INSIGHTS INTO THE ETIOLOGY, PREVENTION, AND TREATMENT OF FOOD ALLERGY

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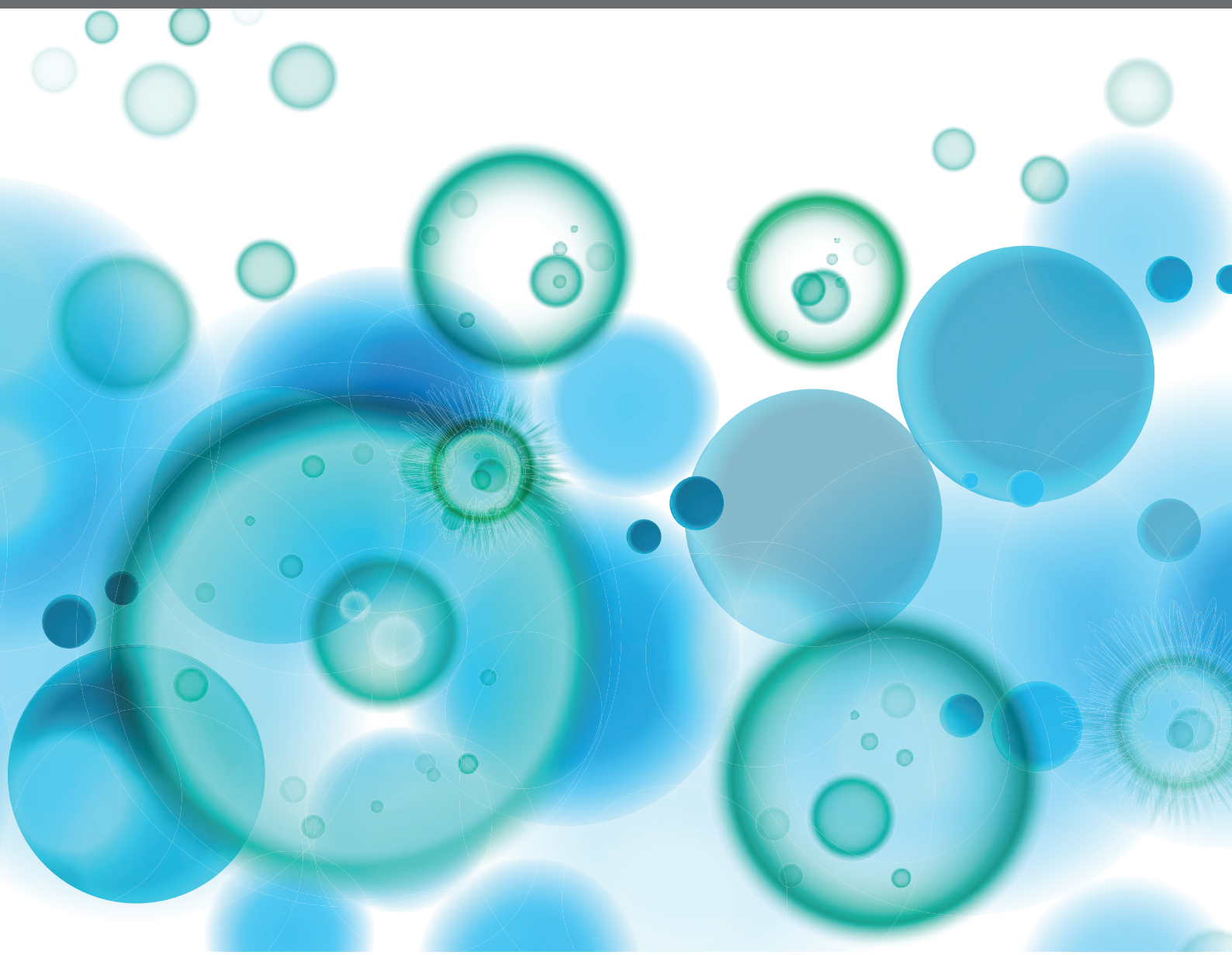

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INSIGHTS INTO THE ETIOLOGY, PREVENTION, AND TREATMENT OF FOOD ALLERGY

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EDITORIAL
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Editorial: Insights Into the Etiology, Prevention, and Treatment of Food Allergy

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Keywords: food allergy, immunology, allergic diseases, immunotherapy, food hypersensitivity

Editorial on the Research Topic

Insights Into the Etiology, Prevention, and Treatment of Food Allergy

Food allergy (FA) is a growing public health concern affecting nearly up to 6–8% in children and 3–5% in adults with life-threatening potential. The increase in prevalence of FA in recent years has implicated environmental influences related to a modern life style in disease pathogenesis. This Frontiers Research Topic was designed to provide a timely collection on animal and clinical studies on new research directions in the etiology, prevention, and treatment of FA. We received a series of 14 original and review articles on the topic that span a wide range of exciting, new areas that will inform the research community on FA.

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ETIOLOGY AND PREVENTION

FA can be defined as clinical immune responses to normally harmless food allergens. The disease is typically associated with $CD4^+$ T cells that secrete pathogenic T helper (Th) 2 cytokines and by allergen-specific immunoglobulin (Ig) E antibodies that trigger the release of inflammatory mediators from mast cells (MCs) and circulating basophils. Normally, ingestion of foods results in oral tolerance, therefore FA is thought to be a result of a failure of oral tolerance. Alterations in regulatory T cell functions, Th2 responses, microbiota, and/or food sensitizations via alternative routes, such as the skin, likely contribute to the failure of oral tolerance and to the development of FA. However, the mechanisms responsible for breakdown in oral tolerance remain poorly understood.

A series of articles were directed at the immune regulatory mechanisms in FA tolerance. Satitsuksanoa et al. reviewed the range of immune regulatory mechanisms of tolerance to FA. Ingested food proteins may cause allergic immune responses leading to FA but how these proteins become immunogenic and cause food allergies is not completely understood. Tolerance to food is mainly acquired by dendritic cells, epithelial cells in the gut, and the gut microbiome. These authors found that a subset of CD103+ DCs is capable of inducing T regulatory cells (Treg cells) that express anti-inflammatory cytokines. Anergic T cells also contribute to oral tolerance by reducing effector cells. Similar to Treg cells, regulatory B cells (Breg cells) suppress effector T cells and contribute to the immune tolerance to food allergens. Saunders et al. reviewed allergic IgE responses and found that IgE memory response has unique features that distinguish it from classical B cell memory. Burton et al. investigated tissue-specific expression of FcγRIIb, a low-affinity

IgG receptor on MCs in allergy. The authors combined flow cytometry, quantitative PCR, and immunofluorescence staining of MCs derived from the tissues of humanized mice, human skin, or fixed paraffin-embedded sections of human tissues, and demonstrated that FcγRIIb is absent from dermal MCs but expressed by MCs throughout the gastrointestinal tract. IgE-induced systemic anaphylaxis in humanized mice is strongly inhibited by antigen specific IgG; thus, these authors concluded that IgG signaling via FcγRIIb plays a role in suppressing hypersensitivity reactions. Krajewski et al. found that epigenetic regulation of MC activation during immune responses may occur via altered histone acetylation, and that exposure to dietary substances may induce epigenetic modifications that regulate MC function.

The skin is a major immunologic organ that may induce protection, sensitization, or tolerance. Epicutaneous immunotherapy (EPIT) has been proposed as an attractive strategy to actively treat FA in humans. Dioszeghy et al. demonstrates that EPIT induced tolerance in sensitized mice through the induction of Foxp3+ regulatory T cells (Tregs), especially CD62L+ Tregs. Although both Langerhans cells (LCs) and CD11b+ dermal classical dendritic cells could induce Tregs, the absence of LCs during EPIT impaired treatment efficacy, indicating that LCs play a crucial role in skin-induced tolerance.

