co-culture model, only scFOS/lcFOS was able to further significantly enhance the CpG mediated increase in basolateral IFN-γ and IL-10 production (Figure 3E, F). In the peanut-specific model, IL-13 was only detectable after restimulation of the cells with PMA and ionomycin for 24 hours, and shows a similar pattern as in the aspecific model (analyzed for n = 4 donors, Figure 3G).

IFN-γ and IL-10 concentrations were positively correlated in both the aspecific and peanut-specific co-culture models (Figure 4A-C,F). In the aspecific co-culture model, two distinct populations were observed; population 2 consisted of four patients (N04, N06, N08, N14) for all epithelial stimuli. This indicated that patients can respond differently in terms of cytokine production pattern, however, this was not related to the demographic data from Table 1. In addition, in both populations this positive correlation was observed. In the aspecific model, IFN-γ and IL-13 (Figure 4D) and TNF-α and IL-10 concentrations (Figure 4E) were negatively correlated.

3.2 | Increased galectin-9 production by IECs upon apical exposure to CpG ODN in presence or absence of oligosaccharides

Galectins are soluble type lectins that have a binding specificity for β-galactoside sugars. Galectins among others are expressed and secreted by IECs, and contribute to immunomodulatory functions. Total galectin-9 concentrations were measured in the basolateral compartment after 24 hours (aspecific model) and 6 days (peanut-specific model) (Figure 5A,C). Also, IEC-released galectin-9 was measured in the aspecific co-culture model (Figure 5B). Since the IECs in the peanut-specific model were already cultured for 6 days, we did not measure galectin-9 levels of these IECs separately.

Exposure of IECs to oligosaccharides alone did not influence galectin-9 concentrations, and data are shown as ratio of the intrinsic medium control. No difference in basolateral galectin-9 concentration was observed after 24 hours in the aspecific co-culture (Figure 5A), while IECs after another 24 hours of culture without PBMCs showed an increased galectin-9 production when exposed to CpG ODN (Figure 5B). This was further significantly

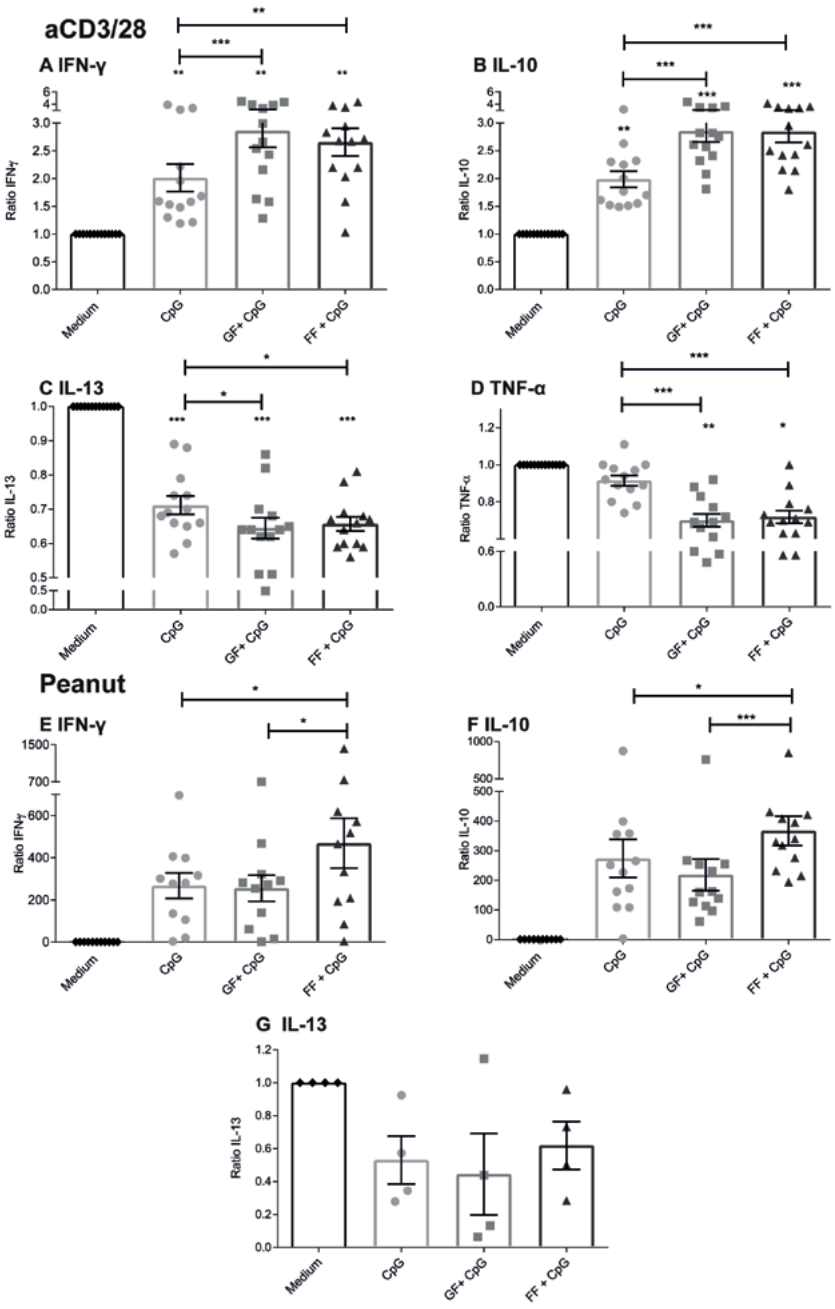

FIGURE 3 | Enhanced production of IL-10 and IFN-γ by aspecific or peanut-specific activated PBMCs upon combined exposure of IECs to CpG ODN and oligosaccharides
Exposure of IECs to CpG ODN in combination with scGOS/lcFOS (GF) or scFOS/lcFOS (FF) enhanced basolateral IFN-γ and IL-10 production in the aspecific model **(A, B)**. >>>

Legend Figure 3 Continued
IL-13 production was decreased in the aspecific model upon exposure of IECs to CpG ODN and was further decreased by oligosaccharides **(C)**. TNF-α production was decreased in the combined presence of scGOS/lcFOS and scFOS/lcFOS **(D)**. Only scFOS/lcFOS was able to enhance basolateral IFN-γ and IL-10 production induced by CpG ODN in the peanut-specific model **(E, F)**. IL-13 was measured after restimulation in four peanut-allergic patients **(G)**. Data are represented as ratios compared to the medium control and represent n = 12–13 peanut-allergic patients, mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by one-way ANOVA.

enhanced by combined exposure of IECs to both CpG and scGOS/lcFOS. In the peanut-specific co-culture model, combined exposure to CpG and both oligosaccharide mixtures significantly enhanced galectin-9, while CpG exposure alone showed a similar tendency.

3.3 | Increased percentage of Treg and Tfh subsets in the peanut-specific co-culture model upon exposure of IECs to CpG ODN

Allergy is caused by a combination of overactivation of Th2 cells and impaired active suppression mediated by Treg and regulatory cytokines or anergy induction.[33] Therefore, T cell polarization was assessed to determine whether this could be affected by the oligosaccharide mixtures. The Treg population ($CD4^+CD25^{high}CD127^-FoxP3^+$, Figure 6A) remained stable in the aspecific co-culture model (Figure 6B), while it significantly increased in the peanut-specific model upon exposure of IECs to CpG ODN (Figure 6C). In addition, the Tfh subset (Figure 6D) in the aspecific model was significantly increased (Figure 6E), and a similar trend in the peanut-specific model was observed (Figure 6F). Tfh can produce IL-21, which can inhibit class switching to IgE.[34] Intracellular IL-21 was measured in the aspecific co-culture model after restimulation with PMA and ionomycin, and was increased after CpG exposure in presence or absence of oligosaccharides (data not shown).

3.4 | Increased Th1 subset in a peanut-specific co-culture model upon exposure of IECs to CpG, while CRTH2 is downregulated

Similar to the Treg population, no changes were observed in the Th1 or Th2 subset (gating Figure 7A) in the different IEC exposure conditions of the aspecific co-culture model (Figure 7B, Th2 data not shown). However, in the peanut-specific model, IECs exposed to CpG ODN in the apical compartment enhanced the percentage of basolateral Th1 cells (Figure 7C). This Th1 polarization was further significantly enhanced when IECs were exposed to both CpG ODN and scFOS/lcFOS, but not with scGOS/lcFOS.

The Th1 subset comprised a significantly lower percentage in peanut-specific stimulated PBMCs compared to aspecifically-activated PBMCs; by contrast the Th2 subset was increased up to two-fold (Figure 7D,E). This indicates that the stimulation with the peanut-extract induced a peanut-specific Th2 response. Although this response is higher in the peanut-specific model, no changes were observed in percentages of Th2 cells in the separate conditions of the peanut- or aspecific co-culture model (data not shown). However, the

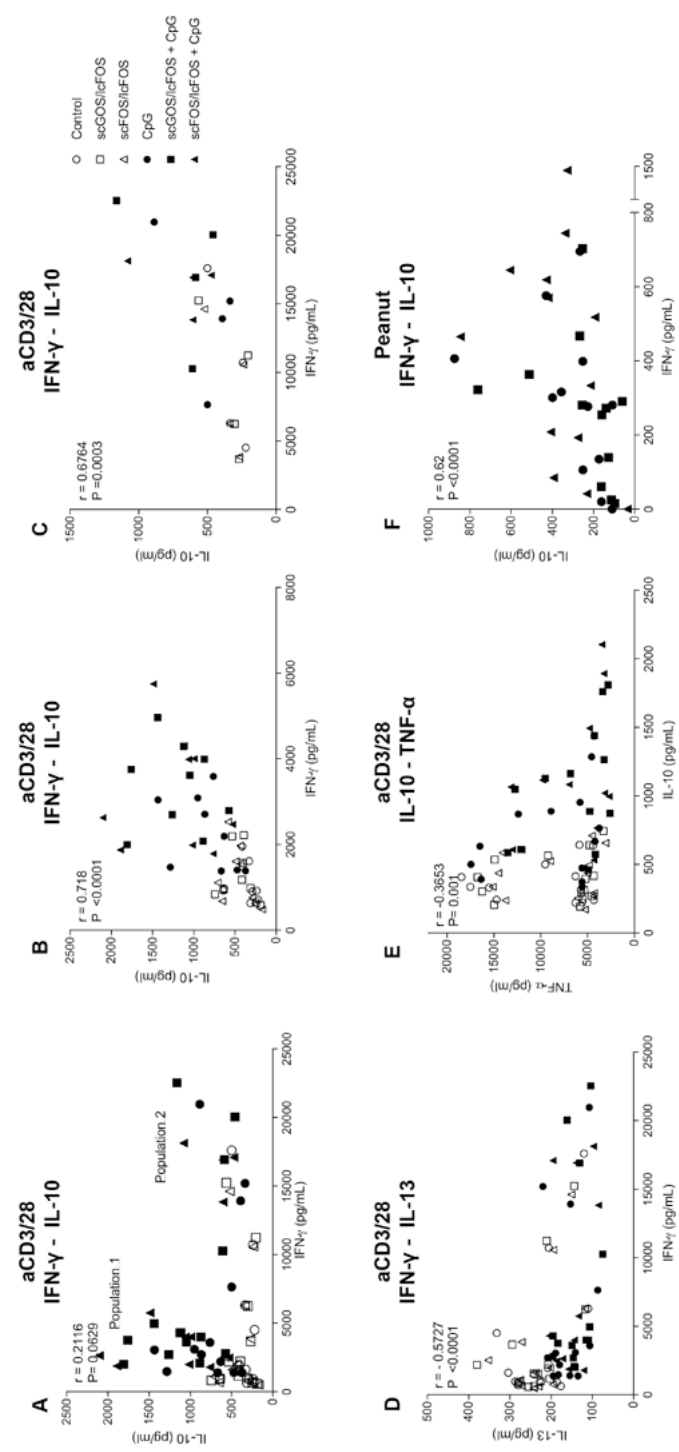

FIGURE 4 | Correlation cytokine production
IFN-γ and IL-10 concentrations were positively correlated in both the aspecific and peanut-specific co-culture models **(A–C, F)**. In the aspecific model, a clear distinction could be made into two populations **(B,C)**. Population 2 was comprised of 4 patients for all data points. A negative correlation existed between IFN-γ and IL-13 concentration **(D)** and TNF-α and IL-10 concentration **(E)**. Data represent n = 12–13 peanut-allergic patients. Correlation was tested with Spearman's rank correlation coefficient.

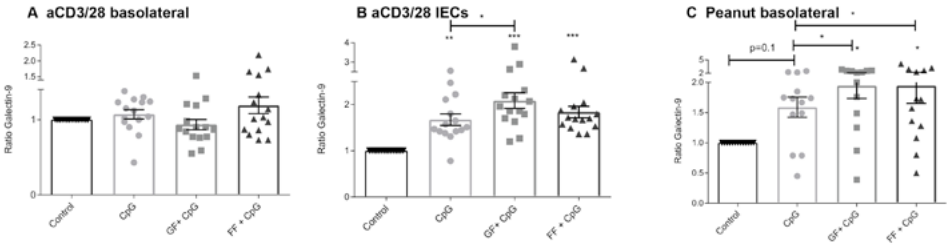

FIGURE 5 | Increased galectin-9 production by IECs upon apical exposure to CpG ODN in presence or absence of oligosaccharides
In the aspecific co-culture model, no differences in basolateral galectin-9 were observed after 24 hours **(A)**. Exposure of IECs to oligosaccharides alone did not influence galectin-9 levels, while CpG exposure influenced galectin-9 release by IECs after 48 hours, which was further enhanced by short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) (GF), but not by scFOS/lcFOS (FF) **(B)**. In the peanut-specific model, both oligosaccharide mixtures further enhanced galectin-9 concentrations **(C)**. Data represent n = 15 peanut-allergic patients, mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by one-way ANOVA.

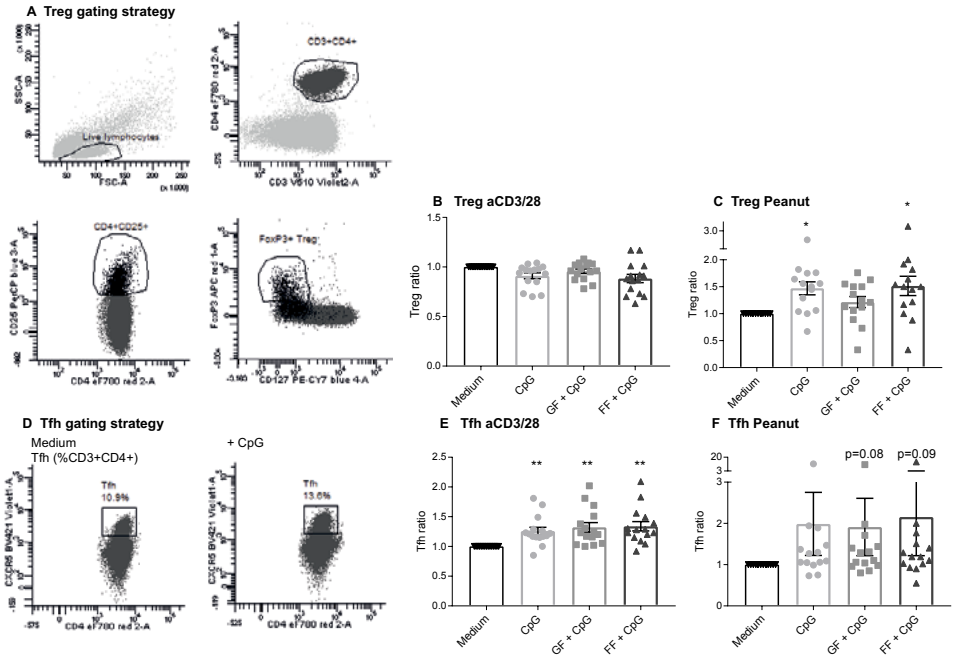

FIGURE 6 | Increased percentage of Treg and Tfh subsets in the peanut-specific co-culture model upon exposure of IECs to CpG ODN
Tregs were gated as indicated **(A)**. Treg polarization in the aspecific co-culture model was not altered upon exposure of IECs to CpG ODN, and no contributions of the oligosaccharides were observed **(B)**, whereas in the peanut-specific model, the Treg population increased upon CpG exposure **(C)**. Tfh were gated as indicated **(D)**. The percentage of Tfh cells increased upon CpG exposure of IECs, but was not further enhanced by the oligosaccharides **(E)**. In the peanut-specific model, Tfh also increased upon CpG exposure of IECs **(F)**. Data represent n = 15 peanut-allergic patients, mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by one-way ANOVA. scGOS/ lcFOS (GF), scFOS/lcFOS (FF).

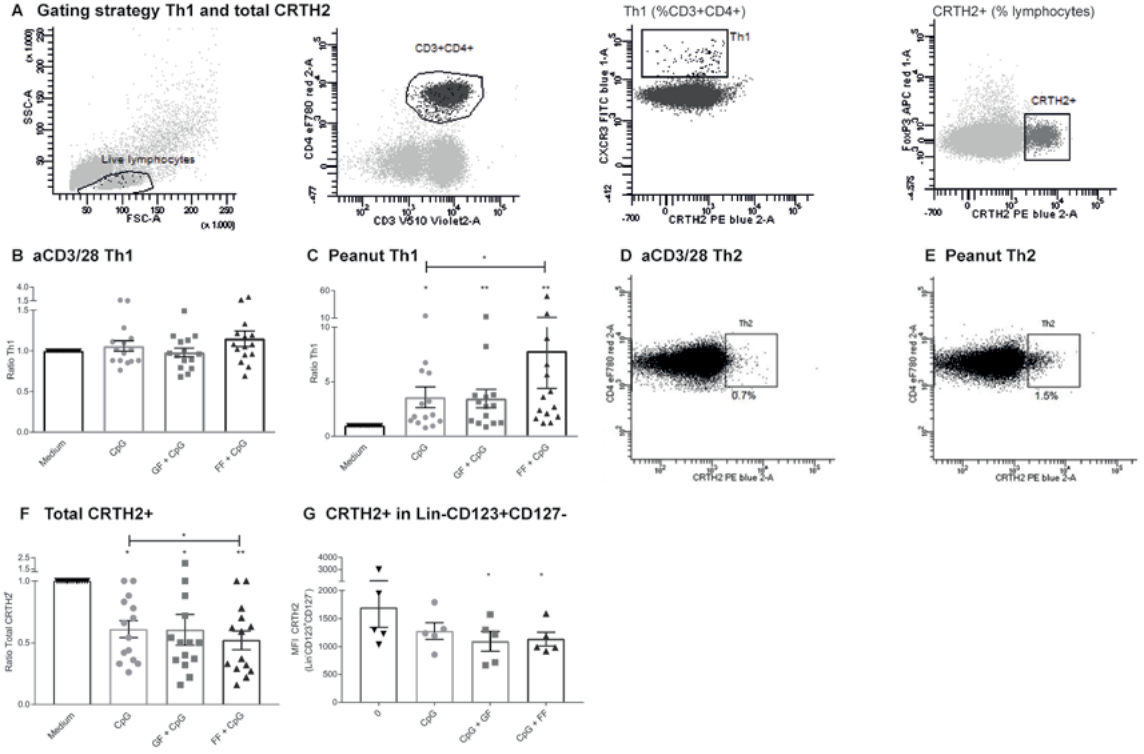

FIGURE 7 | Increased Th1 subset in a peanut-specific co-culture model upon exposure of IECs to CpG, while CRTH2 is downregulated
Gating strategy of Th1 and CRTH2+ cells **(A)**. The Th1 subset of the aspecific co-culture model was not affected after CpG exposure of IECs **(B)**. In the peanut-specific model, IECs exposed to CpG ODN mediated an increase in the basolateral Th1 population, which was further enhanced by scFOS/lcFOS (FF) **(C)**. A representative patient shows that the percentage of Th2 cells was higher in the peanut-specific model **(D, E)**. A significant decrease in surface marker CRTH2 in the peanut-specific PBMC fraction was observed, after apical exposure of IECs to CpG ODN, which was further decreased by scFOS/lcFOS **(F)**. Reduction of $Lin^{-}CD123^{+}CD127^{-}$ coincided with a reduction in CRTH2 expression in this cell subset **(G)**. N = 15 peanut-allergic patients, mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by one-way ANOVA, scGOS/lcFOS (GF).