The regulation of gut antibody responses also plays a role in regulating food allergens. In a review, Hoh and Boyd showed that the gastrointestinal mucosa is a critical environmental interface in which plasma cells and B cells are exposed to orally-ingested antigens such as food allergen proteins. It is unclear how the development of B cells and plasma cells in the gastrointestinal mucosa differs between healthy humans and those with FA, and how B cells contribute to, or are affected by, the breakdown of oral tolerance. Furthermore, the human microbiome is an essential mediator in the induction of oral tolerance or FA. In another study with germ-free mice, Schwarzer et al. showed that microbiota-induced maturation and gut-homing of MCs is a critical step for the development of symptoms of experimental FA. This new mechanistic insight into microbiota-MC-FA axis could be exploited in preventing and treating FA in humans. Hayen et al. found that intestinal epithelial exposure to short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides enhanced the CpG induced Th1 and regulatory IL-10 response in a peanut-specific co-culture model. This suggests that such oligosaccharides are candidate for dietary adjunct in allergen-specific immunotherapy.

While oral immunotherapy (OIT) has shown promise in treating food allergies, gastrointestinal symptoms are experienced by patients and few develop eosinophilic gastrointestinal disease. Wright et al. found pre-existing gastrointestinal eosinophilia is common in adults with IgE-mediated peanut allergy. Eosinophilic inflammation in these subjects may be accompanied by mild endoscopic and histologic findings. Further, Sallis et al. found that eosinophilic esophagitis (EoE) patients with food impaction were indistinguishable from other EoE patients based on their tissue eosinophil count, serum IgE levels, or the mRNA transcriptome-based a probability score for EoE [p(EoE)] but in an elegant analysis, they showed that a distinct esophageal mRNA pattern identified EoE patients with food impactions. The EoE-specific mRNA pattern indicates that impaired motility may be one underlying factor for the development of food impactions in pediatric patients.

Finally, Fujimura et al. reviewed the influences of maternal factors over offspring allergies. They reviewed how food allergens, allergen-specific immunoglobulins, cytokines, genetics, and environmental factors transferred during pregnancy or breastfeeding influence offspring allergies and how such effects may be applicable to FA. They also discussed the mechanisms by which maternal factors, including the impact of immune complexes, transforming growth factor-β, vitamin A, and regulatory T-cell responses, contribute to the induction of neonatal tolerance vs. development of allergic responses to maternally transferred allergens.

TREATMENT

There is currently no cure for FA other than strict avoidance of identified foods. Key problems in this field that remain to be resolved are our insufficient understanding of the mechanisms of the breakdown in oral tolerance in FA and our understanding of the reasons why such mechanisms have recently taken such a strong base in the human population. We are at an exciting point at which discoveries about the etiology, mechanisms, treatment, and prevention of FA are critical to guiding future areas of research and identifying therapeutic options for food allergic patients.

Currently, two forms of peanut immunotherapy, OIT and EPIT, are in Phase III clinical trials and have shown promise to desensitize patients with FA. However, there are several limitations with OIT and EPIT, such as allergic side effects, daily dosing requirements, and the infrequent outcome of long-term tolerance. Next-generation therapies for peanut allergy may overcome these limitations, particularly with adjuvanted immunotherapy. Johnson-Weaver et al. reviewed adjuvants and formulations that have shown pre-clinical efficacy in treating peanut allergies. In an analysis of 428 participants with positive food challenges, Purington et al. found those with a history of asthma, high allergen-specific IgE to total IgE ratio, and/or high values of allergen-specific IgE were also found to be at higher risk for severe reactions during food challenges. Their findings will help optimize food challenge dosing schemes in multi-food allergic, atopic patients, specifically at lower doses where the majority of reactions occur. In an analysis of a large food challenge dataset, baseline population characteristics, biomarkers, and challenge outcomes were studied to develop diagnostic criteria predictive of positive oral food challenges (OFCs) across multiple allergens in our multi-allergic cohorts (Sindher et al.). They found a history of atopic dermatitis and multiple food allergies were significantly associated with a higher

risk of positive OFCs. The majority of food-specific skin prick tests, allergen-specific IgE, and allergen-specific IgE/total IgE thresholds calculated from cumulative tolerated dose (CTD)-dependent receiver operator curves had high discrimination of OFC outcomes and participants with values above the thresholds were more likely to have positive challenges.

Overall, we strove to provide an overview of recent progress in the field of food allergy. Our 14 research articles provided novel findings in the field of environmental and genetic risk factors for food allergy, the mechanisms of food allergy: effector immune cells involved in the initiation, development and manifestations of disease, regulatory immune mechanisms, and insights into the prevention and treatment of food allergy.

AUTHOR CONTRIBUTIONS

RC and MO: wrote and contributed substantial intellectual contribution to this editorial.

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ORIGINAL RESEARCH
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Exposure of Intestinal Epithelial Cells to Short- and Long-Chain Fructo-Oligosaccharides and CpG Oligodeoxynucleotides Enhances Peanut-Specific T Helper 1 Polarization

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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) T helper 1 (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.

Keywords: allergen-specific, immunomodulation, non-digestible oligosaccharides, co-culture, epithelial cells, T cell polarization

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 toward 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 amongst 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 crosstalk 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.

MATERIALS AND METHODS

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).

PBMC Isolation

100 mL blood of peanut-allergic patients was withdrawn in heparin tubes. Blood was diluted 1:1 with 1× 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 (2,400 rpm, 20 min). PBMCs were resuspended in RPMI 1640 (Gibco, Life Technologies, the Netherlands) with 2.5% pooled human AB serum and penicillin/streptomycin (100×, Gibco, Life Technologies).

Culture of IECs 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 5 A medium (Gibco, Life Technologies, the Netherlands) supplemented with 10% heat-inactivated FCS (Gibco, Life Technologies, the Netherlands) and penicillin/streptomycin (100×, 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.

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, NY, 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, 3×10^6 PBMCs from peanut-allergic patients were either stimulated for 24 h 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**). CPE 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 1× PBS. 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 h or 6 days, culture supernatants from the basolateral compartment were stored at −20°C until cytokine measurement. After 24 h 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 h, to determine galectin-9 production by IECs. In the peanut-specific model,

TABLE 1 | Patient characteristics.

Patient	Age (years)	Sex (M/F)	Müller score[b]	SPT peanut (mm)	Subjective eliciting dose (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[a]	27	F	2	4+	4	40	12.8
N07[a]	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[a]	18	F	3	4+	300	1,000	>100
N11	32	F	2	4+	10	3,000	No data
N12[a]	27	M	3	5+	0.1	1,000	66
N13	25	M	1	3+	10	–	11.2
N14[a]	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 double-blind placebo-controlled food challenge (DBPCFC), and specific IgE per peanut-allergic subject.

[a]*Subjects that visited a second time.*

[b]*Müller score 0: symptoms oral cavity, 1: symptoms of the skins and mucous membranes 2: gastro-intestinal symptoms, 3: respiratory symptoms, 4: cardiovascular symptoms. SPT (mm). Diameter of 3 mm (3+) is considered positive. All patients underwent a DBPCFC, subjective, and objective effective doses are displayed.*

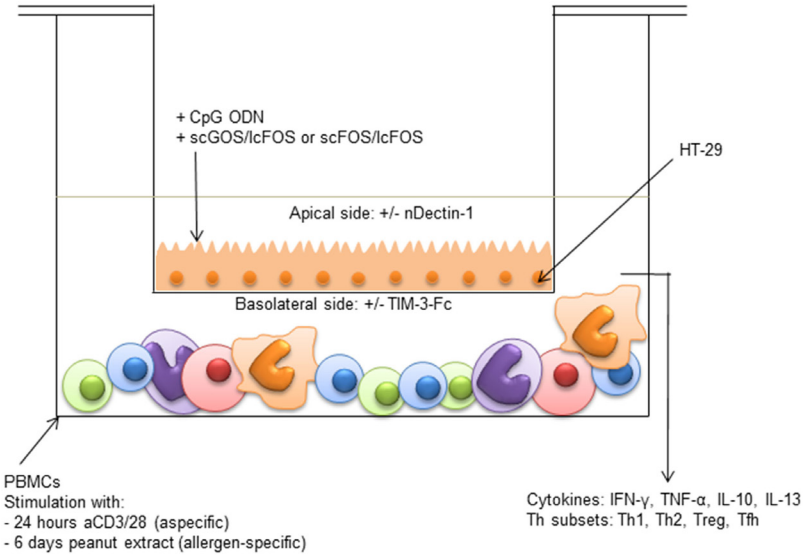

FIGURE 1 | Intestinal epithelial cells (IEC) transwell co-culture model HT-29 cells (IECs) cultured in transwells were apically exposed to synthetic CpG oligodeoxynucleotides in the presence or absence of either short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) or scFOS/lcFOS. Simultaneously, 3×10^6 peripheral mononuclear cells (PBMCs) in the basolateral compartment were either stimulated aspecifically for 24 h with aCD3/CD28, or for 6 days with peanut extract. Basolateral cytokine production and T cell polarization were measured. After 24 h, 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.

galectin-9 was measured directly in the basolateral compartment after 6 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)).

Flow Cytometric Analysis

After 24 h (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 (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).

Cytokine Production of PBMCs in the Basolateral Compartment

In the basolateral supernatants, IFN-γ, TNF-α, IL-10, and 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, 10 ng/mL, Sigma-Aldrich, the Netherlands) and ionomycin (1 µg/mL, Sigma-Aldrich, the Netherlands) for 24 h which did yield detectable levels of IL-13. Galectin-9 production was analyzed using human-galectin-9 polyclonal and biotinylated polyclonal antibodies (BioTechne). Data were analyzed by 4-parametric curve fitting using Microplate Manager software.

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 $p < 0.05$.

RESULTS

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 (6 male and 9 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 ratio's 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 (**Figures 2A,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 (**Figures 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 (**Figures 2D,H** and **3D**). 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 (**Figures 3E,F**). In the peanut-specific model, IL-13 was only detectable after restimulation of the cells with PMA and ionomycin for 24 h, 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 (**Figures 4A–C,F**). In the aspecific co-culture model, two distinct populations were observed; population 2 consisted of four patients (N04, N06, N08, and 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.

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 h (aspecific model) and 6 days (peanut-specific model) (**Figures 5A,C**). Also, IEC-released galectin-9 was measured in the aspecific co-culture model

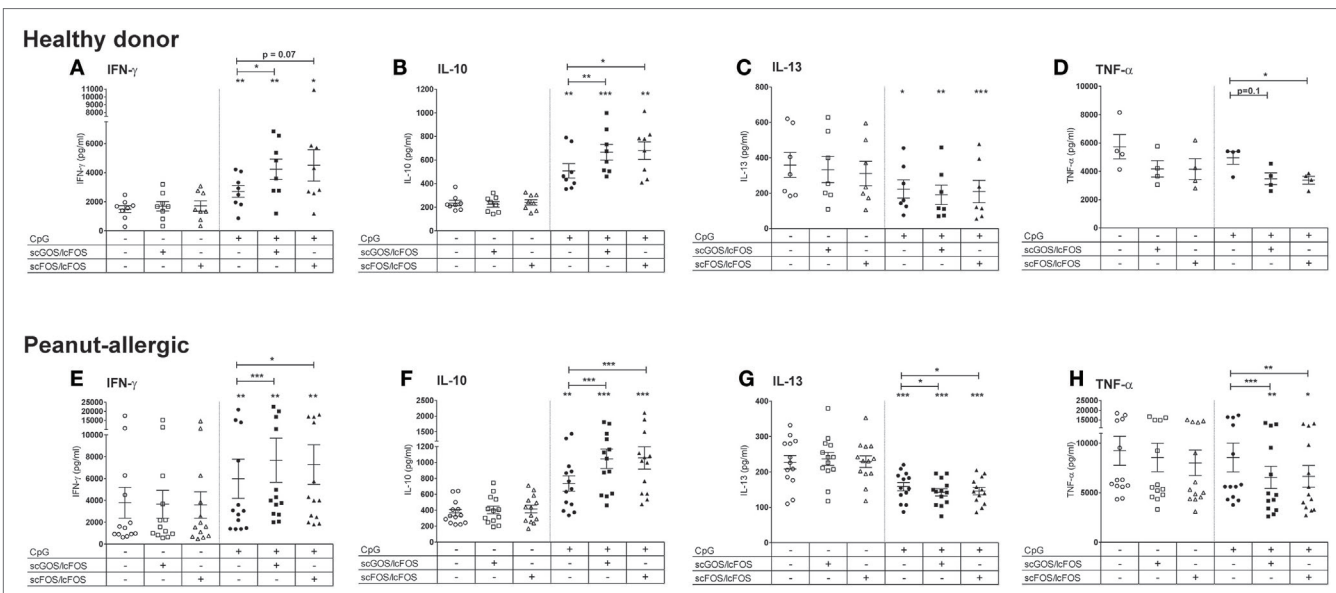

FIGURE 2 | Comparison aspecific co-culture model between healthy donors and peanut-allergic donors. Apical exposure of intestinal epithelial cells (IECs) to non-digestible oligosaccharides in absence of CpG oligodeoxynucleotides (ODN) did not affect cytokine concentrations in the basolateral compartment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