CRTH2 expression significantly decreased in the peanut-specific PBMC fraction when IECs were apically exposed to CpG ODN (Figure 7F). This was further significantly decreased by scFOS/lcFOS. CRTH2 is a prostaglandin D2 receptor, and is a surface marker that is selectively expressed on, for instance, Th2 cells, but also on other cells involved in allergy such as basophils and eosinophils.[35,36] This reduction in CRTH2 corresponded with a decrease in a recently described new subset, a Lin^{-} $CD123^{+}$ $CD127^{low}$ population (Figure 7G) which shares some markers with both basophils and ILCs.[37]

3.5 | Neutralization of galectin-9 abrogates IFN-γ production in the peanut-specific co-culture model

Previous research in our group indicated that the neutralization of galectin-9 by TIM-3-Fc in an aspecific co-culture model with CpG ODN and scGOS/lcFOS abrogated the increase in IFN-γ and IL-10 production by PBMCs.[10] To examine the contribution of galectin-9 in the immunomodulatory effects of scFOS/lcFOS in the peanut-specific co-culture model, basolateral galectin-9 was inhibited by TIM-3-Fc. In these donors scFOS/lcFOS tended to increase IFN-γ further than CpG ODN alone, this effect was abrogated with TIM-3-Fc (Figure 8A). This indicates that galectin-9 is involved in the scFOS/lcFOS induced increase of IFN-γ when added together with CpG ODN in the peanut-specific co-culture model. Although combined exposure to both scGOS/lcFOS and CpG ODN did not further increase the IFN-γ concentration compared to CpG ODN, neutralization of galectin-9 by TIM-3-Fc reduced IFN-γ production, hereby also indicating the involvement of galectin-9 in the IFN-γ response in presence of scGOS/lcFOS. IL-10 concentrations were not further increased by scGOS/lcFOS or scFOS/lcFOS, and also no effects of TIM-3-Fc were observed (Figure 8B).

3.6 | Neutralization of the dectin-1 receptor does not affect IFN-γ and IL-10 production in the peanut-specific co-culture in which IECs are exposed to both CpG and oligosaccharides

Since dectin-1 is a C-type lectin receptor and can bind carbohydrates, it may be a possible candidate receptor for oligosaccharides to exert their functions. Neutralization of the dectin-1 receptor (nDectin) showed varying results in the patient samples. IFN-γ and IL-10 production were not affected by neutralization of the dectin-1 receptor on HT-29 cells (Figure 8C, D).

4 | DISCUSSION

This research explored and compared the immunomodulatory capacities of oligosaccharide mixtures scGOS/lcFOS and scFOS/lcFOS to gain insight in the underlying mechanisms of the observed allergy-reducing effects. To our knowledge, the immunomodulatory capacities

Peanut

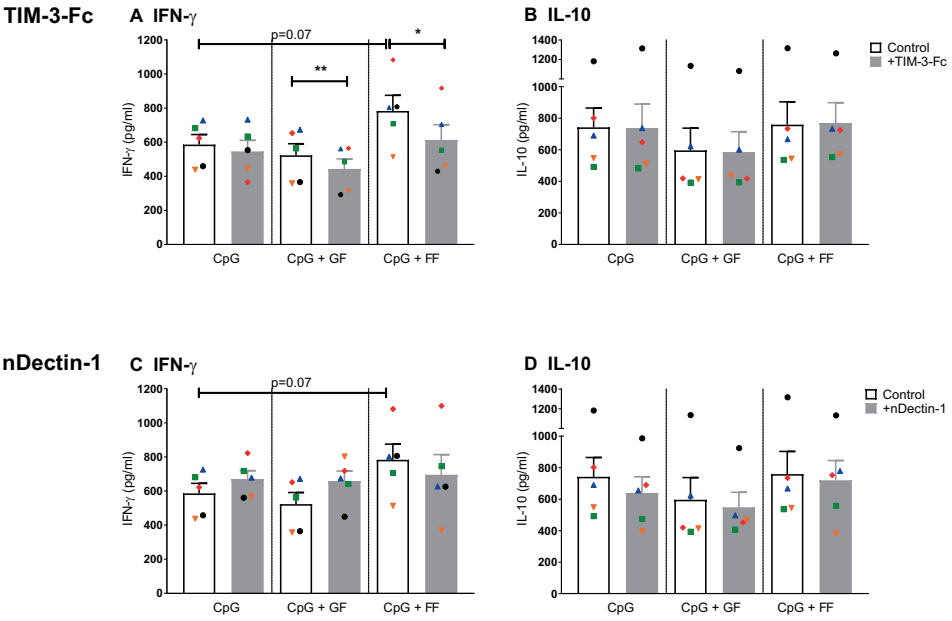

FIGURE 8 | Neutralization of galectin-9 abrogates IFN-γ production in the peanut-specific co-culture model, while neutralization of the dectin-1 receptor does not affect IFN-γ and IL-10 production
Addition of TIM-3-Fc to the peanut-specific co-culture abrogated additional IFN-γ production by scFOS/lcFOS (FF), and also decreased IFN-γ production upon combined exposure to CpG ODN and scGOS/lcFOS (GF) **(A)**. IL-10 production was not influenced by addition of TIM-3-Fc **(B)**. Neutralization of dectin-1 receptor on HT-29 cells did not affect IFN-γ or IL-10 production **(C, D)**. Data represent n = 5 peanut-allergic patients, mean ± SEM, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by one-way ANOVA or Student's paired *t*-test.

of scFOS/lcFOS in *in vitro* models has not been investigated previously. In addition, this study was performed with cells of peanut-allergic patients instead of healthy donors. In the aspecific co-culture model with PBMCs of peanut-allergic patients both oligosaccharide mixtures were effective in significantly enhancing IFN-γ and IL-10, while decreasing IL-13 and TNF-α production. By contrast, in presence of TLR-9 ligation with CpG ODN, the combination with scFOS/lcFOS rather than scGOS/lcFOS was effective in enhancing this Th1 and regulatory IL-10 response in a peanut-specific model. This correlated increase in both IFN-γ and IL-10 production was described previously,[13] and depended on the presence of the IECs in the co-culture model. These IECs can modulate immune responses, and under the influence of TLR9 ligand CpG ODN, both IFN-γ and IL-10 were upregulated and this was further enhanced by oligosaccharides. Although the IL-13 production in the peanut-specific model could only be determined in a small sample size, it showed a similar trend as the aspecific model. In addition, a significant decrease in prostaglandin receptor

CRTH2 expression was observed in the peanut-specific model when IECs were exposed to both scFOS/lcFOS and CpG ODN. This receptor is associated with allergy and inflammation, since activation of this receptor can induce chemotaxis of Th2 cells, eosinophils or basophils to sites of inflammation.[38-40] Therefore, we can conclude that the overall cytokine balance of the observed effector response of CpG ODN combined with scFOS/lcFOS in a peanut-specific model is favored towards a Th1 and regulatory IL-10 response, driving away from the inflammatory allergic phenotype. The latter is supported by the observed decrease of TNF-α, and the negative correlation between IL-10 and TNF-α. In the aspecific model of both healthy as well as peanut-allergic donors, scGOS/lcFOS and scFOS/lcFOS significantly enhanced the effect of CpG ODN. Typically in the peanut-specific model, only scFOS/lcFOS was capable of enhancing the regulatory Th1 response when combined with CpG ODN in terms of increased IFN-γ and IL-10 production and Th1 polarization. This may be related to structural differences between these oligosaccharides. scGOS is synthesized from lactose by β-galactosidase, and consists of galactose polymers in combination with a glucose moiety on the reducing terminus, with a degree of polymerization (DP) of less than 10 monomers.[41] In contrast, scFOS is derived from inulin, and consists of fructose polymers with a DP of 2-6.[41] Currently it is not known why scGOS/lcFOS did not enhance CpG effects in the peanut-specific model; however this may be related to the allergen-specific way of stimulation of the PBMCs. These differences in stimulation indicate the importance of confirming the effects in an allergen-specific model beyond the use of aspecific stimulation models. The differences between cytokine responses of scGOS/lcFOS and scFOS/lcFOS in this transwell co-culture model could be evaluated more in depth with a concentration-response study, however due to the limited amount of PBMCs obtained from peanut-allergic patients, this was not possible in this study.

Although additional cytokine effects of the prebiotic mixtures in combination with CpG were observed in the aspecific co-culture model, these additional effects could not be directly linked to the Th1 cell polarization like was previously shown using PBMCs derived from healthy donor buffy coats.[22] However, in the peanut-specific model the additional effect of scFOS/lcFOS on top of the CpG ODN effect on IFN-γ production could be linked to increased Th1 percentages. An explanation for missing this direct link between the additional cytokine production by the oligosaccharides and T cell polarization is that cytokines IFN-γ and IL-10 can be produced by other cell subsets than Th1 cells or Tregs within the PBMCs. For instance, NK cells, CTLs and ILCs can produce IFN-γ,[42] whereas monocytes and B cells can also produce IL-10.[13,43] The decrease in IL-13 was not associated with a reduction in the Th2 subset, but may be explained by the decrease in the total CRTH2 population, or the increase of IFN-γ. This cytokine is known to be able to inhibit Th2-type responses.[44] It could be possible that the non-digestible oligosaccharides exert their functions not only on T cell level, but also influence other cells in the co-culture model, which should be further investigated. Cytokine production can also be influenced by age.

This study depended on patients that voluntarily donated blood, therefore the age of patients was not homogeneous. However, no correlations between age and cytokine production were observed (data not shown). The choice for using HT-29 cells in this study was based on previous research. For the future, it would be interesting to validate these results with for instance primary epithelial material from (allergic) patients.

In the peanut-specific model an additional increase in basolateral galectin-9 concentration was observed when IECs were exposed to the combination of oligosaccharides and CpG ODN. This coincided with a decrease in IFN-γ production in the peanut-specific co-culture model when galectin-9 was neutralized by TIM-3-Fc. Therefore, we assume that also in an allergen-specific setting, galectin-9 may mediate the immunomodulatory effect in the case of scFOS/lcFOS, as was described previously.[10] Next to the role of galectin-9, we assessed whether oligosaccharide mixtures exert their functions via C-type lectin receptor dectin-1 which is present on human IECs and HT-29 cells.[27] IFN-γ production was not significantly affected after neutralization of this receptor, indicating that dectin-1 might not be important in the recognition of non-digestible oligosaccharides. However, there are also studies indicating that dectin-1 can collaborate with other TLRs or complement receptor 3 (CR3).[45,46] Further investigation into the possible role of dectin-1 might be necessary to rule out any collaboration with other receptors. In conclusion, this *in vitro* study indicates that combined exposure of IECs to CpG ODN and scFOS/lcFOS in a peanut-specific co-culture model contributes to an effector response that is favored towards a Th1 and regulatory IL-10 response and is less prone to the Th2 milieu. To improve efficacy and safety of currently developing protocols for immunotherapy, scFOS/lcFOS may be an interesting candidate for dietary adjunct therapy in allergen-specific immunotherapy, since the final efficacy goal of immunotherapy is the suppression or recovery of the allergen-specific Th2 response which may contribute to acquiring long lasting tolerance induction.

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CHAPTER 6

Non-digestible oligosaccharides can suppress basophil degranulation in whole blood of peanut-allergic patients

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ABSTRACT

Background

Dietary non-digestible oligosaccharides (NDOs) have a protective effect against allergic manifestations in children at risk. Dietary intervention with NDOs promotes the colonization of beneficial bacteria in the gut and enhances serum galectin-9 levels in mice and atopic children. Next to this, NDOs also directly affect immune cells and low amounts may reach the blood. We investigated whether pre-incubation of whole blood from peanut-allergic patients with NDOs or galectin-9 can affect basophil degranulation.

Methods

Heparinized blood samples from 15 peanut-allergic adult patients were pre-incubated with a mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS), scFOS/lcFOS, or galectin-9 (1 or 5 μg/ml) at 37°C in the presence of IL-3 (0.75 ng/ml). After 2, 6 or 24 hours, a basophil activation test (BAT) was performed. Expression of FcεRI on basophils, plasma cytokine and chemokine concentrations before degranulation were determined after 24 hours.

Results

Pre-incubation with scGOS/lcFOS, scFOS/lcFOS or galectin-9 reduced anti-IgE mediated basophil degranulation. scFOS/lcFOS or 5 μg/mL galectin-9 also decreased peanut-specific basophil degranulation by approximately 20%, mainly in whole blood from female patients. Inhibitory effects were not related to diminished FcεRI expression on basophils. Galectin-9 was increased in plasma after pre-incubation with scGOS/lcFOS, and both NDOs and 5 μg/mL galectin-9 increased MCP-1 production.

Conclusion & Clinical relevance

The prebiotic mixture scFOS/lcFOS and galectin-9 can contribute to decreased degranulation of basophils *in vitro* in peanut-allergic patients. The exact mechanism needs to be elucidated, but these NDOs might be useful in reducing allergic symptoms.

1 | INTRODUCTION

In Westernized countries, the prevalence of food allergies has increased over the years and is still increasing.[1,2] The prevalence of food allergy is currently estimated between 6%-10%.[3] Among children, less is known about the prevalence, although peanut allergy is one of the most common food allergies. Allergic reactions develop as a result of a hampered tolerance mechanism towards harmless antigens.[4] When patients are sensitized, B cells start to produce antigen-specific IgE molecules that can sensitize the high affinity FcεRI on mast cell or basophils.[5] Upon a second encounter with the specific allergen, these IgE molecules can crosslink and will induce degranulation of mast cells and basophils, leading to clinical symptoms due to the release of histamine and other mediators. Currently there is no curative treatment available to re-establish tolerance against these harmless food antigens, although progress is made in terms of immunotherapy and dietary adjunct therapy with for example probiotics that can improve the efficacy of immunotherapy.[6]

Previous research has indicated a role of prebiotic non-digestible oligosaccharides (NDOs) in decreasing the incidence of atopic dermatitis in children at risk of developing allergy.[7-9] The exact mechanism of action of these NDOs is not fully understood, however it is known that they can promote the colonization of beneficial bacteria in the gut, similar as human milk oligosaccharides (HMOs) in breast milk.[10,11] Whey-allergic mice receiving oral immunotherapy (OIT) in combination with a diet of short- and long chain fructo-oligosaccharides (scFOS/lcFOS) experienced enhanced serum galectin-9 levels.[12] Galectin-9 is a soluble type lectin which can, among others, be released by intestinal epithelial cells (IECs). It can bind to carbohydrate moieties located on the heavy chains of IgE [13,14] , hereby suppressing degranulation of mast cells and basophils by the inhibition of the formation of the IgE-allergen complex, which could be abrogated in the presence of lactose.[14,15] In addition, galectin-9 can support tolerance via the induction of Tregs.[15,16] Next to induction of galectin-9, these NDOs might also have a direct effect on immune cells. Earlier research indicated that HMOs (normally present in concentrations of 5-23 g/L in human milk) could be traced with ^{13}C labeling, HPLC and other techniques in plasma and urine.[17-20] Approximately 0.05-0.1% of these oligosaccharides could be traced back to plasma, while 4% was traced back in urine.[18-21] Additionally, a study with fructo-oligosaccharides demonstrated that FOS, and hereby most likely more prebiotic structures, could reach the plasma compartment and were excreted in the urine.[21] For this research, we were interested in both the direct and indirect (galectin-9) effects of NDOs on basophil degranulation in whole blood of peanut-allergic patients. Therefore, two different mixtures of NDOs were tested; scGOS/lcFOS and scFOS/lcFOS. In addition, the effects of galectin-9 on basophil degranulation were assessed. Next to determining the effects on basophil degranulation, the expression of FcεRI on basophils and mediator release in whole blood exposed to NDOs or galectin-9 was determined.

2 | MATERIALS & METHODS

2.1 | Study design and study population

Fifteen peanut-allergic patients (six male and nine female; age 18-50; mean 32) were recruited from the department of Dermatology/Allergology at the University Medical Center Utrecht. Inclusion criteria consisted of a type I allergic reaction to peanut, previously confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC) and serum peanut-specific IgE (Table 1). Exclusion criteria were pregnancy or the continuous use of systemic immune-suppressants, such as prednisone. Total IgE levels were measured by ELISA (Euroimmun, Lübeck). In addition, average basophil percentages are shown per patient (normal range 0% - 2%). All patients gave written informed consent before enrollment in the study. The study was reviewed and approved by the Ethics Committee of the University Medical Center Utrecht (NL51606.041.15).

2.2 | Basophil activation test

Whole heparinized blood was obtained from fifteen peanut-allergic patients. Blood was incubated for 2, 6 or 24 hours at 37°C with galectin-9 (1 μg/mL (~28 nM) or 5 μg/mL (~140 nM), R&D Systems, Minneapolis, USA), 0.05% (w/v) of a 9:1 mixture of scGOS (Vivinal GOS, Borculo Domo, the Netherlands) and lcFOS (scGOS/lcFOS) (Raftiline HP, Orafti) or 0.05% of a 9:1 mixture of scFOS (Raftilose P95, Orafti) and lcFOS (scFOS/lcFOS). To maintain basophil viability, 0.75 ng/mL IL-3 (R&D Systems) was added to the blood during pre-incubation and control samples were included. After different pre-incubation periods, a basophil activation test (BAT) was performed. Basophils in the different blood samples were stimulated for 30 minutes with increasing concentrations of anti-IgE (0.1, 0.3, 1 μg/mL, Vector Laboratories, Burlingame, USA) or crude peanut extract (0.1, 0.3, 1, 3, 10, 100, 1000 ng/mL) in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 1 ng/mL IL-3). Control samples included RMPI + IL-3 and formyl-methionyl-leucyl-phenylalanine (1 μM fMLP, Sigma-Aldrich). Leukocytes were stained with an antibody cocktail of CD45-PO (Life Technologies), CD123-FITC (Biolegend), HLA-DR-PB (Biolegend), CD63-PE (Monosan), CD41-PE-Cy7 (Beckman coulter), CD203c (Biolegend). Basophils were defined as $CD45^+$ $CD203c^+$ $CD123^+$ and $HLA\text{-}DR^-$ $CD41^-$, and degranulation was determined as $CD63^+$ cells. Results are expressed as percentage of $CD63^+$ cells. Per patient, the dose of peanut-allergen or anti-IgE that induced maximal degranulation in the control sample was used to normalize the data.