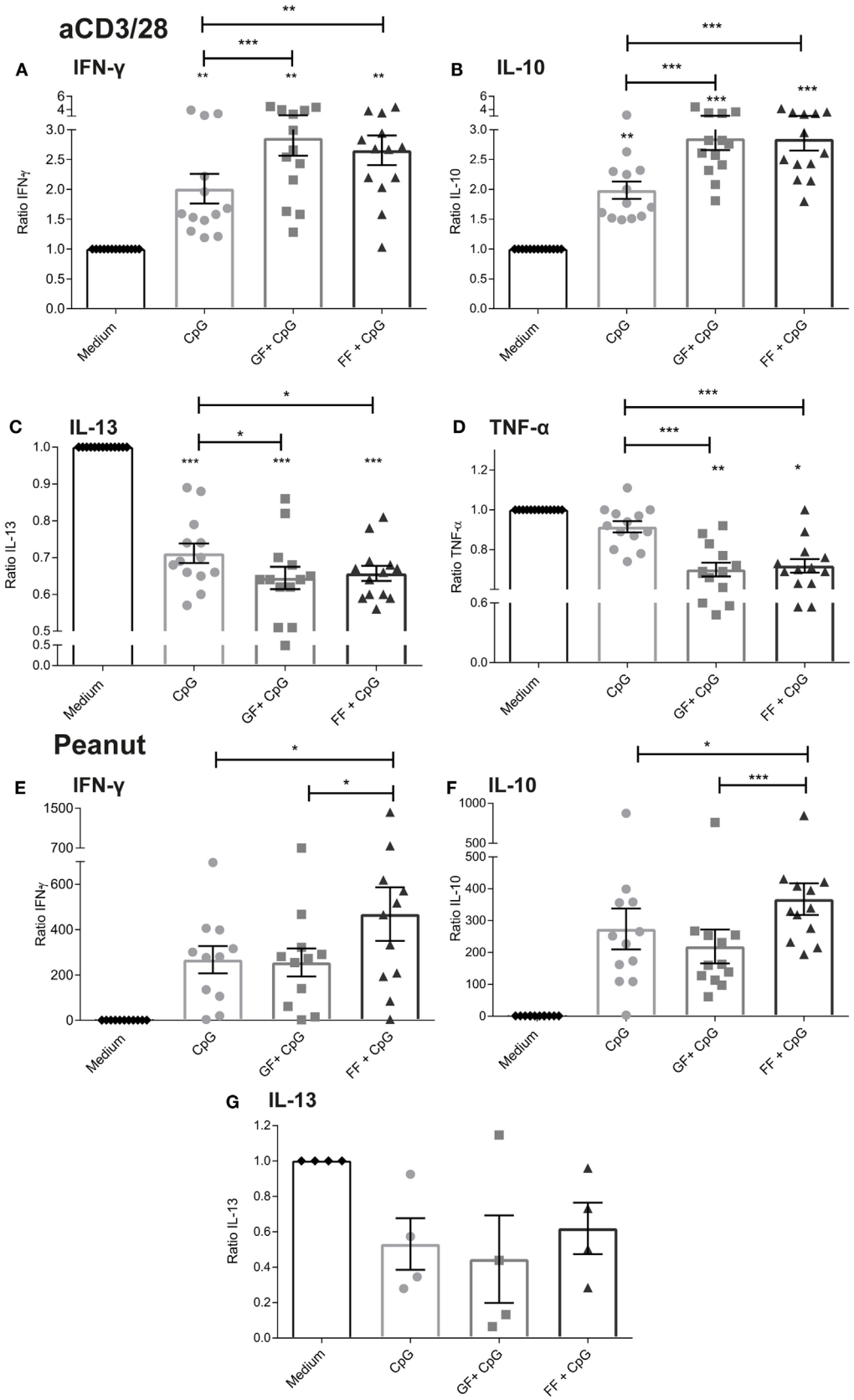

FIGURE 3 | Continued

FIGURE 3 | Enhanced production of IL-10 and IFN-γ by aspecific or peanut-specific activated peripheral mononuclear cells upon combined exposure of intestinal epithelial cells (IECs) to CpG oligodeoxynucleotides (ODN) and oligosaccharides. Exposure of IECs to CpG ODN in combination with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) (GF) or scFOS/lcFOS (FF) enhanced basolateral IFN-γ and IL-10 production in the aspecific model **(A,B)**. 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 ratio's 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.

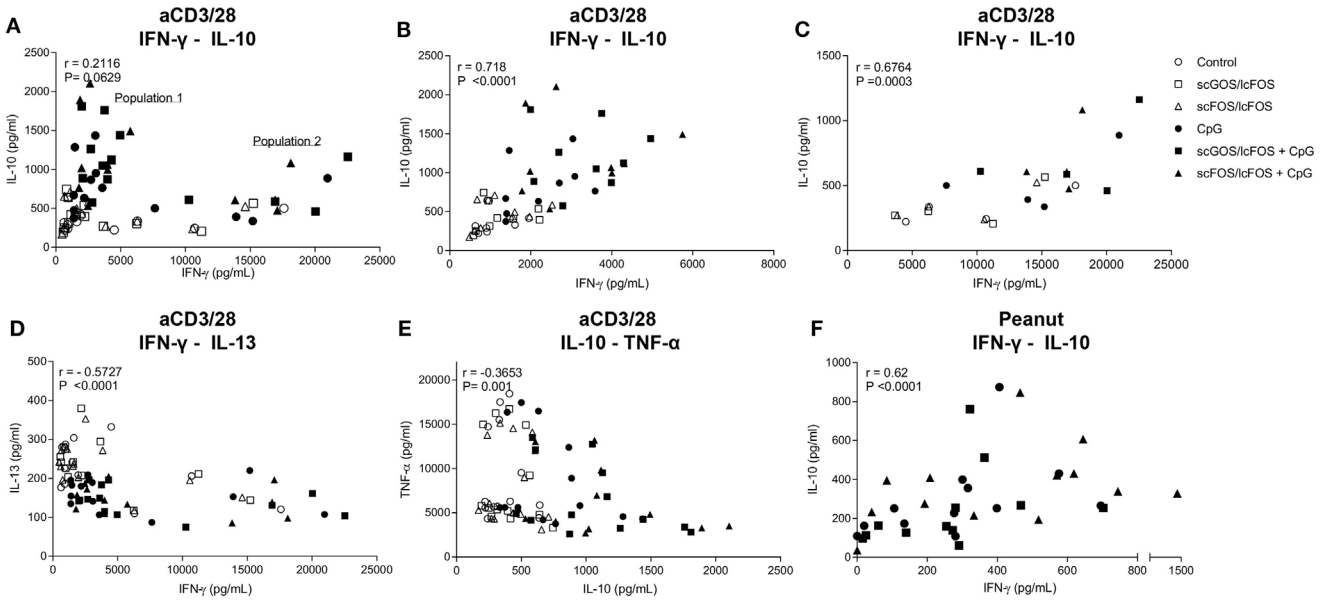

FIGURE 4 | Correlation of cytokine production. IFN-γ and IL-10 concentrations were positively correlated in both the aspecific and peanut-specific co-culture models **(A–C,F)**. In the aspecific model, a clear distinction could be made into two populations **(B,C)**. Population 2 was comprised of four 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 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 h in the aspecific co-culture (**Figure 5A**), while IECs after another 24 h of culture without PBMCs showed an increased galectin-9 production when exposed to CpG ODN (**Figure 5B**). This was further significantly 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.

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).

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

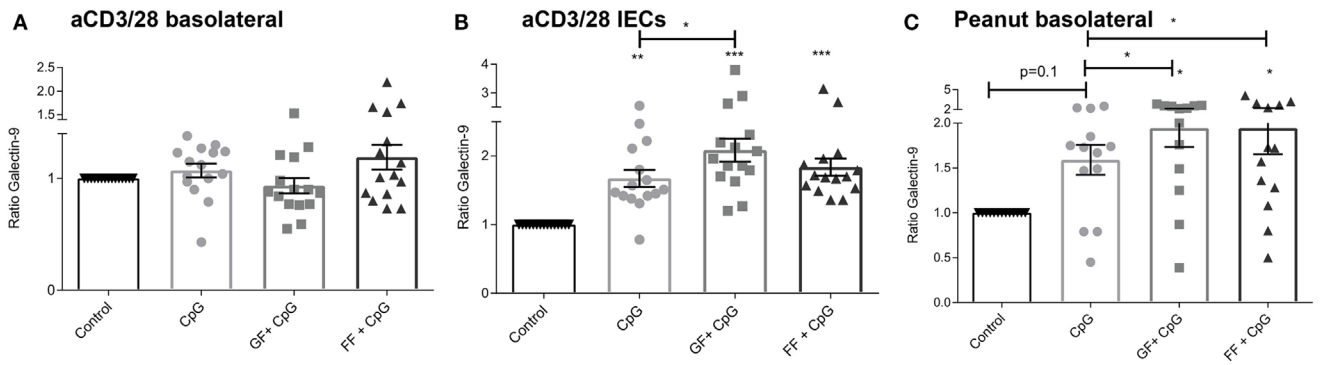

FIGURE 5 | Increased galectin-9 production by intestinal epithelial cells (IECs) upon apical exposure to CpG oligodeoxynucleotides in the presence or absence of oligosaccharides. In the aspecific co-culture model, no differences in basolateral galectin-9 were observed after 24 h **(A)**. Exposure of IECs to oligosaccharides alone did not influence galectin-9 levels, while CpG exposure influenced galectin-9 release by IECs after 48 h, 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.