2.3 | Determination of FcεRI on the cell surface of basophils

FcεRI expression was quantified in seven random patients using a QIFIKIT (Dako) according to the manufacturer's protocol. In short, a small sample of blood was first

TABLE 1 | Patient characteristics

Patient	Age (years)	Sex (M/F)	Müller score*	SPT peanut	Subjective ED (mg)	Objective ED (mg)	Total IgE (IU/mL)	CAP peanut (kU/L)	Percentage basophils (% total)
N01	41	F	2	3+	10	-	238	1.7	0.35%
N02	37	M	4	3+	0.1	300	1,482	44	0.63%
N03	45	M	2	4+	100	-	101	1.8	0.40%
N04	50	F	3	4+	10	10	1,537	12	0.38%
N05	35	F	4	4+	0.1	-	535	85	0.62%
N06	27	F	2	4+	4	40	3,786	12.8	0.26%
N07	42	M	3	5+	not known	300	599	42.7	0.30%
N08	24	M	1	4+	100	>3,000	2,054	1.9	0.20%
N09	24	F	3	3+	not known	>3,000	1,062	1	0.34%
N10	18	F	3	4+	300	1,000	5,823	>100	0.40%
N11	32	F	2	4+	10	3,000	3,941	No data	0.40%
N12	27	M	3	5+	0.1	1,000	59,272	66	0.41%
N13	25	M	1	3+	10	-	2,302	11.2	0.53%
N14	26	F	2	4+	0.1	100	3,287	9.7	0.53%
N15	34	F	2	4+	40	12,000	1,347	1.55	0.25%

Age, sex, Müller score, SPT, subjective and objective ED as established by DBPCFC, total and peanut-specific IgE and average basophil count per subject. * Müller score 0: symptoms oral cavity, 1: symptoms of the skins and mucous membranes, 2: gastro-intestinal symptoms, 3: respiratory symptoms, 4: cardiovascular symptoms. Skin prick test (mm), diameter of 3 mm (3+) is considered positive. All patients underwent a DBPCFC, subjective and ED are displayed in mg.

incubated with a primary mouse monoclonal against FcεRI (clone CRA-1), or an isotype. CRA-1 binds to both the open and occupied FcεRI receptor,[22] Next, the blood samples, set-up and calibration beads were labeled with a FITC-conjugated anti-mouse secondary antibody, followed by further staining of the samples with an antibody cocktail to identify basophils (CD45, CD123, HLA-DR, CD203c, CD41). FcεRI expression was quantified based on the calibration curve from the calibration beads by Graphpad Prism 7.0. Values were calculated relative to the control sample.

2.4 | Determination of cytokines and chemokines in plasma pre-incubated samples

Residual plasma was collected after pre-incubation of the blood samples. Mediators secreted by basophils or involved in basophil activation or degranulation (IL-4 ,[23] IL-5,[24] GM-CSF, [25] MCP-1,[26] MDC[27] and TARC (upregulated by basophil derived IL-4 and IL-13).[28] Chemoattractants or mediators in basophil degranulation Eotaxin-3,[29] RANTES,[30] galectin-3[31,32] and galectin-9[14]) were measured in these plasma samples with a luminex assay, performed by the luminex facility located in the UMC Medical Center, Utrecht. Values were calculated relative to the control sample.

2.5 | Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was analyzed with GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Normally distributed data were analyzed with a one-way repeated measures ANOVA followed by Bonferroni *post hoc* analysis. Data were considered significant when $p < 0.05$. Demographic data were analyzed with the non-parametric Mann-Whitney test. Correlation was determined with Pearson's correlation coefficient.

3 | RESULTS

3.1 | Time-dependent kinetics basophil degranulation influenced by NDOs and galectin-9

In a pilot experiment, kinetics of the effects of basophil degranulation pre-incubated by NDOs and galectin-9 were determined. A time curve was performed in 1 mL whole blood of three patients. Whole blood samples were pre-incubated for 2, 6 or 24 hours at 37°C, with 0.05% scFOS/lcFOS, 1 μg/mL galectin-9 or were left untreated as control. Figure 1A shows the relative IgE-mediated degranulation of basophils of three patients. After 2 and/or 6 hours, some decrease in IgE-mediated basophil degranulation and peanut-specific (Figure 1B) was observed in the pre-incubated samples. After 24 hours, the differences between the pre-incubated and untreated control sample were most pronounced, therefore we continued with the 24 hours pre-incubation.

3.2 | Pre-incubation of blood with NDOs or galectin-9 decreases basophil degranulation in peanut-allergic patients

Blood samples pre-incubated with NDOs or galectin-9 showed a reduced basophil degranulation after the IgE-mediated BAT compared to the controls after 24 hours (Figure 2A). Pre-incubation with scGOS/lcFOS or scFOS/lcFOS resulted in an average decrease in anti-IgE-mediated basophil degranulation of 11 ± 3.5% or 13 ± 4%, respectively. Pre-incubation with 1 μg/mL galectin-9 resulted in an average decrease of 16 ± 6%, while the highest concentration of galectin-9 could reduce basophil degranulation on average with 30 ± 7%.

In the peanut-specific BAT (Figure 2D) only scFOS/lcFOS and the highest dose of galectin-9 reduced basophil degranulation significantly compared to the control sample, with an average reduction of 20 ± 6% (scFOS/lcFOS) and 17 ± 9% (galectin-9).

Not all patients were responsive to all pre-incubations, and also differences in response between the aspecific (anti-IgE) and peanut-specific BAT were observed in blood samples of individual patients. In addition, the oligosaccharides did not alter the fMLP-mediated degranulation, indicating an IgE-specific inhibition rather than a general inhibition (data not shown).

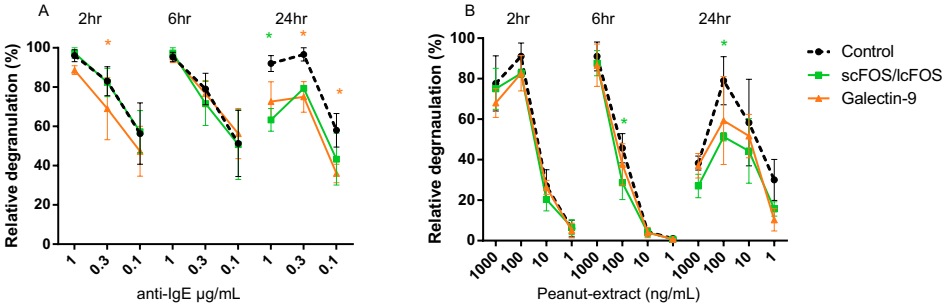

FIGURE 1 | Time-dependent kinetics of NDOs or galectin-9 on anti-IgE or CPE induced basophil degranulation of a representative donor

Blood of peanut-allergic patients (n = 3) was pre-incubated for three different time-periods with scFOS/lcFOS or 1µg/mL galectin-9 either in the aspecific **(A)** or peanut-specific **(B)** BAT. Data represent n = 3 peanut-allergic patients. Significance is indicated compared to the control sample. **P* < 0.05 by two-way ANOVA. Green: control compared to scFOS/lcFOS, orange: control compared to galectin-9.

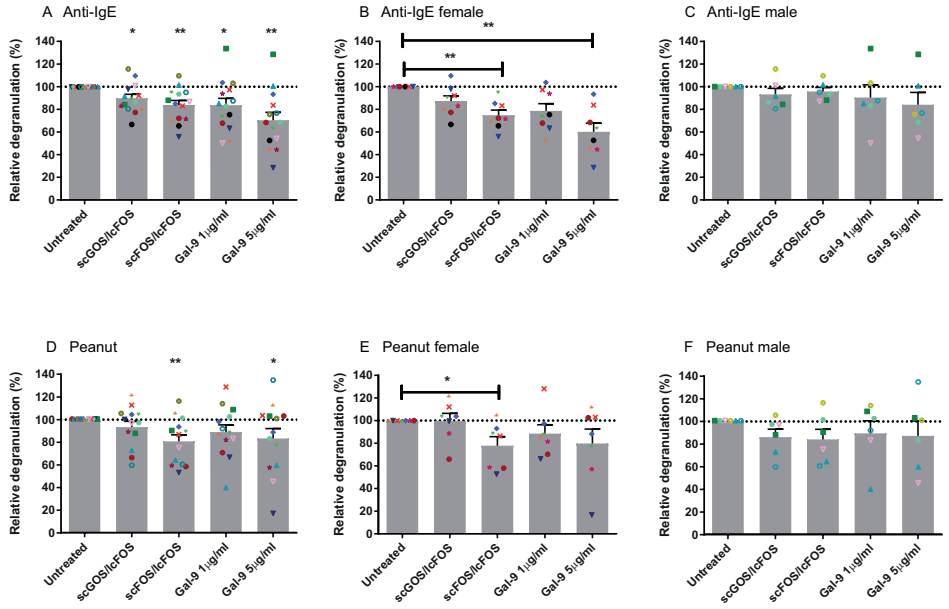

FIGURE 2 | Reduced basophil degranulation after pre-incubation with NDOs or galectin-9

Pre-incubation of whole blood for 24 hours with NDOs of galectin-9 resulted in a decrease in IgE-mediated basophil degranulation **(A-C)**. Pre-incubation with scFOS/lcFOS and 5 µg/mL galectin-9 reduced peanut-specific basophil degranulation **(D-F)**. Females tended to have a higher decrease in basophil degranulation than males after pre-incubation **(B, C, E, F)**. Data represent the mean ± SEM of n = 15 peanut-allergic patients, two patients were unresponsive (< 5% basophil degranulation) in the peanut-specific BAT, one of these two patients was also non-responsive in the IgE-mediated BAT. **P* < 0.05, ***P* < 0.01 by one-way ANOVA.

3.3 | Pre-incubation of blood with NDOs or galectin-9 reduces basophil degranulation more effective in female patients and does not correlate with FcεRI expression

Next to the role of pre-incubation or the specificity of the BAT (anti-IgE or peanut) on basophil degranulation, differences in degranulation were observed between male and female blood samples. Female blood samples pre-incubated with NDOs or galectin-9 showed less basophil degranulation compared to their control samples than males. This was observed in both the peanut-specific and the anti-IgE BAT (Figure 2 B-C, E-F). In the IgE-mediated BAT, significant differences were observed between the female and male samples for scFOS/lcFOS ($P < 0.01$) and a similar trend for galectin-9, 5µg/mL ($P < 0.1$). In addition, other demographic variables as indicated in Table 2 could not explain the differences between males and females, as they were not statistically different.

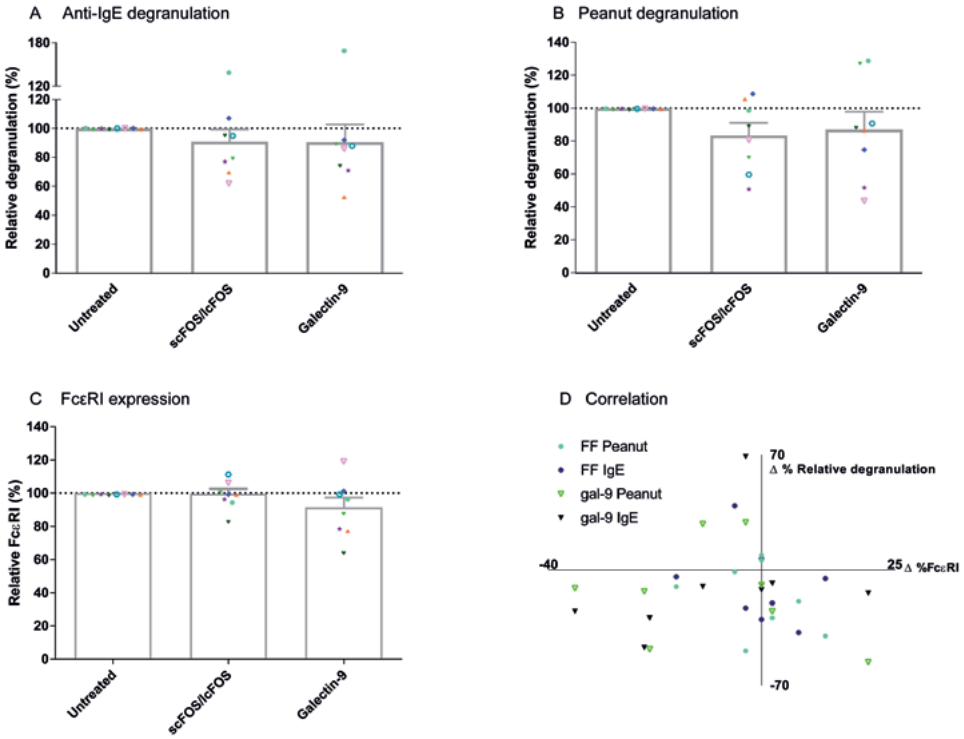

FIGURE 3 | Correlation relative expression of FcεRI and basophil degranulation
Anti-IgE mediated and peanut-specific basophil degranulation after 24 hours of pre-incubation with scFOS/lcFOS or galectin-9 **(A, B)**. FcεRI expression on basophils was not decreased after pre-incubation with scFOS/lcFOS or 1µg/mL galectin-9 **(C)**. Δ% relative degranulation was calculated based on Figure A and B, whereas Δ%FcεRI was calculated based on Figure C, both relative to the control samples. No correlation was found between expression of ΔFcεRI on the cell surface and the corresponding Δbasophil degranulation **(D)**. (FF = scFOS/lcFOS). Data represent n = 8 patients. Correlation was tested with Pearson's correlation coefficient.

Table 2 | Demographic data male and female patients

Characteristic	Male (n = 6)	Female (n = 7)	*P* value
Age (years)			
Mean ± SD	33.3 ± 9.2	31.4 ± 11	0.70
Median (25th,75th percentile)	32 (25, 43)	27 (24, 41)	
Müller score			
Mean ± SD	2.3 ± 1.2	2.4 ± 0.5	0.88
Median (25th,75th percentile)	2.5 (1, 3.3)	2 (2, 3)	
CAP peanut (kU/L) ± SD			
Mean ± SD	27.9 ± 26.7	19.8 ± 35.7	0.36
Median (25th,75th percentile)	26.9 (1.88, 49.5)	9.7 (1.5, 12.8)	

Table 3 | Average concentration mediators (pg/mL) in blood plasma after 24 hours of pre-incubation with NDOs

Mediator	Control ± SEM	scGOS/lcFOS ± SEM	scFOS/lcFOS ± SEM	Galectin-9 (1 µg/mL) ± SEM	Galectin-9 (5 µg/mL) ± SEM
IL-4	4 ± 0.8	3 ± 0.9	3 ± 0.7	3 ± 0.4	4 ± 0.6
IL-5*	13 ± 6	11 ± 5	9 ± 3	9 ± 2	11 ± 3
GM-CSF*	28 ± 5	23 ± 4	23 ± 3	20 ± 2	25 ± 3
MDC	444 ± 39	458 ± 36	454 ± 33	472 ± 44	507 ± 42
TARC	15 ± 2	15 ± 2	15 ± 2	15 ± 2	15 ± 2
Eotaxin-3	254 ± 48	245 ± 50	251 ± 51	256 ± 53	262 ± 52
RANTES	39,679 ± 4,131	40,454 ± 5,155	39,055 ± 4,537	37,172 ± 4,396	36,854 ± 4,178
Galectin-3	42,093 ± 3,210	35,832 ± 2,878	46,998 ± 9,034	45,240 ± 6,155	37,298 ± 2,584

* Values out of range below

To determine whether scFOS/lcFOS or galectin-9 suppressed basophil degranulation via directly or indirectly affecting FcεRI expression, these expression levels on basophils were determined in blood samples of seven patients relative to the expression of this receptor on untreated positive control samples of the same donor (Figure 3). First, anti-IgE (Figure 3A) and peanut-specific basophil degranulation (Figure 3B) of blood pre-incubated with either scFOS/lcFOS or galectin-9 was determined as described earlier. In addition, quantitative expression of FcεRI on basophils was determined before basophil degranulation (Figure 3C). No correlation was observed between the difference in expression of FcεRI and the corresponding difference in basophil degranulation of the pre-incubated samples (Figure 3D). Therefore, the mechanism of action of NDOs and galectin-9 cannot be ascribed to effects on expression of FcεRI on the basophil cell surface.

3.4 | Luminex analysis plasma samples

To determine whether NDO or galectin-9 pre-incubation of blood affects mediator secretion by cells, remaining plasma samples after the pre-incubation were collected and were analyzed on cytokine and chemokines levels. Table 3 indicates the mean baseline levels per

mediator and the mean levels ± SEM when blood samples were pre-incubated with scGOS/lcFOS, scFOS/lcFOS or galectin-9. IL-5 and GM-CSF were for several samples extrapolated from the standard curve or below the detection limit of the luminex assay, therefore no conclusions can be drawn on the contribution of these mediators in the observed decrease in basophil degranulation.

Significant differences compared to the untreated control were found for Galectin-9 and MCP-1 (CCL2) levels (Figure 4). Galectin-9 levels in blood pre-incubated with scGOS/lcFOS increased relative to the untreated control sample which was set to 100%, while pre-incubation with scFOS/lcFOS did not result in an increase (Figure 4A). Galectin-9 levels in the galectin-9 pre-incubated samples were above the detection limit of the luminex and are therefore not displayed. MCP-1 (CCL2) levels were increased in plasma samples after pre-incubation with scGOS/lcFOS, scFOS/lcFOS and the highest dose of galectin-9 relative to untreated controls (Figure 4B). However, no correlations were found between levels of galectin-9, MCP-1 and anti-IgE or peanut-specific basophil degranulation (Figure 4C-F). Only a trend between galectin-9 and IgE-mediated basophil degranulation was observed for the control sample and after pre-incubation with scFOS/lcFOS (Figure 4C).

4 | DISCUSSION

Basophil degranulation is an important event in allergic reactions. This study demonstrates that IgE-mediated and peanut-specific basophil degranulation can be reduced by pre-incubation of blood with NDOs scGOS/lcFOS and scFOS/lcFOS, but also by their indirect product galectin-9, which can be enhanced among others under influence of NDOs.[33] These effects on basophil degranulation were mainly observed in female peanut-allergic subjects. The reduction in basophil degranulation was not correlated with altered FcεRI expression levels on the basophil cell surface. The differences between males and females could not be related to other demographic variables. An explanation for differences between males and females might be hormone-related. While the exact working mechanism of hormones is not elucidated, estrogen receptors for instance are found on several immune subsets and are described to have effects on the allergic sensitization pathway including promotion of basophil degranulation.[34-37] More differences in innate and adaptive immune responses between males and females have been studied and described, indicating that sex is an important parameter to consider for the observed differences in basophil degranulation.[38] No other relations between the effects of NDOs and other demographic variables, for example age, were observed. However, we cannot exclude this, due to the limited number of patients and their inhomogeneous age distribution.