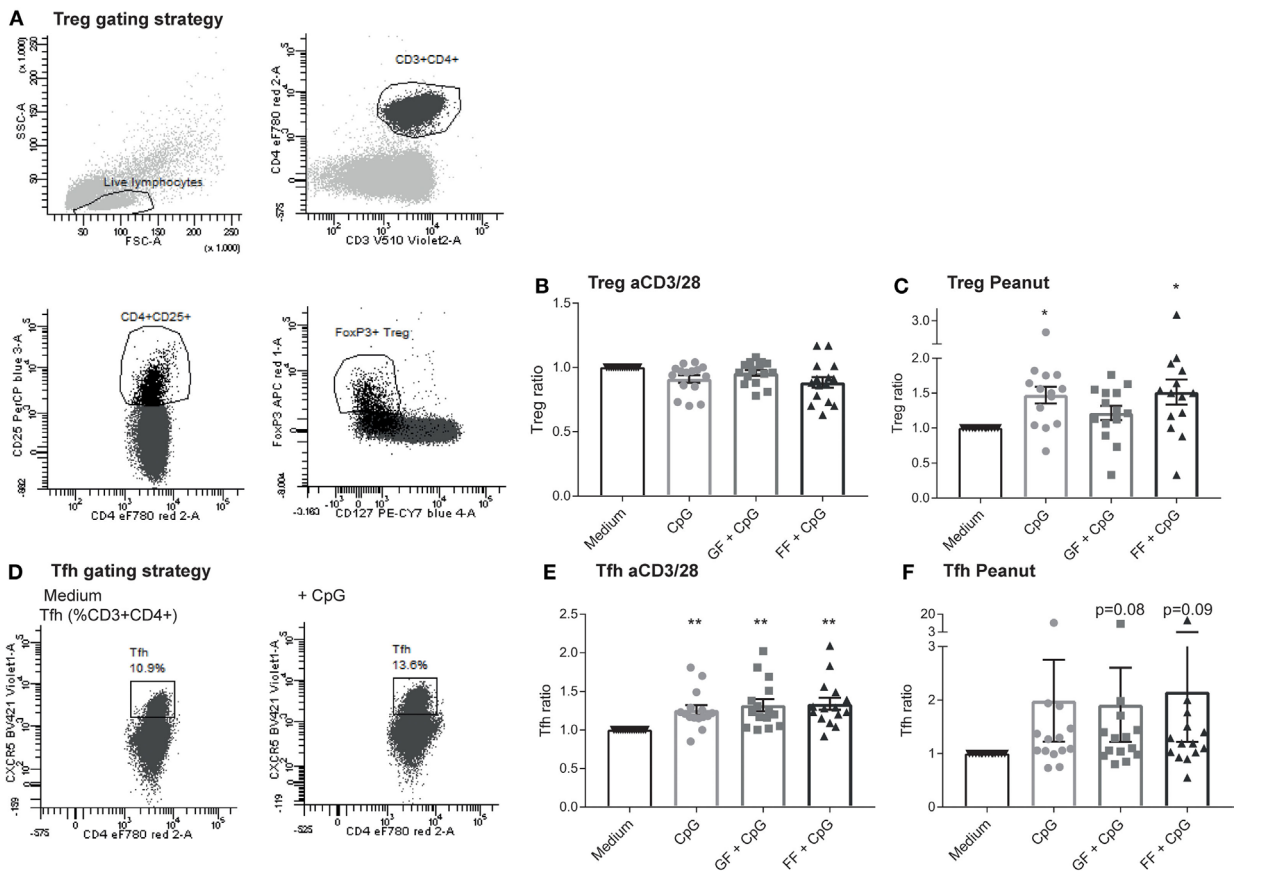

FIGURE 6 | Increased percentage of regulatory T cells (Treg) and Tfh subsets in the peanut-specific co-culture model upon exposure to intestinal epithelial cells (IECs) to CpG oligodeoxynucleotides (ODN). Tregs were gated as indicated **(A)**. Treg polarization in the aspecific co-culture model was not 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. Short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) (GF), scFOS/lcFOS (FF).