When blood was pre-incubated with scGOS/lcFOS, an increase in galectin-9 was observed. Soluble type lectin galectin-9 is a small (36 kDa) glycoprotein that can bind to glycans

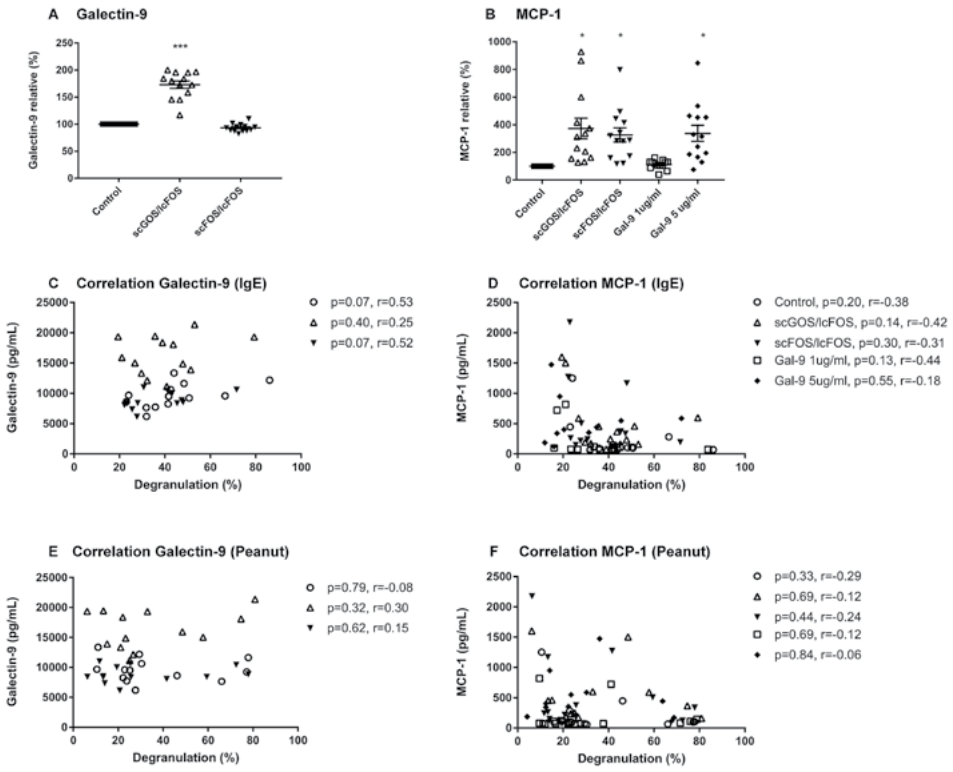

FIGURE 4 | No correlation between relative basophil degranulation and galectin-9 or MCP-1 production Galectin-9 increased in scGOS/lcFOS pre-incubated samples, and were above the detection limit for galectin-9 treated samples **(A)**. MCP-1 (CCL2) was increased after pre-incubation with both NDOs and 5µg/mL galectin-9 **(B)**. This was not correlated with anti-IgE or peanut-specific basophil degranulation **(C-F)**. Data is represented as mean ± SEM of n = 15 patients. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by one-way ANOVA. Correlation was tested with Pearson's correlation coefficient.

containing galactose and derivatives.[39] The carbohydrate recognition domains (CRDs) can only fit galactose residues; other sugar residues do not fit into these CRDs due to steric hindrance.[40] Interactions between galactose residues and galectin-9 can result in the formation of so-called lattices which play an important role in the regulation of immune responses.[41] We hypothesize that pre-incubation with scGOS/lcFOS stimulates cells in blood, such as T cells, B cells, eosinophils or basophils[14] to release galectin-9. This may result in the reduced basophil degranulation that was observed, since galectin-9 may have bound to IgE on the basophil cell surface, hereby hindering the formation of an IgE-antigen complex. Similar effects of IgE-binding capacities of (recombinant) galectin-9 have previously been described, and here galectin-9 was able to reduce mast cell degranulation in RBL-2H3 cells or HMC-1 cells, which was also explained by steric hindrance by galectin-9.[14,42] In the allergen-specific BAT, basophil degranulation was not significantly

decreased upon pre-incubation with scGOS/lcFOS and the lowest dose of galectin-9. This might be explained by the difference in size between anti-IgE (~150 kDa) and peanut allergens (~15-60 kDa). Peanut-allergens are smaller and have multiple IgE-binding epitopes,[43] and therefore may circumvent the steric hindrance caused by galectin-9. The higher concentration of galectin-9 might overcome this steric hindrance in the peanut-specific BAT, while scFOS/lcFOS probably acts via a different mechanism than scGOS/lcFOS and galectins.

Although the concentration of galectin-9 was upregulated by scGOS/lcFOS to a concentration of 16 ng/mL, it is relatively low when compared to the recombinant galectin-9 used in this study (1 and 5 μg/mL). No data is available whether such low concentrations of galectin-9 could influence basophil degranulation. Studies that have been performed used higher concentrations, and indicated a dose-response curve for galectin-9 and subsequent degranulation.[14,42] However, these concentrations are always much higher than physiological galectin-9 concentrations in serum of healthy controls (5-12 ng/mL).[44] In addition, most studies performed on basophil degranulation are using recombinant galectin-9, which may also generate different responses than natural galectin-9 that was induced by scGOS/lcFOS. In summary, galectin-9 might be a contributing factor in the observed decrease in degranulation after pre-treatment with scGOS/lcFOS, but since there was no correlation between galectin-9 and degranulation and not all patients were responsive to scGOS/lcFOS, other factors are probably involved.

scFOS/lcFOS was most effective in reducing basophil degranulation in both the IgE-mediated and the peanut specific BAT, with a similar reduction as 5 μg/mL galectin-9 (approximately 20%). In contrast to scGOS/lcFOS, scFOS/lcFOS did not affect plasma galectin-9 concentrations. This indicates that scFOS/lcFOS may exert its functions in a different manner than scGOS/lcFOS. One of the possible mechanisms by which scFOS/lcFOS may exert its effect is via modulation of FcεRI expression. However, no correlation was observed between scFOS/lcFOS induced effects on basophil degranulation and relative FcεRI expression on these cells.

In addition to differences in galectin-9 levels, changes in MCP-1 chemokine levels after pre-incubation were observed. MCP-1 is described in literature as a potent activator for basophil degranulation at concentrations of 3-10 nM and can be produced by various cell types.[26,45] This increase in MCP-1 is in contrast to our findings, since basophil degranulation was reduced in the pre-incubated blood samples. Both NDO mixtures and the highest concentration of galectin-9 increased MCP-1 levels in the blood plasma. However, these concentrations of MCP-1 are not elevated enough to induce basophil degranulation, since they are approximately 0.02-0.05 nM. In addition, no correlation was observed between MCP-1 levels and basophil degranulation, and no spontaneous CD63 release was observed after 2, 6 and 24 hours of pre-incubation with NDOs or galectin-9 (data not shown). This might indicate that NDOs and galectin-9 can have direct effects on basophils. A previous

study investigating the effects of galectin-9 on degranulation of HMC-1, a mast cell line that does not express FcεRI, also indicated an increase of MCP-1, which was dose-dependently correlated to increased galectin-9 levels.[42] In this study, galectin-9 reduced PMA and ionomycin-induced degranulation of HMC-1 and induced the phosphorylation of the ERK1/2 pathway. Based on these results, it would be interesting to investigate whether activation of this ERK1/2 signaling pathway is also involved in the reduction in degranulation like we observed in human basophils.

This study was an *in vitro* model, and it is therefore important that the effects observed in this study are validated *in vivo* in humans. For future research, it would be interesting to focus on the effects of NDOs and other components on other cells subsets, such as eosinophils, that play an important role in allergy and also have degranulation capacities. In addition, this study was performed in whole blood and results shown may be caused by either a direct or indirect effect of NDOs. To be able to investigate the mechanism of action of these NDOs and galectin-9 on basophil degranulation, and to disseminate between these paths, it would be interesting to perform these experiments on isolated basophils of allergic patients, to exclude the involvement of surrounding cells.

In conclusion, this study indicated that NDOs can decrease basophil degranulation in an *in vitro* model. Indirect mediator galectin-9, which may be released by different kinds of cell types in response to exposure to NDOs, was also able to decrease basophil degranulation. No modification of the FcεRI receptor expression, cytokines or chemokines was found in relation to this effect. The exact mechanism of action by which these NDOs can exert their immunomodulatory effects still needs to be elucidated, and *in vivo* validation of these results is necessary. However, it indicates that these NDOs are interesting dietary immunomodulatory agents, and might be useful as adjunct-therapy in allergen-specific immunotherapy.

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CHAPTER 7

Direct immunomodulatory effects of fructo-oligosaccharides in a peanut-specific autologous dendritic cell and T cell co-culture

Submitted for publication

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ABSTRACT

Background

Dendritic cells (DCs) play an important role in the presentation of antigens, and are an interesting target for immune-modulation in allergies. Non-digestible oligosaccharides (NDOs), such as short- and long-chain fructo-oligosaccharides (scFOS/lcFOS) have immunomodulatory capacities, and may influence the outcome of DC antigen presentation.

Objective

This study investigated the effect of scFOS/lcFOS during DC maturation and allergen presentation using cells of peanut-allergic patients in an autologous DC-T cell assay.

Methods

$CD14^+$ and $CD4^+$ T cells were isolated from peanut-allergic patients. $CD14^+$ monocytes were differentiated into immature DCs (imDCs), and matured (matDCs) with cytokines (IL-1β, IL-6, TNF-α, PGE2) in the presence or absence of crude peanut-extract (CPE) and/or scFOS/lcFOS (FF), and co-cultured in an autologous DC-T cell assay. After 6-7 days, T cell polarization, proliferation and cytokine production was measured.

Results

IL-10 production of matDCs increased compared to imDCs, and combined exposure to CPE and FF enhanced IL-10 compared to CPE alone. IP-10 secretion increased in these CPE/FF-matDCs. CPE-matDCs induced IL-13 secretion by T cells, which remained unaffected by FF. Although CPE and/or FF did not affect Th1 and Th2 polarization, Treg polarization was further increased by CPE-matDCs in presence or absence of FF. CPE/FF-DCs tended to increase the Treg/Th1 and Treg/Th2 ratios compared to matDCs. The proliferation of Th2 and Treg cells tended to increase when T cells were co-cultured with CPE-matDCs compared to matDCs. This became significant when CPE-matDCs were also exposed to FF and the same trend was observed for Th1 cells.

Conclusion

Only in the presence of FF, CPE-matDCs produced increased regulatory and Th1-related mediators. CPE-matDCs modified T cell polarization and proliferation, however only CPE/FF-matDCs tended to enhance Treg/Th2 and Treg/Th1 ratios. This indicates an immunomodulatory role for FF during maturation of DCs in the presence of allergens and modification of the successive T cell response.

1 | INTRODUCTION

Food allergies are the result of a loss of tolerance towards harmless antigens, since these antigens are recognized by the immune system as harmful. The incidence of food allergies worldwide is still increasing, and antigen-specific immunotherapy strategies are still developing. Until now, little data is available about the induction of long-lasting unresponsiveness towards specific food allergens, although there are studies showing the induction of unresponsiveness in part of the patient population[1]. More interest is gained towards the use of adjuvants, such as pro- and prebiotics during these forms of immunotherapy to improve existing protocols.[2, 3] These adjuvants can amongst others influence the growth of beneficial bacteria in the gut, and can induce the release of beneficial mediators, such as mucosal tissue derived galectin-9 or microbial derived short-chain fatty acids (SCFAs). An example of such a prebiotic mixture is a mixture composed of short-and long-chain fructo-oligosaccharides (scFOS/lcFOS). Previous studies have indicated that a dietary intervention with fructo-oligosaccharides in humans indeed can increase the numbers of beneficial bifidobacteria.[4, 5]

These prebiotics can exert their functions as a result of fermentation by gut bacteria and they also become available systemically.[6-9] Traces of fructo-oligosaccharides and human milk oligosaccharides (HMOs) were retrieved in plasma and urine of newborns, indicating that several hundred milligrams can circulate in the blood daily.[8-10] Previous research indicated that indeed a prebiotic mixture of short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS) was able to affect dendritic cells (DCs) of healthy donors directly.[11] DCs play an important role in the development of food allergies. They are one of the most important antigen-presenting cells (APCs) which are involved in priming the innate and adaptive immune responses. DCs can take up antigens, process them into peptides and present them via the major histocompatibility complex (MHC) to T cells.[12] This antigen presentation to T cells is an important aspect in the development of food allergies. Under certain conditions, these T cells can develop into Th2 cells, which produce cytokines such as IL4, IL-5 and IL-13 and in turn can induce class-switching of B cells.[12] These B cells start to produce antigen-specific IgE, which can opsonize the high-affinity IgE receptor FcεRI on basophils and mast cells.[13] Upon a second encounter of the specific allergen, the IgE molecules on basophils and mast cells can crosslink, which will result in the subsequent degranulation of mast cells and basophils. Mediators such as histamines are released, which will eventually lead to clinical symptoms, such as, swelling of the oral mucosa, urticaria, wheezing and even fatal anaphylaxis.

To interfere with allergies, DCs would be an interesting target and oligosaccharides may alter their function. When antigen presentation to T cells is influenced this might affect the development of allergies, by helping to prevent sensitization or restore tolerance by the induction of for instance Tregs. In addition to Tregs, Th1 cells can counterbalance over-

reactive Th2 cells. The previously used scGOS/lcFOS mixture however may pose risks in patients with severe cow's milk allergy, since scGOS is produced from cow's milk derived lactose.[14] Therefore, the prebiotic mixture scFOS/lcFOS might be an interesting alternative. The goal of this study was therefore to determine whether scFOS/lcFOS can affect antigen presentation of DCs to T cells. This was investigated with use of a peanut-specific autologous DC-T cell assay, using cells of peanut-allergic patients.

2 | MATERIALS & METHODS

2.1 | Study population

From the clinic of Dermatology/Allergology at the University Medical Center Utrecht, 15 peanut-allergic patients were recruited. Patients were considered peanut-allergic based on their history, a positive skin prick test (SPT) and double-blind placebo-controlled food challenge (DBPCFC). Patients between 18-65 years of age with a type I allergic reaction to peanut and a positive DBPCFC were included in the study, whereas pregnant patients or patients using systemic immunosuppressants, such as prednisone were excluded. Demographic data, SPT, Müller score and the eliciting dose (ED) as established by DBPCFC are described previously.[2] Before patients were enrolled in the study, they gave written informed consent. The study was reviewed and approved by the Ethics Committee of the University Medical Center Utrecht (NL51606.041.15).

2.2 | PBMC isolation

100mL blood of peanut-allergic patients was withdrawn in heparin tubes. Blood was diluted 1:1 with 1x PBS (Sigma-Aldrich, the Netherlands), followed by isolation of PBMCs using a Ficoll-Paque PLUS (GE Healthcare Life Sciences, Sweden) density gradient centrifugation (2400rpm, 20 min).

2.3 | Isolation of monocytes and $CD4^+$ T cells

After PBMC isolation, cells were resuspended in MACS buffer (1x PBS, 2% FCS (Biowest, France) 0.1 mM EDTA (Thermofisher Scientific)). First, monocytes were isolated from this PBMC fraction by positive selection using MACS beads and a magnetic cell separator (CD14 Microbeads, Miltenyi Biotec, Germany). The flowthrough, containing $CD14^-$ cells, was used to isolate $CD4^+$ cells using negative selection (Miltenyi Biotec). $CD4^+$ T cells were frozen using freezing medium (90% FCS and 10% DMSO (Sigma-Aldrich, the Netherlands)), and kept at -80 ^{0}C until further use.

2.4 | Culture of monocyte-derived dendritic cells (moDCs)

$CD14^+$ monocytes isolated from the PBMC fraction were brought to a concentration of 1 x 10^6 cells/mL, and were differentiated to immature DCs (imDCs) with 20 ng/mL IL-4 and

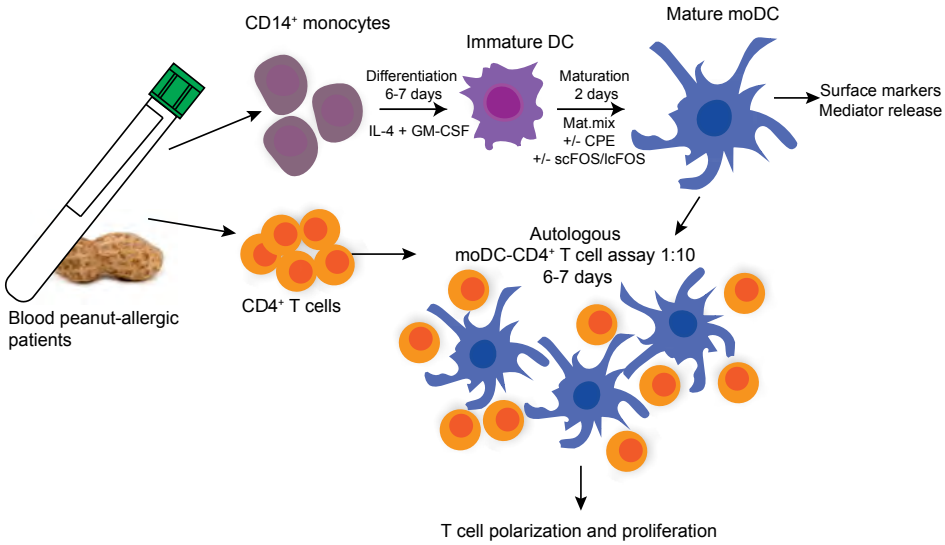


FIGURE 1 | Experimental setup autologous DC-T cell assay
CD14+ monocytes and CD4+ T cells were isolated from blood of peanut-allergic patients by MACS. CD4+ T cells were frozen until use. CD14+ monocytes were differentiated with IL-4 and GM-CSF. After 6-7 days, immature DCs were matured for 2 days with a maturation mix (IL-6, TNF-α, IL-1β and PGE2) either or not combined with 10 μg/mL peanut extract (CPE) and/or scFOS/lcFOS (FF). Mature moDCs were harvested and used in an autologous DC-CD4+ T cell assay in a ratio of 1:10. CD4+ T cells were stained with CellTrace Violet and DCs and T cells were co-cultured for 6-7 days, after which T cell polarization and proliferation were determined.

20 ng/mL GM-CSF (both Miltenyi Biotec) in XVIVO 15 medium (Lonza, Switzerland) in a 24-wells plate (Corning Costar, Sigma Aldrich) for 6-7 days. Medium was refreshed at day 3 or 4. After differentiation, imDCs were matured for 2 days with a Th2-skewing maturation mix (10 ng/mL IL-1β, 10 ng/mL TNF-α, 10 ng/mL IL-6 and 1μg/mL PGE2 (Pfizer, USA)), in presence of crude peanut-extract (CPE, 10 μg/mL) and/or scFOS/lcFOS (0.05% w/v (0.5g/L)) (scFOS: Raftilose P95, Orafti, lcFOS: Raftiline HP, Orafti) (Figure 1). The CPE extract was prepared as previously described.[15] In five patients, the time-point of addition of scFOS/lcFOS was investigated. scFOS/lcFOS was added during the differentiation and CPE-maturation (d), or only during CPE-maturation. No differences were observed between Treg, Th2 and Th1 polarization (Figure S1A) and proliferation (Figure S1B). DCs were analyzed on expression of surface markers with use of flow cytometry. DCs were identified as $CD14^{-}$ $CD11c^{+}$ $HLA\text{-}DR^{+}$. Maturation of DCs was assessed by markers CD80 (BD), CD83 (Biolegend) and CD86 (BD). Dendritic cell-specific ICAM-3-grabbing non integrin (DC-SIGN, BD) which can recognize mannose type carbohydrates was measured as possible receptor for the oligosaccharides. In addition, tolerogenic markers such as Immunoglobulin-like transcript-3 and 4 (ILT3 and ILT4, both Biolegend), PD-L1 (eBioscience) and DC2 marker OX40L were measured. After two days of maturation,

supernatant was harvested and analyzed on the production of IL-10, IL-12, IFNα, IFNβ, CCL17 and IP-10 by means of a luminex assay. CCL17 was below the detection limit of the luminex assay. IL-12 production by DCs can promote the development of Th1 cells, while the production of type-I IFNs by DCs can increase upon dectin-1-mediated signaling by, for example, β-glucans.[16] IL-10 is a cytokine involved in tolerance and IP-10 is a Th1 related chemokine.

2.5 | Autologous DC-T cell assay

DCs were harvested from the wells, and washed two times with PBS. Simultaneously, $CD4^+$ T cells from the same patient were thawed and washed two times with PBS. $CD4^+$ T cells were stained with CellTrace Violet according to the manufacturer's protocol (Invitrogen, USA). DCs and T cells were combined in a round-bottom 96-wells plate (Corning Costar, Sigma Aldrich) in a ratio of 1:10 in triplo. As positive control, T cells were stimulated with 1:10 CD3/28 beads (Thermo Fisher Scientific) (Figure 1).
After six days, supernatant was pooled for analysis with ELISA, and T cell polarization (Th1 ($CD4^+CXCR3^+$), Th2 ($CD4^+CRTH2^+$) and Treg ($CD4^+CD25^+CD127^-FoxP3^+$)) and proliferation were measured with flow cytometry. Flow cytometry data was analyzed with FACS DIVA software (BD).

2.6 | ELISA

In the supernatant of the DC-T cell co-culture, IFN-γ, TNF-α, IL-10 and IL-13 were measured by ELISA, according to the manufacturer's protocol (Ready-Set-Go, eBioscience). IFN-γ, TNF-α and IL-10 levels were below the detection limit of the ELISA. Data analysis was performed by 4-parametric curve fitting using Microplate Manager Software.

2.7 | Statistics

Normally distributed data was analyzed by one-way ANOVA for repeated measures, with Bonferroni *post hoc* test. If the data was not distributed normally, the data was first transformed (LOG). Data were analyzed with Graphpad Prism 7.0.

3 | RESULTS

3.1 | CPE exposed matDCs induce IL-13 secretion by autologous T cells

To first determine the ability of DCs to present allergens to T cells, differentiated DCs of five patients were either treated with medium (control), CPE (10 μg/mL), or matured with the cytokine maturation mix (IL-6, TNF-α, IL-1β and PGE2) either or not combined with CPE. CD83 (Figure 2A) and CD86 (Figure 2B) maturation marker expression was determined. In contrast to immature moDCs, DCs matured with the maturation mix

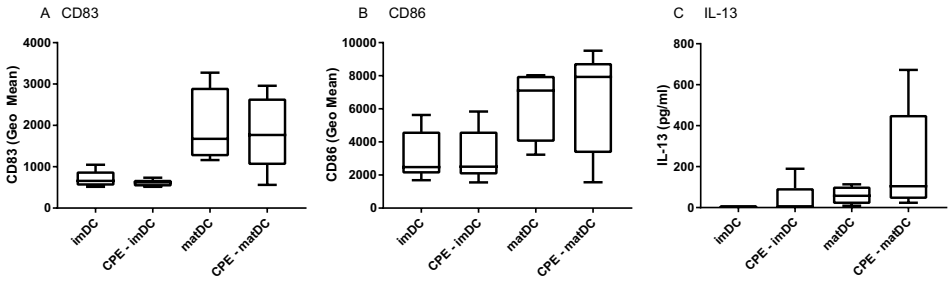


FIGURE 2 | Pilot experiment to determine maturation status of DCs
In five patients, DCs were differentiated with IL-4 and GM-CSF, followed by incubation for two days with either medium (control), 10 µg/mL peanut extract (CPE), or were matured with the maturation mix, or maturation mix in combination with CPE. Expression of CD83 **(A)**, CD86 **(B)** and IL-13 production by $CD4^+$ T cells in the following autologous DC-T cell assay **(C)** were determined. Data are presented as box plot summary, n = 5.

(matDC) showed increased expression of maturation markers CD83 and CD86 in presence or absence of CPE. In addition, IL-13 production by the autologous DC-T cell co-culture was measured after six days of co-culture between matDCs and autologous $CD4^+$ T cells. IL-13 production was absent in imDCs, and low in DCs that were solely exposed to CPE or the cytokine maturation mix. IL-13 production was highest when DCs were matured with the cytokine mix and were simultaneously loaded with CPE, indicating that maturation of the DCs in necessary to induce an autologous allergen-specific T cell response. Based on these results, further autologous DC-T cell cultures were performed with DCs matured with the cytokine maturation mix.

3.2 | Exposure of DCs to scFOS/lcFOS or CPE during maturation does not alter the surface expression of maturation markers

To determine whether the NDO mixture scFOS/lcFOS or the peanut extract were able to alter the maturation status of DCs, DCs were either left untreated (imDC), matured (matDC), or matured in the presence of scFOS/lcFOS (FF), CPE or a combination of CPE and scFOS/lcFOS (CPE/FF) (Figure 3). DCs matured with the cytokine mix showed increased expression of maturation surface markers CD80 (Figure 3A), CD83 (Figure 3B) and CD86 (Figure 3C). Expression of DC-SIGN, a C-type lectin receptor surface molecule that recognizes mannose type carbohydrates and is mainly expressed on immature DCs[17, 18] was decreased (Figure 3D). ILT3 and ILT4 are membrane proteins with cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and are involved in the down-regulation of immune responses. ILT3 and ILT4 are mainly expressed by tolerogenic DCs.[19, 20] ILT3 expression was decreased after maturation (Figure 3E) while ILT4 showed a tendency to increase (Figure 3F), which was significant in the presence of CPE during maturation. Expression of OX40L on DCs can activate cells expressing OX40, such as activated T cells.[21]

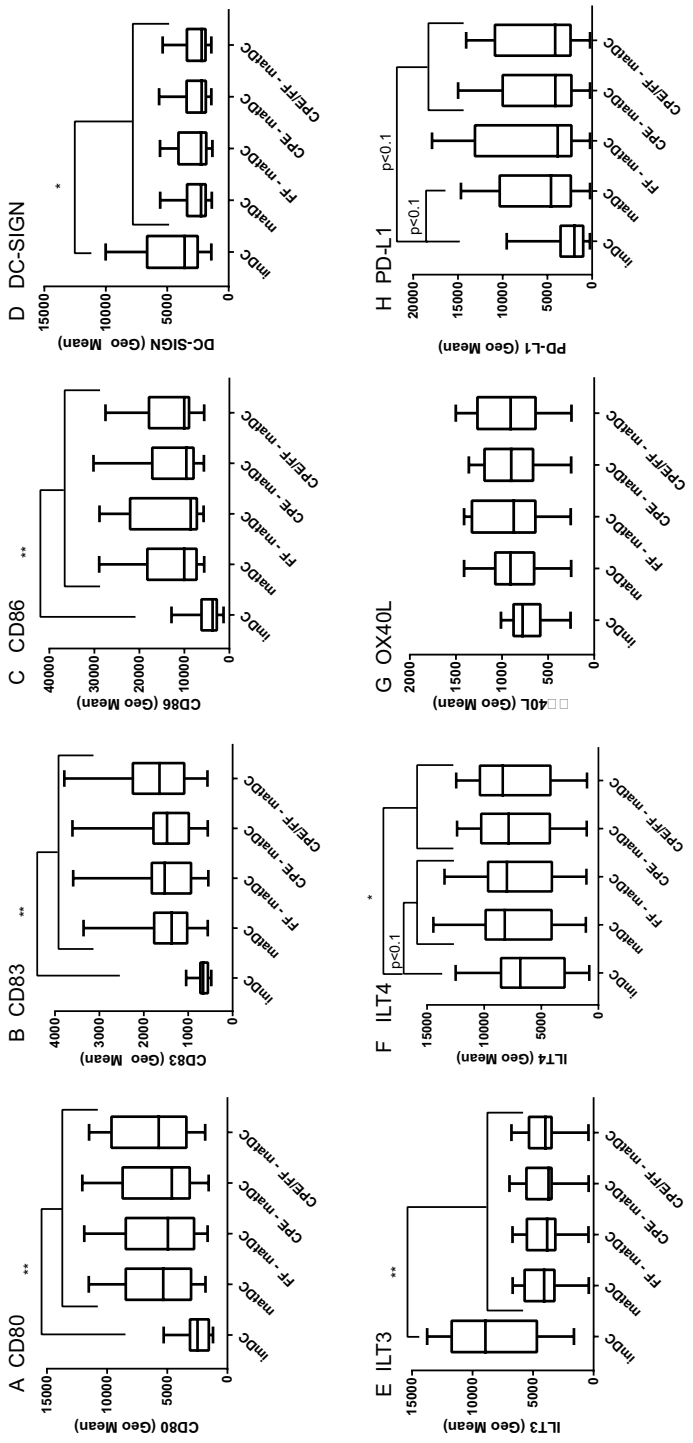


FIGURE 3 | Maturation status of DCs in the presence of CPE and scFOS/lcFOS
Compared to a control sample treated with medium, DCs matured with the maturation mix containing IL-6, TNF-α, IL-1β and PGE2 showed an increase in expression of CD80 **(A)**, CD83 **(B)**, CD86 **(C)**, while DC-SIGN **(D)** and ILT3 **(E)** decreased. An increased trend was observed for ILT4 **(F)** and PD-L1 **(H)**. No changes were observed for OX40L expression **(G)**. CPE and scFOS/lcFOS (FF) did not alter expression levels of matured DCs. Data are presented as box plot summary. $*P < 0.05$, $**P < 0.01$, n = 12.

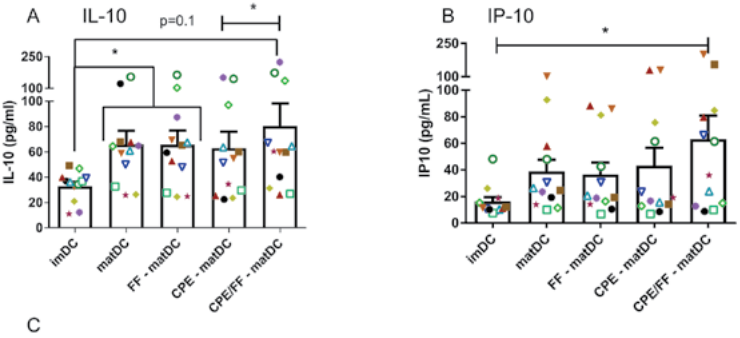
Enhanced expression of OX40L induces a Th2 cell-promoting effector DC (DC2).[22] In the experiments performed, no changes were observed in OX40L expression (Figure 3G). Lastly, PD-L1 expression of DCs was assessed, and showed a tendency to increase when DCs were matured, which was not affected by CPE and/or FF (Figure 3H). PD-L1 plays a role in proliferation of T cells and cytokine production; blocking PD-L1 resulted in enhanced T cell proliferation and an increase in IL-10 and IFN-γ.[23] In conclusion, maturation of DCs resulted in increased expression of CD80, CD83, CD86, ILT4 and PD-L1, while decreasing DC-SIGN and ILT3. Exposure to FF or CPE did not further alter expression levels. This indicates that CPE and FF do not alter the maturation status of the DCs, or expression of tolerogenic or Th2-related expression markers.

3.3 | Production of IL-10 and IP-10 is enhanced in DCs matured with CPE and FF

After maturation, mediator production by DCs was analyzed (Figure 4). IL-10 concentrations increased significantly in matDCs in presence or absence of FF (Figure 4A), which might indicate a more tolerogenic DC phenotype. CPE exposure during DC maturation did not result in significantly increased IL-10 levels in DCs compared to matDCs. However, combining CPE with FF during maturation showed a significant increase in IL-10 compared to CPE alone, but was not different from IL-10 production by matDCs. In addition to an increase in IL-10, also production of Th1-associated chemokine IP-10 by DCs increased when DCs were matured with the combined presence of CPE and FF (Figure 4B), while maturation alone with or without only CPE or FF did not result in significant increases in IP-10. No differences were observed in IL-12, IFN-α and IFN-β secretion in the supernatant (Figure 4C). This indicates that the combined exposure of DCs to both CPE and FF during maturation induces a phenotype with both regulatory and Th1-like features.

3.4 | CPE/FF-matDCs tend to enhance Treg/Th1 and Treg/Th2 ratios in an autologous DC-T cell co-culture

Matured DCs were co-cultured for 6-7 days with autologous $CD4^+$ T cells. In this DC-T cell co-culture, IL-13 cytokine production, T cell polarization and proliferation were measured (Figure 5). T cell subsets were gated as indicated (Figure 5A). Similar as described in Figure 1, only T cells that were co-cultured with CPE-matured DCs secreted significant amounts of IL-13 compared to the controls (Figure 5B). Th2 ($CD4^+CRTH2^+$) and Th1 ($CD4^+CXCR3^+$) polarization were not significantly affected (Figure 5C, D). Treg polarization increased significantly in the presence of CPE-matDCs or CPE/FF-matDCs compared to matDCs without CPE (+/- FF) (Figure 5E). Overall, only DCs matured with both CPE and FF tended to increase Treg/Th1 and Treg/Th2 ratios (Figure 5F, G), indicating that combined exposure of DCs to CPE and FF during maturation can favor the T cell balance towards a more tolerogenic phenotype.



C

Mediator	Control ± SEM (pg/mL)	Mat.DC ± SEM (pg/mL)	Mat.DC + FF ± SEM (pg/mL)	Mat.DC + CPE ± SEM (pg/mL)	Mat. DC + CPE & FF ± SEM (pg/mL)
IL-12	9 ± 0.3	12 ± 1	12 ± 1	13 ± 1	13 ± 1
IFN-α	4 ± 0.4	6 ± 1	6 ± 1	7 ± 1	7 ± 1
IFN-β	10 ± 0.5	12 ± 0.5	11 ± 0.6	11 ± 0.7	11 ± 0.5

FIGURE 4 | Cytokine production by DCs
After maturation, supernatant of DCs was harvested and analyzed by luminex. IL-10 produced by DCs increased when DCs were exposed to the maturation mix **(A)**. Although CPE and scFOS/lcFOS (FF) did not further enhance IL-10, the combination of CPE and FF increased IL-10 concentrations compared to CPE alone. IP-10 production by DCs increased after combined maturation with CPE and FF **(B)**. No effects of maturation, CPE and FF were observed for IL-12, IFN-α and IFN-β **(C)**. Data are presented as mean ± SEM, $*P < 0.05$, $**P < 0.01$, n = 12.

3.5 | T cell proliferation is enhanced when co-cultured with matDCs in the presence of CPE and FF

In addition to T cell polarization, the corresponding T cell proliferation was assessed (Figure 6). Cell subsets were gated as in Figure 5, and the proliferation of these subsets was measured as indicated. Controls included were T cells co-cultured with imDCs (Figure 6A), which did not show any proliferation as expected (since these DCs were not matured and do not present CPE to the T cells) and T cells stimulated with aCD3/28 beads (Figure 6B) to induce a full proliferative response. In addition, representative T cell proliferation of one patient was shown for T cells co-cultured with matDCs (Figure 6C) or T cells co-culture with CPE/FF-matDCs (Figure 6D). Similar to Treg polarization, Treg proliferation was also significantly enhanced after co-culture with CPE/FF-matDCs compared to matDCs (+/- FF), while for CPE-matDCs a similar trend was shown (Figure 6E). In addition, these CPE/FF-matDCs tended to induce Th1 proliferation compared to matDCs (Figure 6F). Th2 proliferation (Figure 6G) tended to increase compared to matDCs (+/- FF) in the presence of CPE-matDCs, and was significantly enhanced by co-culturing T cells with CPE/FF-matDCs. Furthermore, Survival of T cells assessed by the live lymphocyte gate was not affected by the exposure to CPE or FF (data not shown).These proliferation studies indicate that the T cells of peanut-allergic patients are proliferating upon exposure to CPE presented by their DCs, which establishes that the model is indeed allergen-specific. Exposure of DCs to FF during CPE-specific maturation was not

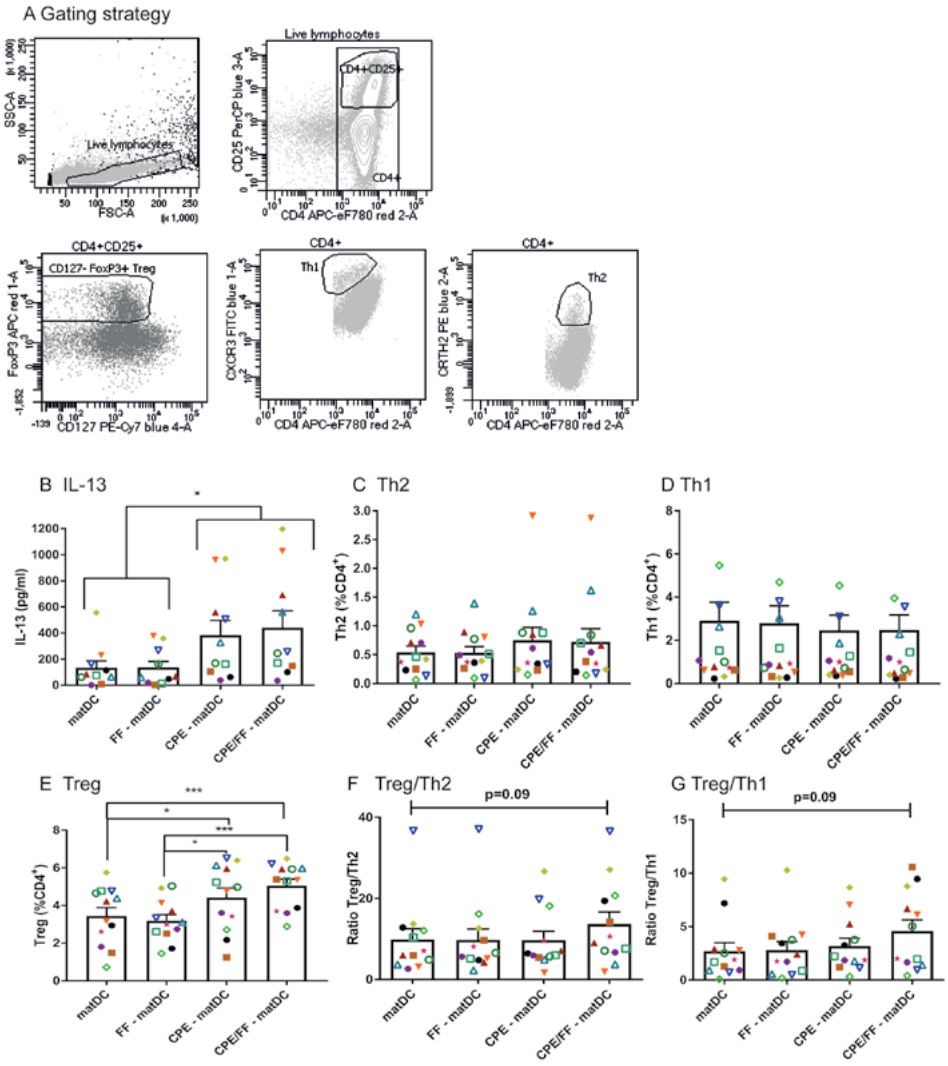


FIGURE 5 | T cell polarization
Matured DCs were co-cultured for 6-7 days with autologous $CD4^+$ T cells. Populations were identified as indicated **(A)**. Only T cells co-cultured with CPE-matured DCs secreted IL-13 **(B)**. Th2 polarization **(C)** and Th1 polarization **(D)** were not significantly affected. Treg polarization increased in the presence of CPE-matDCs +/- FF compared to matDCs and FF-matDCs **(E)**. CPE/FF-matDCs showed a trend in increasing the ratios between Treg/Th1 and Treg/Th2 **(F, G)**. Data are presented as mean ± SEM, $*P < 0.05$, $**P < 0.01$, $*** P < 0.001$. n = 10 for IL-13 production (for two patients, IL-13 production was below the detection limit of the ELISA), n = 12 for T cell polarization.

able to reduce Th2 proliferation, although also Treg and Th1 proliferation were enhanced or showed a tendency to enhanced proliferation.