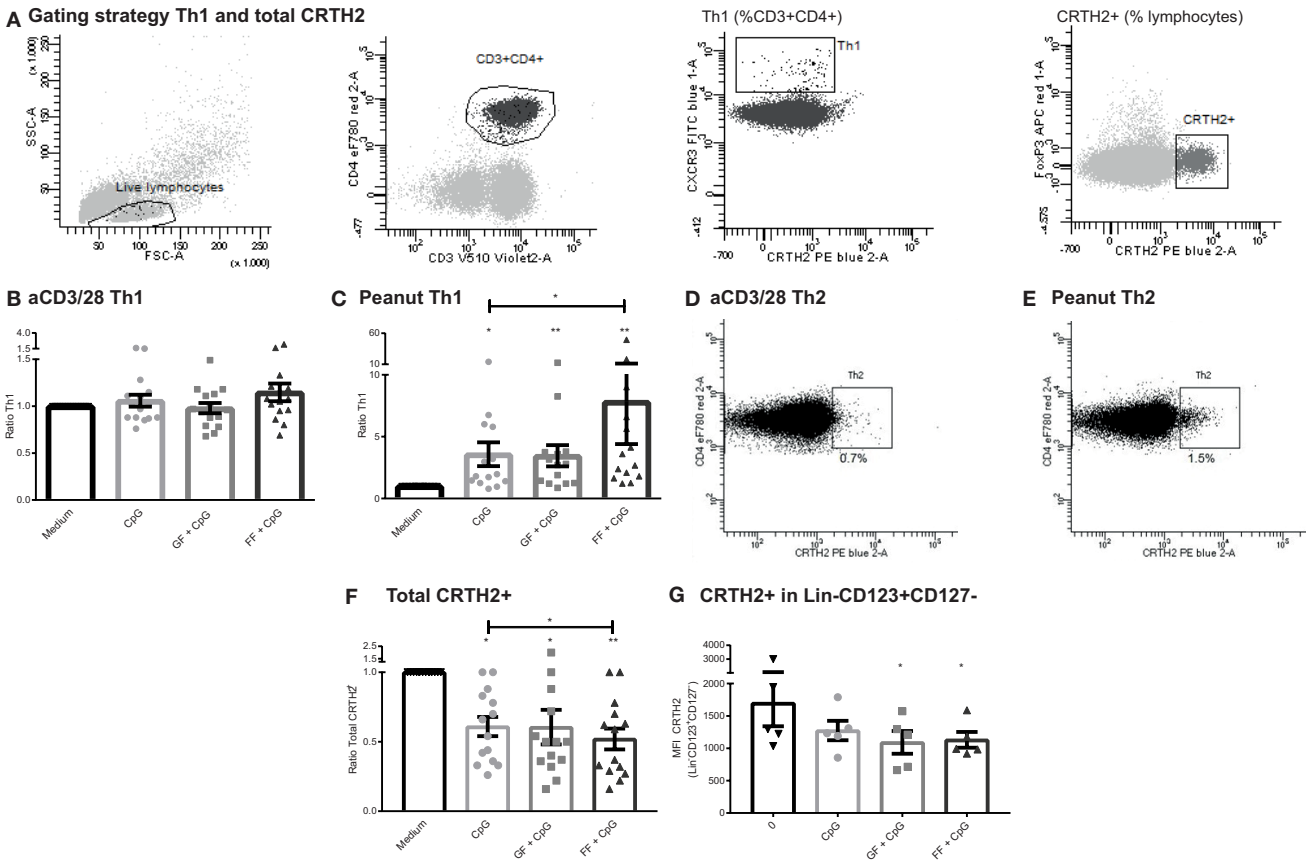

FIGURE 7 | Increased T helper 1 (Th1) subset in a peanut-specific co-culture model upon exposure of intestinal epithelial cells (IECs) to CpG, while CRTH2 is downregulated. Gating strategy of Th1 and CRTH2$^+$ cells **(A)**. The Th1 subset of the aspecific co-culture model was not affected after CpG exposure of IECs **(B)**. In the peanut-specific model, IECs exposed to CpG oligodeoxynucleotides (ODN) mediated an increase in the basolateral Th1 population, which was further enhanced by short- and long-chain fructo-oligosaccharides (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 peripheral mononuclear cells 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).

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 twofold (**Figures 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 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).

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

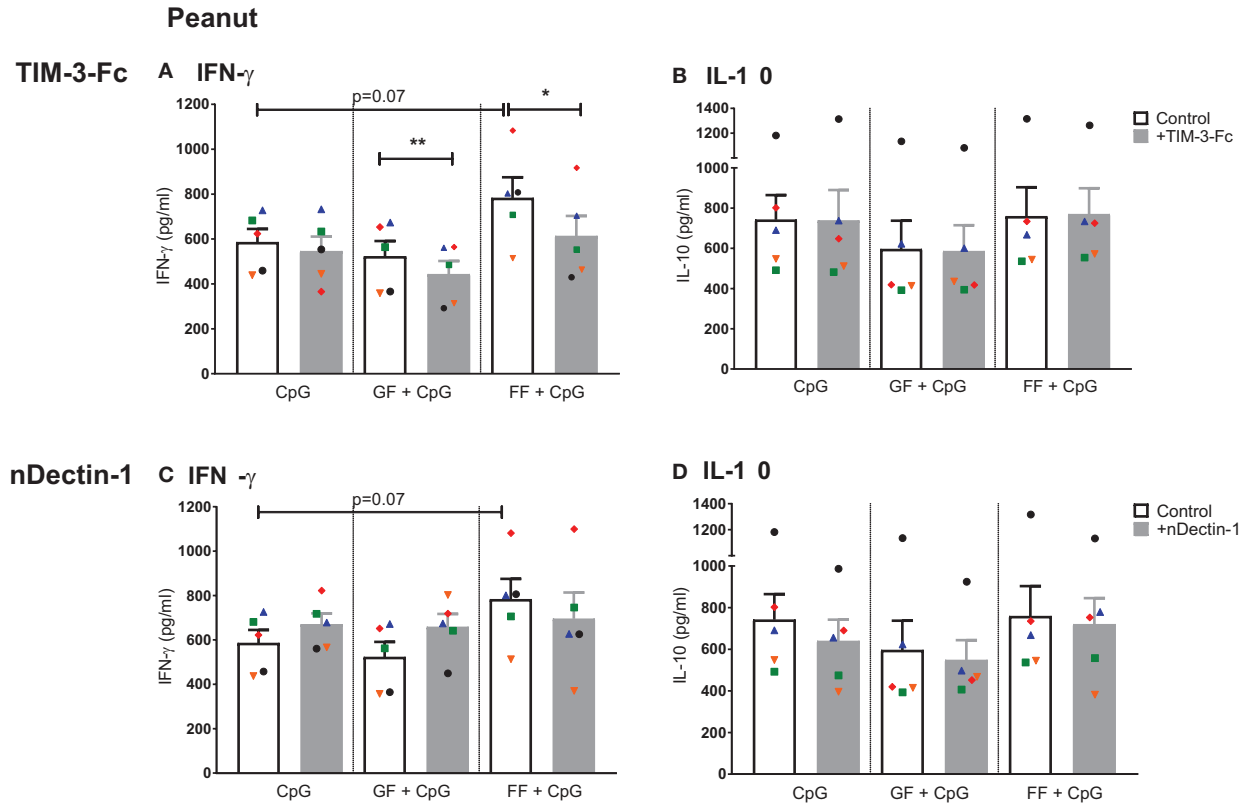

FIGURE 8 | Neutralization of galectin-9 abrogates IFN-γ production in the peanut-specific co-culture model, while neutralization of the dectin-1 receptor does not affect IFN-γ and IL-10 production. Addition of TIM-3-Fc to the peanut-specific co-culture abrogated additional IFN-γ production by short- and long-chain fructo-oligosaccharides (scFOS/lcFOS) (FF), and also decreased IFN-γ production upon combined exposure to CpG oligodeoxynucleotides 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.

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**).

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 (**Figures 8C,D**).

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 of scFOS/lcFOS in *in vitro* models have 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 toward 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 which 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 (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 toward 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.

ETHICS STATEMENT

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the University Medical Center Utrecht (NL51606.041.15).

AUTHOR CONTRIBUTIONS

SH, LW, and HO designed the experiments. AK assisted in recruitment of patients. SH and SO performed the experimental procedures. SH performed data collection and analyses and drafted the manuscript. JG, AK, SO, LW, and HO contributed to data interpretation and critically revised the manuscript.

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Tissue-Specific Expression of the Low-Affinity IgG Receptor, FcγRIIb, on Human Mast Cells

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Immediate hypersensitivity reactions are induced by the interaction of allergens with specific IgE antibodies bound *via* FcεRI to mast cells and basophils. While these specific IgE antibodies are needed to trigger such reactions, not all individuals harboring IgE exhibit symptoms of allergy. The lack of responsiveness seen in some subjects correlates with the presence of IgG antibodies of the same specificity. In cell culture studies and *in vivo* animal models of food allergy and anaphylaxis such IgG antibodies have been shown to exert suppression *via* FcγRIIb. However, the reported absence of this inhibitory receptor on primary mast cells derived from human skin has raised questions about the role of IgG-mediated inhibition of immediate hypersensitivity in human subjects. Here, we tested the hypothesis that mast cell FcγRIIb expression might be tissue specific. Utilizing a combination of flow cytometry, quantitative PCR, and immunofluorescence staining of mast cells derived from the tissues of humanized mice, human skin, or in fixed paraffin-embedded sections of human tissues, we confirm that FcγRIIb is absent from dermal mast cells but is expressed by mast cells throughout the gastrointestinal tract. IgE-induced systemic anaphylaxis in humanized mice is strongly inhibited by antigen-specific IgG. These findings support the concept that IgG, signaling *via* FcγRIIb, plays a physiological role in suppressing hypersensitivity reactions.