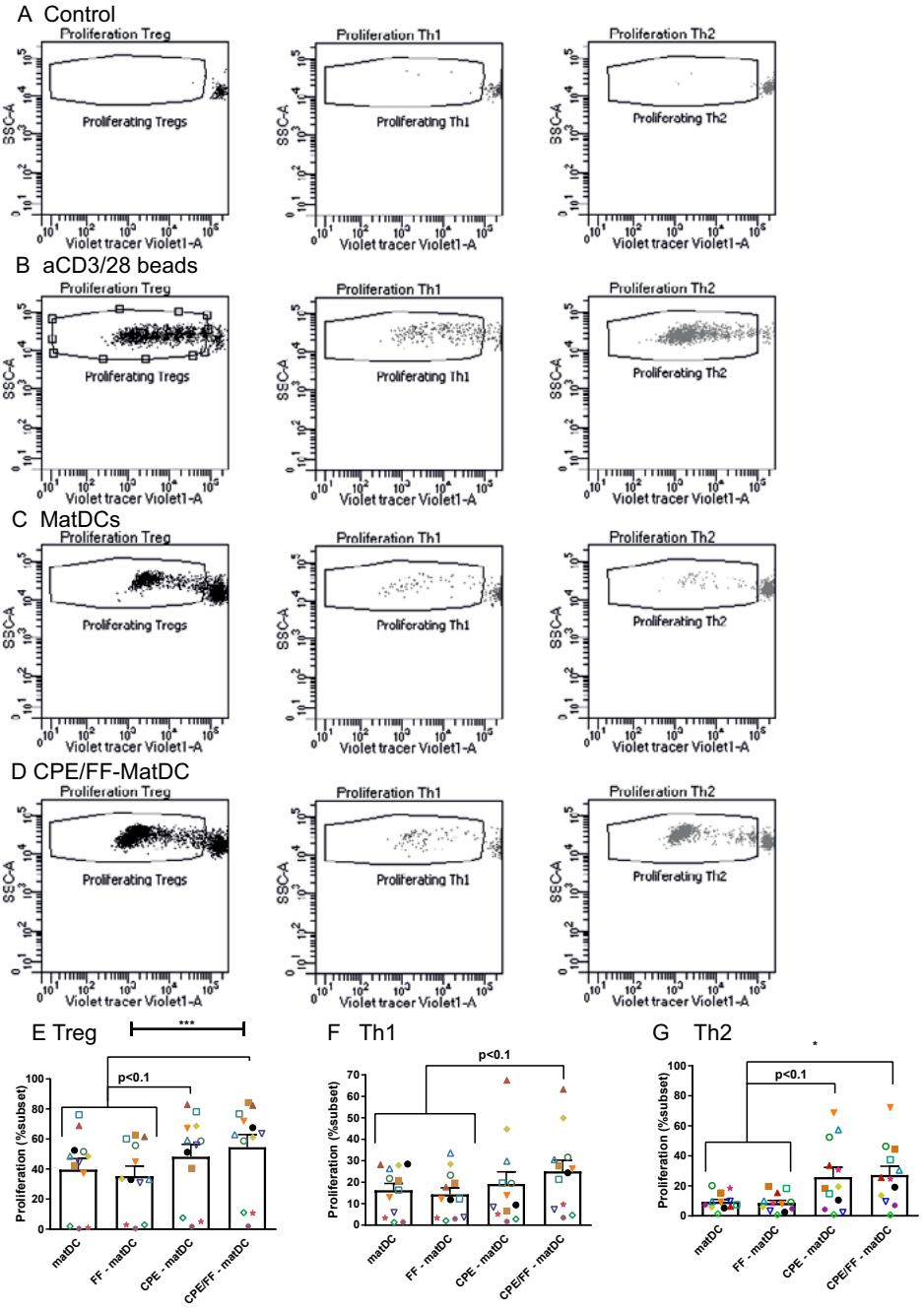


FIGURE 6 | T cell proliferation
Proliferation of the different subsets was identified as indicated **(A-D)**. Treg proliferation was enhanced after co-culture with CPE/FF-matDCs, and exposure to CPE during DC maturation showed a similar trend **(E)**. >>>

Legend Figure 6 Continued
An increased trend in enhanced Th1 proliferation was also observed in T cells co-cultured with CPE/FF-matDCs **(F)**. Th2 proliferation was significantly enhanced by co-culture with CPE/FF-matDCs, while co-culture with CPE-matDCs showed the same trend **(G)**. Data are presented as mean ± SEM, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n = 12.

4 | DISCUSSION

The direct influence of prebiotic mixture scFOS/lcFOS on maturation of immature moDCs and subsequent antigen presentation to autologous $CD4^+$ T cells in a peanut-specific manner was investigated in this study. imDCs exposed to CPE were not able to induce a peanut-specific response by autologous T cells (hallmarked by IL-13), whereas DC maturation with a cytokine mix and combined exposure to CPE did induce an allergen-specific response. In this allergen specific model, the most important findings are that the combination of exposure to CPE and FF during DC maturation can induce a more tolerogenic/DC1 phenotype hallmarked by increased production of IL-10 and IP-10 in presence of FF. Although IL-10 production was already increased in matured DCs, FF significantly further increased the IL-10 production of CPE-matDCs. However, the question remains whether this increase in IL-10 by FF has further functional effects on the outcome of the T cell response. For future experiments, it would be interesting to neutralize IL-10 to determine if IL-10 released by FF exposed CPE-matDCs is involved in Treg polarization and proliferation. In the subsequent DC-$CD4^+$ T cell co-culture, an allergen-specific response in terms of IL-13 and enhanced Th2 proliferation could be induced in the presence of CPE-matured DCs, which could not be decreased by the presence of FF. However, overall T cell proliferation (Treg and Th1) increased significantly compared to matDCs when DCs were exposed to both CPE and FF during maturation, and a similar trend was observed for CPE exposure alone. In addition, there was a tendency of increased ratios of Treg/Th1 and Treg/Th2 only when DCs were exposed to both CPE and FF during maturation, indicating that FF during allergen-specific maturation of DCs might tip the balance toward a more regulatory T cell response, even though Th2-specific proliferation and cytokines were not affected.

To the best of our knowledge this is the first autologous DC-T cell co-culture using cells of peanut-allergic patients that shows a peanut-specific response in terms of allergen-specific IL-13 secretion. Although other studies have been performed with similar co-culture models using peanut,[24] these studies focused more on proliferation rather than polarization and cytokine production. Intracellular production of IL-4 could be measured, but no differences were observed between healthy donors and allergic patients. Other co-culture studies focusing on food allergens in DC-T cell co-cultures were also unable to measure cytokines such as IL-13.[25] This might be related to the maturation of the dendritic cells.

In these studies, DCs were pulsed only with food allergen, without the addition of maturation cytokines. When the concentration of allergen-extract is high enough, an induction in maturation status of the DCs was observed, however this could not be translated into an allergen-specific response by T cells. In contrast, a study using inhalant allergens was also able to induce an allergen-specific response in T cells of poly-sensitized individuals. In the latter study, also allergen-pulsing was combined with maturation of moDCs by TNF-α and IL-1β.[26] In contrast to a recent study where direct effects of a different prebiotic mixture scGOS/lcFOS were observed,[11] we were not able to find immunomodulatory effects of scFOS/lcFOS when directly added during maturation of the DCs without further maturation with cytokines. This can be related to the concentration of scFOS/lcFOS used in this study which was lower and approaches physiological circulating levels.[8-10] A limitation of this study was that due to the limited amount of patient material, we were not able to compare scFOS/lcFOS with the previously studied scGOS/lcFOS.

It appears that the combination of scFOS/lcFOS and CPE during DC maturation plays an important role in the outcome of parameters involved in T cell polarization and proliferation. Where scFOS/lcFOS alone did not modulate the DC maturation status, the induction of IL-10 in DCs matured with the combination of FF and CPE indicates that a more tolerogenic phenotype arises. This coincided with the observation that in the subsequent DC-T cell assay, T cell polarization was more prone to a Treg phenotype in terms of a tendency towards increased Treg/Th2 and Treg/Th1 ratios. Although cytokine production of IL-10 and IFN-γ by T cells could not be detected, this might indicate a supportive role for scFOS/lcFOS in phenotypic changes of DC maturation. The allergen-specific response in terms of IL-13 production and Th2 cell polarization and proliferation remained unaltered when exposing matDCs to FF and CPE compared to CPE maturation alone. However, a skewing towards a more tolerogenic phenotype of the DCs and similar tendency for the subsequent T cell balance suggests immunomodulatory properties for FF via modulation of DC function. These results with regard to a tolerogenic phenotype correspond to recent results in a murine cow's milk allergy model, where oral immunotherapy (OIT) was combined with a diet of scFOS/lcFOS. An increase in the Treg population in the mesenteric lymph nodes (MLN) was observed and the efficacy of OIT was enhanced by scFOS/lcFOS.[27] In addition, a different oligosaccharide mixture scGOS/lcFOS also was able to induce Tregs in an allogeneic DC-T cell culture with healthy donors.[11]

Unfortunately, the direct mechanism of action for scFOS/lcFOS has not been elucidated. Type-I IFN production by matDCs was not increased after exposure to scFOS/lcFOS. This indicates that scFOS/lcFOS does not act via the dectin-1 receptor, since activation of this receptor is known to enhance type-I IFN release by DCs. Also previous research in our group indicated that neutralizing dectin-1 did not affect the immunomodulatory effects of FF.[28] In a previous study, a possible mechanism via TLR4 was proposed, where a high dosage of scGOS/lcFOS induced IL-10 release by moDCs which was abrogated in the presence of

a TLR4 antagonist.[11] Unfortunately, due to restrictions in patient material, we were not able to study this in the current peanut-specific model. A recent study postulated that the observed effects of these oligosaccharides via TLR4 most likely can be ascribed to contamination of the samples with LPS.[29] Although previous effects of scGOS/lcFOS resulted from a solution with endotoxin levels lower than 3 ng/mL,[11] the study of Perdijk *et al* indicated that levels of >0.5 EU/mL (1 EU~0.1 ng endotoxin) already can influence DCs and can result in tolerogenic DCs. The oligosaccharide mixture that was used in this study was also analysed for endotoxin content by means of a LAL-assay. The measured endotoxin content of these mixtures was <0.07 EU/mL in the concentration used and therefore we believe that the observations in this study cannot be ascribed to endotoxin contamination.

In conclusion, in a peanut-specific autologous DC-T cell co-culture assay, the addition of scFOS/lcFOS during maturation of CPE-exposed DCs enhanced regulatory and Th1-related mediator release and subsequently tended to increase the Treg/Th2 and Treg/Th1 ratios. scFOS/lcFOS might alter the DC phenotype during maturation in presence of the allergen and in this way adapt the successive T cell response. Therefore, scFOS/lcFOS might be an interesting candidate to support immunotherapeutic approaches for food allergy.

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SUPPLEMENTAL FIGURE

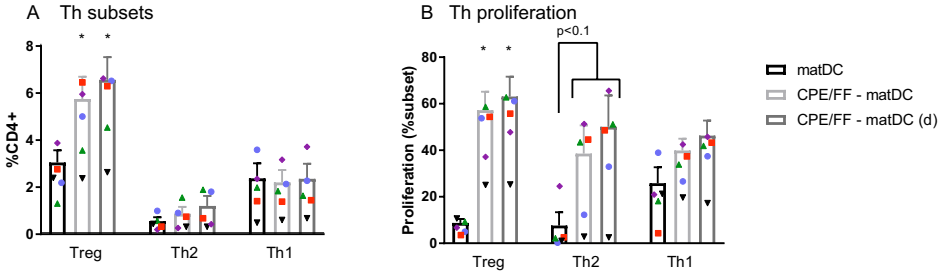

FIGURE S1 | Effect of scFOS/lcFOS during maturation or differentiation and maturation (d)
scFOS/lcFOS (FF) was added to DCs during the 2 days of maturation in the presence of CPE or during differentiation (d) and maturation in combination with CPE. No differences were found in time-point of addition of scFOS/lcFOS on T cell polarization **(A)**, or T cell proliferation **(B)**. *$P < 0.05$, n = 5.

CHAPTER 8

General discussion

This thesis describes molecular and cellular aspects of peanut allergy and the immunomodulatory effects of non-digestible oligosaccharides that might help in supporting allergen-specific immunotherapy protocols which are currently in development. These immunomodulatory effects were investigated in *in vitro* models using blood of peanut-allergic patients.

1 | MAIN FINDINGS OF THE THESIS

I - Diagnosis of peanut allergy and hereditary factors

- In addition to 2S proteins Ara h 2 and 6 as predictors for peanut allergy, isoform Ara h 7.0201 may be of additional value in diagnosing peanut allergy, since it has unique epitopes compared to Ara h 2 and 6 - **Chapter 2**
- Univariate, rather than multivariate relations between HLA-DQB1, HLA-B and peanut-allergy were found in a peanut-allergic cohort. Univariate significance was lost after correction for multiple comparisons, indicating the need for a larger cohort. High-resolution genotyping as performed in this study is recommended, as associations are easily missed with low-resolution typing. Since there are indications that the genetic background in terms of HLA might be one of the factors contributing to the development of peanut allergy, further investigation is needed - **Chapter 3**

II - Immunotherapeutic approaches

- Specific oral immunotherapy (OIT) for peanut, egg or cow's milk allergy thus far results in good desensitization, although sustained unresponsiveness remains difficult to achieve. To improve safety and efficacy and to achieve sustained unresponsiveness, using (adapted) allergens, tolerogenic peptides or IgE neutralizing antibodies may be essential. In addition, dietary components such as non-digestible oligosaccharides (NDOs) should be explored as adjunct therapy in terms of their immunomodulatory capacities - **Chapter 4**

III – Immune modulation by non-digestible oligosaccharides

- In a co-culture model mimicking the crosstalk between intestinal epithelial cells (IECs) and immune cells, combined exposure of IECs to short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) or scFOS/lcFOS and CpG oligodeoxynucleotides (ODN) enhances Th1 polarization and production of IFN-γ and IL-10 by anti-CD3/28 or peanut-stimulated PBMCs on top of the effects of CpG ODN alone. In addition, IL-13 production was decreased. Under peanut-specific stimulation conditions, especially scFOS/lcFOS was effective and decreased expression of Th2-related cell surface marker CRTH2. This indicates a potential role for scFOS/

lcFOS in skewing the immune response away from the allergic phenotype and in this way it may contribute to enhancing the efficacy of OIT - **Chapter 5**

- Exposure of whole blood of peanut-allergic patients to scGOS/lcFOS, scFOS/lcFOS or indirect NDO mediator galectin-9 can contribute to decreased IgE-mediated basophil degranulation. For peanut-specific basophil degranulation, especially scFOS/lcFOS and galectin-9 were effective. Therefore, scFOS/lcFOS might be useful in reducing allergic symptoms and improving safety of allergen-specific immunotherapy - **Chapter 6**
- DCs matured with both crude peanut extract (CPE) and scFOS/lcFOS produced increased Th1-related mediators and the combination of CPE with scFOS/lcFOS significantly enhanced IL-10 secretion by DCs when compared to CPE alone. In addition, there was a tendency in enhanced Treg/Th2 and Treg/Th1 ratios in the subsequent DC-T cell co-culture of DCs exposed to both CPE and scFOS/lcFOS. This indicates an immunomodulatory role for scFOS/lcFOS during DC maturation for modification of the T cell response which may contribute to enhancing immunotherapy efficacy - **Chapter 7**

2 | DISCUSSION

2.1 | Immunotherapy: victories and obstacles

The most important focus of this thesis was the investigation of the immunomodulatory effects of non-digestible oligosaccharide (NDO) mixtures scGOS/lcFOS and scFOS/lcFOS. Based on previous studies, it was hypothesized that they might be able to contribute to the safety and efficacy of developing approaches for allergen-specific immunotherapy which was further studied using several *in vitro* assays. **Chapter 4** discusses the state of the art of immunotherapeutic approaches available for food allergy including peanut allergy. Over the past 4 years new progress has been developed in terms of optimizing existing protocols with or without the use of adjunct therapies. Oral immunotherapy (OIT) so far remains the most investigated and effective method to induce desensitization for peanut allergens, while sublingual immunotherapy (SLIT) is less efficacious in terms of desensitization.[1-3] With SLIT, lower allergen doses are provided during immunotherapy and consequently desensitisation is achieved for lower doses of the allergen. Published clinical results of studies using epicutaneous immunotherapy (EPIT) show promising results in terms of desensitization and efficacy, and results of multiple studies have to indicate whether this approach is favorable for tolerance induction.[4-6] Both SLIT and EPIT are safer with lower incidence and less severe side effects than OIT. SLIT and EPIT are also safer than subcutaneous immunotherapy (SCIT) which is currently not recommended for food-allergen immunotherapy.[3] However, alternatives for food-allergen SCIT have been

investigated, by replacing food-extracts with recombinant hypoallergenic allergens.[7] These different immunotherapeutic approaches can induce desensitization via different routes. OIT can activate DCs located in the gut mucosa, whereas the allergens in SLIT are primarily interacting with Langerhans cells located in the oral mucosa.[8, 9] Lastly, the mechanism of EPIT relies on the capture of the allergens by Langerhans cells located in the epidermis. These differences in localization of antigen uptake might also play an important role in the efficacy of each approach, although their exact contribution is not completely understood. New trials in the area of peanut allergy have been performed, and a recent article summarized the observations demonstrated in OIT for peanut allergy over the past 8.5 years in a cohort of 270 patients with a mean age of 8 years in North Texas.[10] The standard protocol consisted of a rush dose escalation therapy, followed by maintenance therapy for at least three years. One of the side effects observed for OIT was the incidence of eosinophilic esophagitis (EoE), which was reported in approximately 14% during dose escalation and in one patient during the maintenance phase. More severe adverse events that required epinephrine were reported by 23% of the patients, whereas minor adverse events were reported by 58%. 79% of the patients completed the desensitization protocol and were able to maintain desensitized with daily maintenance, whereas 14 patients (6.5%) passed a challenge to determine sustained unresponsiveness (6000 mg peanut). These patients had followed maintenance therapy for at least three years, followed by 30 days avoidance of peanut and subsequent challenge to evaluate whether the treatment effect was lasting.[10] Although progress has been created in terms of enhancing the effect of desensitization, long-term unresponsiveness is still difficult to achieve and studies with long-term follow ups are scarce. Moreover, major obstacles are the frequent and sometimes severe side effects especially of OIT. These and other studies have indicated that younger age is an important parameter of successfully completing OIT and inducing sustained unresponsiveness, and that peanut-specific IgE was decreased in desensitized subjects.[10-12]

2.2 | Adjunct therapies

As described in **chapter 4,** strategies in adjunct therapy are being developed to reduce side effects and to improve the safety and efficacy of OIT for peanut and other food allergies. One example to enhance safety is the pre-treatment of patients with anti-IgE antibody omalizumab,[11, 13-15] which has shown to support the fast escalation of allergen dosing and was able to decrease side effects during desensitization in among others peanut and cow's milk OIT.[13-15] The mechanism of action of omalizumab is related to its ability to bind free IgE, hereby decreasing free IgE in the serum. Moreover, whereas binding of IgE to FcεRI is stabilizing its plasmamembrane expression, after IgE depletion FcεRI is internalized to the cytosolic compartment. [16, 17] Although omalizumab can improve safety during the rush protocol, it was unable to eliminate the occurrence of EoE. Other molecules with IgE-binding capacities such as ligelizumab,[18] quilizumab,[19] or DARPin[20] have not been studied yet in food allergies,[3] but might have similar capacities as omalizumab.

A recent study showed sustained unresponsiveness four years after oral immunotherapy (OIT) for peanut allergy in combination with the use of probiotics (*Lactobacillus rhamnosus).*[21, 22] 67% of the participants were able to continue consuming peanut after treatment, compared to 4% of the placebo group that did not receive OIT and probiotics. The downside of this trial was that there was no control group that solely underwent OIT without the addition of the probiotic, which would be necessary to establish the efficacy of the probiotics on top of OIT alone. Clinical outcomes of this study revealed smaller wheals after skin prick tests (SPT) and higher ratios of specific IgG4:IgE than the placebo group. IgG4 antibodies have the capacity to act as blocking antibodies,[23] and are often measured as parameter for tolerance induction. Although more of these combined therapies should be performed with adequate control groups, this might indicate that there indeed is potential for immunomodulatory agents to support immunotherapeutic approaches for food allergy. Especially when comparing it to OIT studies mentioned previously, where only 6.5% of patients could maintain sustained unresponsiveness.[10]

2.3 | Immunomodulatory effects of non-digestible oligosaccharides

Instead of using probiotics as adjunct therapy for peanut-OIT, adjunct therapy with the non-digestible oligosaccharides described in this thesis might contribute to overcome the current limitations of OIT or other immunotherapeutic approaches. These NDOs can influence the gut microbiota since they can enhance the growth of selective groups of bacteria, such as lactobacilli and bifidobacteria, and have been shown to reduce the incidence of atopic dermatitis in children at risk.[24-27] Using prebiotics instead of probiotics would lead to more long-lasting changes in the gut microbiota, rather than inducing a short-lived increase of specific bacteria that possibly cannot be sustained. In addition, NDOs have shown to exert immunomodulatory effects in *in vitro* models, which are described in **chapters 5-7**. As the gut plays an important role in discriminating between harmful and harmless antigens and oral tolerance induction, IECs may contribute to tolerance induction via its cross-talk with underlying innate and adaptive immune cells. In the peanut-specific co-culture, scFOS/lcFOS was able to enhance the CpG ODN (model for bacterial DNA) mediated cytokine release in terms of increased IFN-γ and IL-10 production by basolateral peanut-activated PBMCs, while lowering IL-13 production. In addition, combined exposure to scFOS/lcFOS and CpG ODN reduced the expression of CRTH2 on PBMCs, a marker that is expressed by cell subsets involved in allergy (e.g. Th2, basophils). These results suggest that NDOs in combination with a bacterial component can drive away from the allergic phenotype and in this way can support immunotherapeutic approaches for food allergy.

As both NDO mixtures can also become available in the bloodstream they might have direct effects on the immune system. Two *in vitro* models focused on direct effects of NDOs on both basophils and DCs. DCs play an important role in antigen presentation to immune

subsets and immune polarization, and the clinical symptoms of allergy are mediated by among others basophils. **Chapter 6** described an assay where whole blood of peanut-allergic patients was pre-incubated with oligosaccharides or their indirect mediator galectin-9 which can be secreted by IECs. Previous research indicated that serum galectin-9 levels were enhanced in whey-allergic mice receiving a diet of scFOS/lcFOS during immunotherapy.[28] Furthermore, infants affected with IgE- mediated atopic dermatitis that were supplemented with scGOS/lcFOS and *B.breve* experienced enhanced serum galectin-9 levels while their symptoms improved.[29-31] This is an indication that such a type of dietary intervention can modulate galectin-9 levels in human. As galectin-9 is known to be able to bind IgE and hereby prevents the formation of IgE-allergen complexes,[32] a diet of NDOs can indirectly influence basophil degranulation by enhancing galectin-9 levels, as was shown in a study in mice. [28]

Pre-incubation with scFOS/lcFOS or indirect induced mediator galectin-9 reduced peanut-specific basophil degranulation. Plasma of these pre-incubated samples showed increased levels of endogenous galectin-9 (for scGOS/lcFOS pre-treatment) and increased MCP-1 levels, although there was no correlation with the reduction in basophil degranulation. In addition, no correlation was found between the reduction in basophil degranulation and expression of FcεRI on the basophil cell surface, indicating that the mechanism behind this decreased degranulation remains to be elucidated in more detail. However, this reduction in basophil degranulation is an indication that these NDOs can contribute to the safety of immunotherapeutic approaches. In **chapter 7**, the role of exposure of scFOS/lcFOS to DCs was investigated, since DCs are important for antigen presentation to T cells. moDCs were differentiated and during maturation, they were exposed to crude peanut-extract (CPE) with or without scFOS/lcFOS. After maturation, DCs matured in the combined presence of CPE and scFOS/lcFOS produced more regulatory (IL-10) and Th1-related (IP-10) mediators. In addition, this type of response was found in the subsequent autologous DC-T cell co-culture, where there was even a detectable tendency in increased Treg/Th2 and Treg/Th1 ratios. In addition, T cell proliferation was enhanced. These results also indicated that exposure to scFOS/lcFOS during DC maturation can alter the successive T cell response, and therefore might be able to enhance the efficacy of immunotherapeutic approaches.

2.4 | Mechanism of action of non-digestible oligosaccharides

The mechanism of action of the studied non-digestible oligosaccharides is not completely understood yet and most studies focus on the effects of NDOs on the microbiome as a prebiotic effect. It is known that NDOs can reduce the risk of infections as they have the ability to prevent the attachment of harmful bacteria to the cell surface.[33] In addition, several studies have indicated that these NDOs can influence the gut microbiota, since they can be fermented by a selected group of bacteria. NDOs are fermented into short-chain fatty acids (SCFAs), such as butyrate, acetate and propionate.[34] These SCFAs in turn can directly

affect immune cells and IECs via G-protein coupled receptors.[35] For improving safety and efficacy of immunotherapeutic approaches, both the effects of NDOs on the microbiome as well as the effects on other immune cells are of equal importance. However, instead of focussing on the microbiome related effects, this thesis focused on the effects of NDOs on IECs and immune cells by themselves, as less is known about their intrinsic immunomodulatory abilities. The results of **chapter 5** indicated that NDOs can collaborate with bacterial components to skew the immune responses further away from the allergic phenotype, while **chapter 6** and **7** discussed more direct effects of NDOs on basophil degranulation and DCs.

Even though some mechanisms of action of NDOs as mentioned above are known, it is yet unclear if and which receptors are involved. Receptors that are able to bind carbohydrate-like structures might be optional candidates. Examples of such receptors are for instance C-type lectin receptors. During this study, we investigated C-type lectin receptor dectin-1, which is a glycan receptor that is among others expressed on IECs and thus may be involved in the actions of NDOs (**chapter 5**). In the co-culture model, no contribution to the immunomodulatory effects of NDOs was observed. Although no contribution of dectin-1 was found, another suggestion to investigate in the future would be the contribution of galectins as receptors for NDOs. In this thesis we focused more on galectins as mediators, as neutralization of galectin-9 in the IEC-PBMC co-culture model abrogated the additional IFN-γ production (**chapter 5**). However, these galectins might also be able to bind NDOs directly.

Previously NDOs also showed to exert their immunomodulatory effect by enhancing CpG ODN induced galectin-9 release by IECs, which was important for the increase in IFN-γ in the aspecific co-culture model.[36] In **chapter 5** it is shown that also in the peanut-specific model, galectin-9 is involved in IFN-γ upregulation. Next to IECs, other cell subsets are able to express galectin-9. In the BAT model of **chapter 6**, pre-incubation of whole blood with scGOS/lcFOS increased endogenous galectin-9 levels. Higher levels of recombinant galectin-9 decreased both IgE- and peanut-specific basophil degranulation. In addition to decreased degranulation, galectin-9 can contribute to immune polarisation and the induction of Tregs[37] and in mice a reduction in degranulation was found.[28] Since galectin-9 can be upregulated by a diet intervention in both human and mice with a diet intervention ofscGOS/lcFOS or scFOS/lcFOS with or without *Bifidobacterium breve,*[28-30] this indicates that these NDOs can contribute to both safety (reduction basophil degranulation) and efficacy (induction of Tregs) when used during immunotherapy.

2.5 | Personalized medicine

In *in vitro* models using patient samples, variation in responses occurs frequently. It is normal that there is a natural variation between baseline responses of patients, but this can pose difficulties when interpreting data. In the co-culture model of **chapter 5**, often variation

was observed in the magnitude of the cytokine response of the PBMCs. However, all patients followed the same pattern of increased IFN-γ and IL-10 production by PBMCs when IECs were exposed to the combination of oligosaccharides and CpG ODN. The differences in patient variation were more pronounced in the other two models. In the BAT assay (**chapter 6**), pre-incubation of blood samples of some patients only showed a dampening effect of one of the oligosaccharide mixtures on degranulation, whereas some blood samples were responsive to both mixtures, and others were unaffected by the oligosaccharide mixtures. We were not able to elucidate why there is variety between patients on this level, but it might be receptor-related or the influence of surrounding cells in the blood. In the DC-T cell model (**chapter 7**), these differences were observed as well. Due to limitation in patient material, only scFOS/lcFOS was studied in this assay. The mediator release and the T cell polarization varied in terms of magnitude between patients, and some patients showed better effects in improving the balance between Treg over Th2 or Th1 cells. Since there may be different responses in patients for scGOS/lcFOS or scFOS/lcFOS which was shown most clearly in the BAT, it would be interesting to first determine which prebiotic mixture is most beneficial for a specific patient when used as diet intervention during immunotherapy. Further research needs to be performed to elucidate what influences the working mechanism of NDOs in patients. If this is known, a test can be developed where a small sample of patient's material (blood or faeces) is first screened to determine which NDO mixture would work best in this specific patient. In this way, NDO adjunct therapy may be personalized for patients receiving peanut OIT.

In addition to the observation that cells of individual patients respond differently to these oligosaccharides, also differences were observed between males and females, mainly in the BAT assay. Females tended to respond better in terms of decrease in basophil degranulation. This might be hormone-related, but it would be interesting to determine in trials whether females are indeed more prone to successful outcomes using dietary interventions with NDOs. As most animal models for food allergy in combination with dietary intervention most often use solely male or female mice, no data is yet available from these studies that could give an indication for the observed differences in the human *in vitro* models.

More differences arise between patients with regard to the specific peanut allergens to which they are sensitized and/or allergic to. In **chapter 2**, we described that most patients are sensitized to 2S albumins. The patients of our cohort were most often sensitized to Ara h 2, 6 and (recombinant) isoform Ara h 7.0201, but also monosensitization occurred. These different backgrounds in terms of allergenicity might also be a factor that can influence the outcome of OIT. Although OIT is performed with peanut extract, it might be possible that patients that are allergic to multiple peanut allergens have more difficulty in re-establishing tolerance. A study in patients allergic to grass and olive pollen in Spain indicated that molecular diagnosis with specific IgE can help in selecting the most optimal form of immunotherapy for patients.[38] Another factor that has been investigated for its contribution

to the development of food allergy is the hereditary component in terms of HLA, which is discussed in **chapter 3**. Several studies have described the association between peanut allergy and HLA.[39-41] However, in our study, we were not able to find an association between HLA and the development of peanut allergy, although this might be related to the sample size of the allergic cohort and the correction for multiple comparisons. In addition, multiple other factors such as environment, the microbiota and homeostasis in the gastrointestinal mucosa play a role in the development of food allergy.[42]

2.6 | Flashforward: a human study with NDOs as dietary adjunct therapy

Since most immunotherapeutic approaches have focused on OIT for peanut, a future study with NDOs as dietary adjunct therapy for OIT could be considered, as OIT has proven to be thus far the most effective approach to induce desensitization in peanut-allergic patients but safety and efficacy need to be further improved.[1] Taken together data on safety and efficacy of the latest trials in peanut allergy,[13, 43] we propose the pre-treatment of patients with subcutaneous omalizumab or a different IgE-binding mediator for approximately 12 weeks according to European dosing guidelines[13] to be able to enhance dose-escalation and to reduce side-effects. In the meantime, patients can also be provided with scFOS/lcFOS as dietary supplement, since this mixture of NDOs so far appeared to have the best immunomodulatory effects in our *in vitro* models under allergen-specific conditions, and no tests are yet available to determine which NDO mixture would work best as adjunct therapy per patient. To monitor the gut microbiota composition, it would be advisable to take a faeces sample before enrolment and during the study, to determine the possible changes throughout therapy. Throughout the OIT, daily administration of scFOS/lcFOS and omalizumab is continued to support the gut microbiota and enhance efficacy of OIT while reducing side effects. After the rush dose escalation phase and the slower dose escalation phase, omalizumab is discontinued,[13] while the scFOS/lcFOS intervention is continued and patients enter the maintenance phase for approximately 6-10 months.[13, 22] Sustained unresponsiveness will be determined by means of a DBPCFC after at least four weeks of avoidance of peanut. When patients pass the DBPCFC, the maintenance dosing is continued to ensure lasting unresponsiveness.

2.7 | Summarizing proposed effects of NDO adjunct therapy on safety and efficacy of OIT

Figure 1 shows an overview of the proposed immunotherapeutic approach described and the proposed mechanisms of action to facilitate OIT safety and efficacy. We postulated a mechanism where the NDOs exert several favorable immunomodulatory functions in the gut, as well as to support (oral) immunotherapy directly. Hereto, we link the results obtained in **chapter 5-7** and translate them into this proposed OIT setting. As a result of a dietary intervention with, for example, scFOS/lcFOS during OIT, secretion of galectin-9 by among

others the intestinal epithelium may increase and circulate through the bloodstream where it can bind IgE (similar as the omalizumab pre-treatment). Galectin-9 can be measured in the plasma of the patient and will help to reduce mast cell or basophil degranulation during OIT. scGOS/lcFOS might also be able to induce the production of galectin-9 by immune cells in the blood in a different manner (**chapter 6**). This increase in galectin-9 for OIT in combination with scFOS/lcFOS was previously found in a murine model for cow's milk allergy and indeed resulted in decreased mast cell degranulation.[28] Since it is not known whether the upregulated concentrations of galectin-9 in humans are sufficient to capture all free IgE or opsonize IgE bound to effector cells, we still recommend the use of omalizumab, to ensure both safety and efficacy. To assess the effects of the NDO mixture over the effects of omalizumab, one group of patients should undergo OIT with omalizumab, while the other group in addition receives a NDO mixture. The exact mechanism by which both scGOS/lcFOS and scFOS/lcFOS can contribute to decreased basophil degranulation remains to be further investigated, by determining involved receptors and mechanisms.
The overall immunomodulatory effects of NDOs (in combination with a bacterial component) reach further than only the induction of galectin-9. Overall, we observed that in the different models the immune response was skewing away from the allergic phenotype in terms of mediator release by several cell subsets such as DCs and PBMCs, and a similar tendency was observed at the level of T cell polarization and proliferation. The OIT design as proposed should therefore enable Th2-related cytokines to decrease, whereas the regulatory and Th1-related cytokines and other mediators will increase. In addition, presentation of peanut-allergen by DCs will also be more favoured towards a regulatory phenotype, which might help in re-establishing tolerance to peanut during OIT and contribute to long lasting tolerance, since the Treg polarization and proliferation is enhanced.

2.8 | Concluding remarks

The results of this thesis provide valuable insights into the immunomodulatory effects of non-digestible oligosaccharides, as they, for example, skew the immune function towards a more regulatory and Th1-like phenotype in an *in vitro* environment. In addition, NDOs can reduce basophil degranulation and modify antigen presentation by DCs in the presence of peanut-extract. We hypothesize that adjunct therapy with NDOs in combination with immunotherapy might be the next step in safely inducing sustained unresponsiveness in peanut-allergic patients, as they may contribute to enhancing the safety and efficacy of OIT for peanut allergy and most likely also other allergies.