Keywords: IgE, IgG, FcγRIIb, Fc receptors, mast cells, anaphylaxis, immediate hypersensitivity, food allergy

INTRODUCTION

Clinical evaluation for allergy currently centers on detection of allergen-specific IgE antibodies using both skin testing and serum IgE measurements. The presence of specific IgE in patients with histories of allergen-induced reactions is considered confirmatory of allergy. However, while specific IgE is necessary for immediate hypersensitivity reactions, it is not always sufficient. This is particularly true in the setting of food allergy where a significant fraction of patients harboring allergen-specific IgE antibodies to foods can actually ingest those foods with no reaction (1–3). As a result, accurate diagnosis of food allergy can in many cases only be established by ingestion challenge. This poor predictive value of IgE testing offers an important clue regarding the regulation of IgE-triggered mast cell responses to allergens, suggesting the presence of counteracting

mechanisms or inhibitory pathways in individuals producing significant amounts of allergen-specific IgE who are not clinically allergic.

Allergen-specific IgG antibodies can exert such a dampening effect on IgE-mediated responses. Epidemiological studies of cohorts of children have revealed that the prevalence of allergen-specific IgE responses to aeroallergens significantly exceeds that of symptomatic respiratory allergy and that the levels of allergen-specific IgG antibodies correlate with protection from symptoms (4). There is growing evidence for a similar benefit of IgG antibodies in food allergy. Specific IgG levels are inversely correlated with reaction severity in food allergic subjects and increase in parallel with the natural resolution of milk and egg allergy (5–7). Oral immunotherapy (OIT) and early food introduction strategies both elicit food-specific IgG responses (8–14). IgG has been shown to reduce IgE-mediated mast cell activation *via* two distinct mechanisms, (1) antigen interception and steric blockade, blocking binding to IgE or (2) *via* Fc-mediated interactions with the inhibitory receptor FcγRIIb (15). The importance of these IgG pathways in exerting suppression of hypersensitivity *in vivo* has been explored in murine studies in which it has been clearly demonstrated that both are at work but that FcγRIIb ligation is about an order of magnitude more potent in mediating IgE responses than is steric blockade (16–20).

Fcγ receptors (FcγRs) can be classified into activating and inhibitory FcγRs. Mouse mast cells express the activating receptor FcγRIII, while human mast cells express FcγRI and FcγRIIa, but not the low-affinity receptor, FcγRIII. The activating FcγRs, like the high-affinity IgE-receptor FcεRI, signal *via* a cytosolic immunoreceptor tyrosine-based activation motif (ITAM). Upon activation, the ITAMs are transphosphorylated, and a signaling cascade is initiated by the SH2-containing Syk tyrosine kinase. The receptor FcγRIIb is unique as it is the only inhibitory FcγR. It contains an immunoreceptor tyrosine-based inhibitory motif that recruits phosphatases for inhibitory and immunomodulatory downstream signaling. Thus, FcγRIIb is able to attenuate signaling induced by activating FcγRs (21–23). Murine mast cells express FcγRIIb, and genetic models have established that IgG-mediated suppression of IgE-induced anaphylaxis is dependent on its presence (16–19, 24).

The role of FcγRIIb in the suppression of human mast cell activation by IgE *in vivo* has been less clear. Like murine mast cells, human mast cells cultured from hematopoietic progenitors express functional FcγRIIb (25). In contrast, when isolated from the skin, the most accessible tissue from which to obtain them, primary human mast cells lack the receptor (26). This finding along with the observation that subjects who successfully complete food OIT do not exhibit anaphylaxis upon ingestion challenge despite having quite elevated IgE levels but still exhibit positive skin test responses to the same food (27–30) led us to hypothesize that IgG antibodies formed in the course of OIT might suppress the IgE-induced activation of intestinal mast cells (and hence food anaphylaxis) while leaving IgE-induced skin responses unchecked. A corollary of this hypothesis would be that intestinal but not cutaneous mast cells express FcγRIIb. Notably, allergen-specific IgG levels increase by orders of magnitude during OIT (27, 30, 31), and this IgG suppresses basophil degranulation in an FcγRII-dependent manner (18).

In order to test our hypothesis, we used an array of approaches to evaluate the expression of the low-affinity inhibitory Fc receptor, FcγRIIb, in human IgE receptor-bearing cells. We analyzed live cells isolated from human skin and various tissues of humanized mice as well as arrays of fixed tissues from a number of human organs. Our analyses confirm the previously reported absence of FcγRIIb in human skin mast cells but demonstrate its presence in mast cells of the gastrointestinal tract. Using the humanized mouse model, we demonstrate that IgG antibodies suppress IgE-triggered human mast cell-mediated anaphylaxis in an FcγRII-dependent manner.

MATERIALS AND METHODS

Humanized Mice

Humanized mice with robust reconstitution both of human T and B cell adaptive immune compartments and human mast cells were produced as previously described (32, 33). Briefly, non-obese diabetic (NOD).SCIDγc$^{-/-}$ mice transgenic for membrane-bound human stem cell factor (SCF) [NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(PGK1-KITLG*220)441Daw/SzJ] were engrafted with 5×10^4 CD34$^+$ hematopoietic stem cells (HSC) from cord blood (AllCells) for 16–24 weeks. Wild-type BALB/c, C57BL/6J, and FcγRIIb$^{-/-}$ (B6) mice were bred at Boston Children's Hospital. All animal work was conducted under protocols approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital.

Cell Isolation

Peripheral blood was collected from healthy adult volunteers by venipuncture. Neonatal foreskins were obtained through the Human Skin Disease Resource Center. Cells were dispersed from the spleen and bone marrow of (humanized) mice by mechanical disruption. Leukocyte suspensions were prepared from skin and intestine according to established procedures using collagenase digestion (34). Human mast cells were isolated by immunomagnetic selection using CD117 microbeads (Miltenyi Biotec). Human mast cell progenitors were similarly isolated from humanized mouse bone marrow. Human basophils were isolated from Ficoll-separated human peripheral blood mononuclear cells using a Basophil Diamond Isolation Kit (Miltenyi Biotec).

Cell Culture

Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 1% MEM non-essential amino acids, 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, 10 µg/ml gentamicin, 55 µM 2-mercaptoethanol, and 10 mM HEPES buffer (complete RPMI). Isolated human mast cells were cultured in the presence of human SCF (20 ng/ml, Shenandoah Biotechnology). Mast cell phenotypes were assessed by flow cytometry (c-Kit$^+$FcεRIα$^+$) and chloroacetate esterase staining.

RNA Analysis

RNA was extracted using an RNeasy Micro Kit from Qiagen, reverse transcribed using a BioRad iScript cDNA synthesis kit, and analyzed by qPCR using TaqMan probes. GAPDH was used

for normalization, and data are expressed as transcripts per 1,000 GAPDH transcripts.

Immunofluorescence on Formalin-Fixed Paraffin-Embedded (FFPE) Tissues

Human tissue arrays were produced as previously described (35, 36). Paraffin-embedded tissue sections were deparaffinized according to standard procedures, and epitopes retrieved by heating for 20 min in 10 mM sodium citrate pH 6 with 0.05% Tween-20 in a vegetable steamer. Sections were blocked and permeabilized using 0.3% Triton X-100 with 2% BSA and 5% goat serum (all Sigma-Aldrich). The following antibodies were used: anti-human tryptase (clone AA1, mouse IgG1, Santa Cruz Biotechnology), anti-CD32B (rabbit polyclonal ab151497, Abcam), goat anti-mouse IgG1 AlexaFluor488 (Invitrogen), goat anti-mouse IgG2b AlexaFluor568 (Invitrogen), and goat anti-rabbit AlexaFluor568 (Invitrogen). Sections were mounted in Prolong Gold Antifade Reagent with DAPI (ThermoFisher) and imaged on a Nikon E800 microscope.