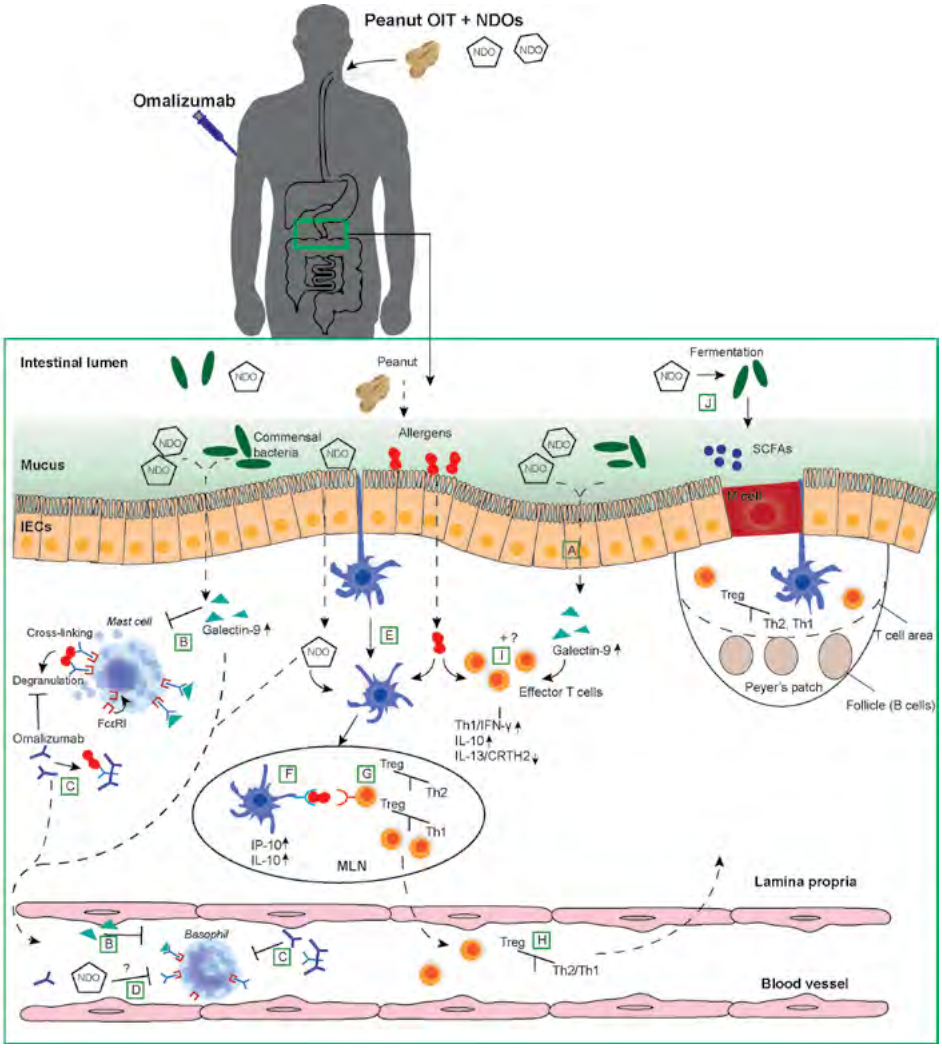

FIGURE 1 | Proposed mechanisms in intestinal mucosa during NDO adjunct therapy for peanut OIT
Dietary intervention during OIT results in increased secretion of galectin-9 by IECs **(A)**. Galectin-9 can inhibit mast cell and basophil degranulation by preventing the binding of peanut allergen to IgE **(B)**. Omalizumab can inhibit basophil and mast cell degranulation by preventing the binding of IgE to FcεRI. IgE-free FcεRI internalizes **(C)**. NDOs can also reduce degranulation themselves by currently unknown mechanisms **(D)**. DCs in the lamina propria can come into contact with NDOs and allergens and maturate **(E)**. DCs exposed to NDOs and allergens during maturation express higher levels of IP-10 and IL-10 and may travel to the mesenteric lymph nodes (MLNs) where they present the peanut allergens to naive T cells (similar in the Peyer's patches) **(F)**. T cells exposed to these DCs matured with allergen and NDOs are more prone to develop into Treg over Th1 or Th2 effector cells **(G)**. These T cells travel via the bloodstream and home back in the intestinal lamina propria **(H)**. Effector T cells in the lamina propria are exposed to galectin-9 and allergen. Partly via galectin-9 and partly via an unknown mechanism, the T cell response is skewed away from the allergic phenotype **(I)**. NDOs are fermented by bacteria into SCFAs such as butyrate and propionate which are known to contribute to generation of Treg and immune homeostasis **(J)**.

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APPENDICES

NEDERLANDSE SAMENVATTING

DANKWOORD

LIST OF PUBLICATIONS

CURRICULUM VITAE

NEDERLANDSE SAMENVATTING

Algemene achtergrond

Voedselallergieën komen wereldwijd steeds meer voor, en ook in de Westerse landen stijgt het aantal personen met een voedselallergie. Bij het ontwikkelen van een voedselallergie vindt er een verstoring in het mechanisme van het immuunsysteem plaats, waardoor ongevaarlijke eiwitten gezien worden als gevaarlijk.

Op dit moment is er nog geen effectieve lange termijn genezing en moeten mensen met een voedselallergie de voeding waarvoor ze allergisch zijn proberen te vermijden. Er zijn wel immuuntherapieën in ontwikkeling, maar de lange-termijn effecten hiervan zijn nog niet goed genoeg. Het doel van dit proefschrift is om te onderzoeken of we de bestaande immunotherapie en de veiligheid daarvan kunnen verbeteren door gebruik te maken van specifieke voedingscomponenten. In deze samenvatting zal eerst meer achtergrond gegeven worden over het ontstaan van een voedselallergie, gevolgd door enig inzicht in hoe een voedselallergie vast te stellen is. Ook wordt er kort stilgestaan bij de risicofactoren die het ontstaan van een voedselallergie in de hand kan werken. Als laatste wordt de strategie besproken waarop de bestaande immunotherapie verbeterd zou kunnen worden met behulp van specifieke fermenteerbare vezels en worden de resultaten van dit proefschrift beschreven.

Ontstaan van voedselallergie

Normaal gesproken herkent het immuunsysteem van de darm een voedingseiwit als veilig (tolerantie), en voorkomt het immuunsysteem dat cellen in actie komen zodra zo'n ongevaarlijk eiwit in hun buurt komt. Als deze tolerantie wordt verbroken, kan er een voedselallergie ontstaan. De eiwitten uit voedsel waar vijandig op kan worden gereageerd, worden ook wel allergenen genoemd. Vaak voorkomende allergenen zijn bijvoorbeeld eiwitten in koemelk, ei, pinda en schaal-en schelpdieren. Het ontwikkelen van een voedselallergie kan worden verdeeld in twee fases (**hoofdstuk 1**). In de eerste fase worden voedingseiwitten die niet volledig verteerd zijn, opgenomen via het epitheel van het darmslijmvlies en komen terecht bij een specifieke groep witte bloedcellen, de dendritische cellen (DCs), die onder het epitheel aanwezig zijn. Als deze DCs een beoordelingsfout maken en het voedseleiwit beoordelen als vijandig slaan ze alarm. Hierna reageert de volgende groep witte bloedcellen op dit alarm: de T cellen. Deze cellen zijn onder andere van belang bij het aanleren van het tolerantiemechanisme, en kunnen eerdere ontmoetingen met eiwitten onthouden en zo snel in actie komen bij een nieuwe blootstelling; ze zijn als het ware de boodschappers voor de rest van het immuunsysteem. Deze T cellen geven het gevaar door aan de B cellen, die antistoffen genaamd immunoglobuline E (IgE) kunnen maken tegen de eiwitten. Deze antistoffen kunnen binden aan basofielen of mestcellen, die

zich door het hele lichaam bevinden op plaatsen waar ons lichaam in contact kan komen met allergenen; de huid, longen, darmen en de bloedbaan. Als het lichaam vervolgens nog een keer in contact komt met het (eigenlijk ongevaarlijke) allergeen (fase 2), dan bindt het allergeen aan de IgEs op de basofielen en mestcellen (cross-linking), die hierop hun inhoud uitscheiden. Deze inhoud bestaat onder andere uit histamine en andere vergelijkbare stoffen, die zorgen voor de bekende allergische symptomen zoals niezen, zwellingen, darmproblemen, huiduitslag of in het ergste geval een levensbedreigende anafylactische shock. Dit proefschrift richt zich specifiek op pinda-allergie, omdat het een allergie is die vaak blijvend van karakter is en vaak ernstige reacties kan veroorzaken.

Voedselallergie en diagnostiek

Een voedselallergie kan worden aangetoond met een huidpriktest, waarbij een kleine hoeveelheid van het voedingsmiddel onder de huid wordt geprikt. Als hierbij een allergische reactie van de huid optreedt, kan dit een indicatie zijn dat iemand inderdaad een voedselallergie heeft voor dit betreffende voedsel. Naast de huidpriktest wordt vaak nog een zogeheten voedselprovocatie uitgevoerd waarbij patiënten het voedingsmiddel waar ze op reageren moeten eten. Patiënten krijgen een steeds hogere dosis van het desbetreffende allergeen om vast te stellen bij welke hoeveelheid allergeen de symptomen tot uiting komen. Het nadeel van deze voedselprovocatie is dat het arbeidsintensief is, en ook voor de patiënt is het vervelend. Op dit moment is er een groeiende interesse voor het bepalen van specifiek IgE tegen de verschillende pinda-allergenen, en de toevoeging die deze zouden kunnen bieden op diagnostisch gebied. **Hoofdstuk 2** laat zien dat naast de pinda allergenen Ara h 2 en Ara h 6, waarvan al langer bekend is dat ze een goede voorspelbare diagnostische waarde hebben, ook Ara h 7 hiervoor een interessant allergeen zou kunnen zijn. Dit allergeen zou daarmee kunnen bijdragen aan het verbeteren van de diagnostiek voor het opsporen van pinda allergie.

Voedselallergie en genetische factoren

Het ontstaan van voedselallergieën is vaak een samenloop van verschillende factoren. De leefomgeving kan een rol spelen en uit onderzoek is gebleken dat ook erfelijke factoren betrokken kunnen zijn. In **hoofdstuk 3** is onderzoek gedaan naar de betrokkenheid van het human leukocyte antigen (HLA) systeem, wat een belangrijke rol speelt bij het presenteren van allergenen aan de rest van het immuunsysteem. Pinda allergenen worden door de eerder beschreven dendritische cellen eerst in kleinere brokstukken geknipt. Deze brokstukken worden door speciale eiwitten (de HLA eiwitten) aangeboden aan de T cellen die hierna geactiveerd kunnen worden en de boodschap van gevaar kunnen doorgeven als hun receptor het eiwit wat aangeboden wordt door het HLA herkend. Er zijn echter veel verschillende types van deze HLA eiwitten en er is maar een kleine kans dat twee willekeurige personen toevallig hetzelfde HLA type hebben. Aangezien elk type HLA eiwit

slechts bepaalde brokstukken van allergenen kan aanbieden aan witte bloed cellen, zou het HLA type wat iemand heeft mogelijk de verklaring kunnen zijn waarom de ene persoon pinda's kan eten terwijl de andere daarvoor allergisch is. In dit hoofdstuk hebben wij daarom het HLA van pinda-allergische patiënten vergeleken met dat van een controlegroep. Er werden geen significante verschillen gevonden op het gebied van HLA tussen deze groepen, maar om met zekerheid de rol van HLA in allergie vast te stellen zal de grootte van de groep van allergische patiënten moeten worden uitgebreid.

Immunotherapie voor voedselallergie

Tot nu toe is er nog geen therapie om pinda-allergische patiënten te genezen en moeten zij proberen voedingsmiddelen waarin pinda verwerkt kan zijn te vermijden. Op dit moment zijn er wel ontwikkelingen op het gebied van therapie om het immuunsysteem te herprogrammeren (immunotherapie, **hoofdstuk 4**). Het doel van deze therapie is om het immuunsysteem weer in evenwicht te krijgen, omdat je niet teveel, maar ook niet te weinig reactie wil hebben. Bij teveel reactie kunnen bijvoorbeeld allergieën zich ontwikkelen, maar bij te weinig reactie is het lichaam vatbaarder voor andere ziektes. Om ervoor te zorgen dat bij mensen met een allergie dit verstoorde evenwicht weer in balans komt, krijgen patiënten tijdens immunotherapie een steeds hogere dosis allergeen toegediend, om zo weer te proberen tolerantie van het immuunsysteem op te bouwen, en de balans terug te brengen. Vooralsnog is deze strategie nog niet effectief genoeg om langdurige tolerantie op te bouwen, vandaar dat in dit proefschrift is gekeken naar aanvullende therapieën die zouden kunnen bijdragen aan het verbeteren van de bestaande immunotherapieën.

De darm speelt een grote rol in het tolerantieproces van allergenen en als dat proces niet goed verloopt kan voedselallergie ontstaan. Bij jonge kinderen wordt de ontwikkeling van het immuunsysteem in de darm sterk beïnvloed door de samenstelling van de darmbacteriën. Specifieke voedingscomponenten zoals niet-verteerbare oligosachariden (een speciaal soort fermenteerbare vezels) kunnen effect kunnen hebben op de bacteriesamenstelling van de darmen. De vezels kunnen worden gefermenteerd door de bacteriën in de darm en er komen bepaalde stoffen vrij, die op hun beurt ook weer invloed kunnen hebben op cellen van het immuunsysteem. Zo zou een dieet interventie met specifieke fermenteerbare vezels mogelijk kunnen bijdragen aan het verbeteren van de immunotherapie. De vezels kunnen echter mogelijk ook zelf een effect hebben op het darmslijmvlies en immuuncellen in de rest van het lichaam. De vezels die gebruikt zijn in dit proefschrift zijn galacto-oligosachariden (GOS) en fructo-oligosachariden (FOS). Van deze vezels is al bekend dat ze selectief de groei van bacteriën zoals bifidobacteriën en lactobacilli kunnen stimuleren. Ook kunnen de vezels in de bloedbaan komen, waardoor ze ook directe effecten op rondzwervende cellen, zoals bijvoorbeeld basofielen kunnen uitoefenen. De directe en indirecte effecten van deze fermenteerbare vezels zijn onderzocht in dit proefschrift.

In **Hoofdstuk 5** onderzochten we deze effecten in een experiment waarin de darmomgeving werd nagebootst en het effect van de vezels via het darmepitheel werd bestudeerd. Hieruit kwam naar voren dat de vezels ervoor konden zorgen dat de allergische reactie van de T cellen werd onderdrukt en de balans van de immuunreactie weer werd verbeterd. Dit zou betekenen dat een dieet met deze fermenteerbare vezels de allergische reactie mogelijk zou kunnen onderdrukken door de werking in de darm. Het precieze mechanisme waarmee deze vezels dit doen is nog niet ontrafeld, maar het eiwit galectine-9 wat gemaakt kan worden door het darmepitheel en immuuncellen speelt hierin een belangrijke rol en de productie hiervan neemt toe in de aanwezigheid van een bacteriële component en de vezels.

Naast een indirect effect op immuuncellen via darmepitheel, kunnen de vezels ook direct beschikbaar komen in de bloedsomloop. Basofielen circuleren rond in het bloed, daarom is het directe effect van de vezels op basofiel degranulatie onderzocht in **hoofdstuk 6**. Hieruit bleek dat wanneer het bloed eerst in aanraking was gekomen met de vezels of het eerder beschreven galectine-9, dit zorgde voor verminderde basofiel activatie. Deze verlaagde activatie kan leiden tot verminderde uitscheiding van onder andere histamine en dit zou de veiligheid van immunotherapie kunnen verbeteren. Dit omdat de kans op allergische reacties, als bijwerking van de immunotherapie die immers gebruik maakt van het gecontroleerd aanbieden van het voedselallergeen, verminderd wordt.

Het aanbieden van allergenen door dendritische cellen aan de T cellen is een ander belangrijk aspect in de ontwikkeling van allergieën. Daarom is in **hoofdstuk 7** het effect van de fructo-oligosachariden (FOS) onderzocht op dendritische cellen (DCs). Deze DCs werden "allergisch" gemaakt in de aanwezigheid van pinda-eiwitten. Hierna werden ze samengevoegd met de T cellen (de boodschappers), om zo te bepalen wat de boodschappers met het ontvangen signaal gingen doen. Hieruit kwam naar voren dat de "allergische" DCs inderdaad ervoor konden zorgen dat het immuunsysteem te actief werd en er een signaal naar de boodschapper T cellen ging om het door te geven aan de andere cellen.
Deze reactie werd vergeleken met "allergische" DCs die naast pinda ook waren blootgesteld aan de fructo-oligosachariden (FOS). Deze DCs lieten zien dat er een lichte verandering was in de reactiviteit van het immuunsysteem; deze ging weer een klein beetje richting balans. Dit liet zien dat er een mogelijkheid is dat deze vezels een rol kunnen spelen bij het bijsturen van het doorgeven van de (foute) allergische immuunrespons.

Dit proefschrift laat zien dat fermenteerbare vezels inderdaad effecten kunnen hebben op witte bloedcellen, zowel indirect in een darmmodel (**hoofdstuk 5**) als direct via blootstelling aan basofielen en DCs (**hoofdstuk 6** en **7**). In **Hoofdstuk 8** wordt een algemene opzet voorgesteld voor een klinische studie, waarin een dieetinterventie met deze vezels gebruikt zou kunnen worden. Daarnaast wordt er een weergave gegeven van mogelijke onderliggende werkingsmechanismen.

Concluderend laat dit proefschrift zien dat in de gebruikte modellen het immuunsysteem weer meer in balans lijkt te komen na blootstelling aan de vezels. Op basis van deze modellen is een dieetinterventie met specifieke fermenteerbare vezels een veelbelovende strategie om de effectiviteit en veiligheid van immunotherapie te kunnen verbeteren om te zorgen dat tolerantie tegen voedselallergenen weer wordt hersteld.

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Als laatste de meest belangrijke persoon: Sjoerd. Wat ben ik de afgelopen jaren ontzettend blij geweest met jouw onvoorwaardelijke steun. Wat was het fijn dat ik altijd op jou kon rekenen en dat jij me altijd weer wist op te peppen. In de tussentijd denk ik dat ik er zelfs in geslaagd ben om te zorgen dat jij (met financiële achtergrond) begrijpt waar ik de afgelopen vier jaar mee bezig ben geweest. Ook al denk ik niet dat ik je, na je verbaasde blik in het lab, er ooit van kan overtuigen waarom we pipetpunten maar 1x gebruiken. Hoogtepunten werden altijd gevierd (vaak onder het genot van bier en bitterballen) en je zorgde ervoor dat ook de laatste loodjes op tijd af waren. Ook ben ik ontzettend blij en bevoordeeld dat ik je sinds twee jaar mijn man mag noemen en ik ben ervan overtuigd dat we nog veel bier en bitterballen-momenten samen gaan hebben.

LIST OF PUBLICATIONS

Hayen SM, Kostadinova AI, Garssen J, Otten HG, Willemsen LEM., *Novel immunotherapy approaches to food allergy*. Curr Opin Allergy Clin Immunol. 2014 Dec; 14(6):549–556

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Hayen SM, Otten HG, Overbeek SA, Knulst AC, Garssen J, Willemsen LEM, *Exposure of Intestinal Epithelial Cells to Short- and Long-Chain Fructo-Oligosaccharides and CpG Oligodeoxynucleotides Enhances Peanut-Specific T Helper 1 Polarization.* Front Immunol. 2018 May 11;9:923.

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In preparation

Hayen SM, Melchers CA, Le T-M, Garrsen J, Willemsen LEM, Knulst AC, Otten HG**,** *No association found between high-resolution HLA-B or HLA-DQB1 alleles and peanut allergy in a West-European cohort*. Submitted 2018

Hayen SM, Knulst AC, Garssen J, Otten HG, Willemsen LEM, *Direct immunomodulatory effects of fructo-oligosaccharides in a peanut-specific autologous dendritic cell and T cell co-culture*. Submitted 2018

CURRICULUM VITAE

Simone Hayen was born on February 24th 1989 in 's Hertogenbosch, the Netherlands. She graduated from secondary school in 2007 at the Elde College in Schijndel. In the same year she started the bachelor program Biotechnology at Wageningen University. After graduation in 2010, she continued with the master program Medical Biotechnology at Wageningen University. As part of this program she conducted her six month internship at the department of Nematology. Here, she investigated the immunomodulatory effects of mushroom beta-glucans. Hereafter, she did another six month internship at Genmab, and investigated the functional potential of a new antibody format for cancer therapy. After graduation in 2012, she started as research analyst in the group of Tuna Mutis at the Laboratory of Clinical Chemistry and Haematology, located at the University Medical Center Utrecht. Here, she continued with cancer research.

In May 2014, she started as a PhD student, also at the University Medical Center Utrecht, under the supervision of dr. Henny Otten and dr. Linette Willemsen (copromotors) and prof. dr. Johan Garssen and prof. dr. André Knulst (promotors). Her research was part of the NUTRALL consortium, where the Utrecht Institute of Pharmaceutical Sciences, Danone Nutricia Research, TNO, UMCU and the Institute of Risk Assessment Sciences collaborated. During her PhD, she investigated the immunomodulatory effects of non-digestible oligosaccharides. The results of this work are described in this thesis. Simone lives in Apeldoorn with her husband Sjoerd Bergsma.