Flow Cytometry

Cells were stained for surface markers in FACS buffer (PBS, 0.5% BSA, 0.01% sodium azide) at 4°C for 30 min. Peripheral blood was subjected to fixation and erythrocyte lysis using BD Phosflow Lyse/Fix reagent for 10 min. All other cells were fixed using BD Cytofix/Cytoperm reagent. After fixation, cells were washed and stained in BD permeabilization buffer.

Non-specific binding was blocked using FcX TruStain (Biolegend) and 10% rabbit serum (Sigma Aldrich). Cells were stained to detect CD45 (clone HI30, Biolegend), CCR3 (clone 5E8, Biolegend), c-Kit (clone 104D2, Biolegend), FcεRIα (clone CRA-1, Biolegend), CD64 (FcγRI) (clone 10.1, Biolegend), CD32A (FcγRIIa) (clone IV.3, Stem Cell Technologies), CD16 (FcγRIII) (clone 3G8, Biolegend), and viability (eBioscience Fixable Viability dyes). CD32B (FcγRIIb) was detected by a rabbit polyclonal antibody directed against the c-terminal (intracellular) portion of CD32B (Abcam, ab151497). This antibody was purified and directly conjugated to PE-Cy7 using a Lightning Link kit (Innova Biosciences). Non-specific rabbit IgG was treated identically. Specific staining for CD32B was accomplished using extensive blocking with unlabeled rabbit IgG (100 μg/ml, 15 min) and validated by comparing the signal obtained on B cells ($CD32B^+$) versus T cells ($CD32B^-$) (Figure S1 in Supplementary Material).

IgG Preparation

IgG was prepared from pooled sera from de-identified peanut-allergic patients that had undergone OIT. IgG was purified over Nab protein G spin columns (Thermo Scientific), concentrated, and dialyzed with Macrosep Advance Centrifugal Devices carrying a 50 kDa cutoff (Pall Corporation) and filter-sterilized with 0.2 μM syringe filters (Millex). Allergen-specific IgG concentrations were determined by Phadia ImmunoCAP (ThermoFisher).

Passive Anaphylaxis

Humanized mice were passively sensitized to peanut by intravenous injection of IgG-depleted serum from highly peanut allergic human donors, containing 70 ng αPN IgE as determined by ImmunoCAP. 24 h prior to allergen challenge, human IgG against PN was injected i.p. Anaphylaxis was evoked by i.p. injection of 1 mg complete peanut extract, which was made as previously described (18). Anaphylactic severity was determined by monitoring the loss of core body temperature using subdermally implanted microchip transponders (Bio Medic Data Systems).

Statistical Analysis

Data were plotted and analyzed in Prism 5.0f (GraphPad Software, Inc.). Anaphylaxis data were analyzed using repeated measures two-way ANOVA; all other data were analyzed using standard ANOVA with Bonferroni post-tests between groups. ELISA values for IgE varied across multiple orders of magnitude and thus were subjected to log transformation prior to statistical analysis; for this purpose, null values were assigned a nominal value corresponding to the limit of detection in the assay. Two-tailed P values are summarized as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Data are represented as mean ± SEM for anaphylaxis curves, and with points showing individual mice overlaid with mean ± SEM elsewhere.

RESULTS

Human Mast Cells Express Both Inhibitory and Activating FcγRII

Murine models have revealed the impact of the inhibitory Fc receptor FcγRIIb on pathogenesis of both allergic and autoimmune diseases (16–18). IgG signaling *via* FcγRIIb potently inhibits IgE-induced anaphylactic shock (16, 19, 24). Since mast cells are thought to be the main drivers of this IgE-mediated hypersensitivity reaction, the mechanisms by which their activation can be modulated by IgG are likely relevant for allergic disease. While it is established that murine mast cells, murine basophils, and human basophils all express high levels of inhibitory FcγRIIb (37, 38), human tissue mast cells are harder to obtain and are less well characterized in this regard. Previous observations that mast cells isolated from human skin express only the activating FcγR, FcγRIIa, whereas mast cells cultured from cord blood have only inhibitory FcγRIIb have suggested potential heterogeneity among human mast cells with respect to the expression of these receptors (25, 26).

Several humanized mouse models have been shown to foster human mast cell development and we reasoned that examination of primary cells from such mice might provide insight into the tissue-specific expression of Fc receptors (32, 33, 39, 40). For this analysis, we used non-obese diabetic (NOD) severe combined immunodeficient (SCID) mice lacking the cytokine receptor common gamma chain ($\gamma c^{-/-}$) and carrying a human SCF transgene that were engrafted with human HSC. We have previously described the engraftment of both a functional adaptive (T and B cell) human immune response as well as abundant human mast cells capable of mediating allergen-specific immediate hypersensitivity responses (33). Leukocytes were isolated from the tissues of such mice and flow cytometric analysis performed to measure FcγR expression on cells positive for c-Kit and human FcεRIα using both an antibody directed at an FcγRIIa (CD32A)-specific surface epitope as well as a peptide-specific polyclonal antibody targeting a sequence unique to the intracellular portion

of FcγRIIb (CD32B) (Figure S1 in Supplementary Material). Consistent with our hypothesis and some prior observations, inhibitory FcγRIIb was expressed by mast cells from the intestine and spleen, but not from the skin of humanized mice (**Figure 1**). FcγRIIa was expressed by all mast cells.

Analysis of primary cells prepared from human skin samples confirmed that skin mast cells express FcγRIIa but not FcγRIIb (**Figure 1**). In contrast, human peripheral blood basophils expressed both FcγRIIa and FcγRIIb as expected (**Figure 1**). Human mast cells from both humanized mice and human skin expressed low levels of the high-affinity IgG receptor FcγRI (CD64), but not the low-affinity IgG receptor FcγRIII (CD16) (Figure S2 in Supplementary Material). Human basophils expressed FcγRIII as previously reported (data not shown) (41).

In order to corroborate our observations we additionally assessed FcγR mRNA expression by quantitative PCR. Consistent with the flow cytometry results, mRNA for FCGR2B was present at reasonably high levels in human mast cells from humanized mouse intestine but was undetectable or nearly so in both human and humanized mouse skin mast cells (**Figure 2**).

For analysis of mast cell FcγR expression in a fully human setting we performed immunofluorescence analysis on normal human tissue arrays, single slides containing FFPE sections from multiple organs, processed in one pass to ensure consistency of staining and reading. FcγRIIb costaining was examined on tryptase-positive cells in human skin and jejunum. FcγRIIb was evident on the mast cells in the intestine, but not the skin (**Figure 3**; Figure S3 in Supplementary Material). Further analysis of the human oral-gastrointestinal tract revealed expression of FcγRIIb in mast cells of the tongue, esophagus, and both small and large intestines (Figure S4 in Supplementary Material and data not shown). Expression was lower in stomach mast cells. Staining was minimal or absent in the abundant mast cells residing in tonsils and the rare mast cells of the lymph nodes and spleen (Figure S4 in Supplementary Material and data not shown).

These analyses of mast cells in normal tissues further support the hypothesis that FcγRIIb expression by mast cells depends on the tissue context, with good expression in the gastrointestinal tract and lack of the receptor in mast cells residing in the skin, spleen, or lymph nodes.

FcγRIIb Inhibits Human Mast Cell Activation

In murine mast cells and human basophils IgG signals delivered *via* FcγRIIb potently suppress IgE:FcεRI-mediated activation. IgG:FcγRIIb-mediated inhibition has similarly been

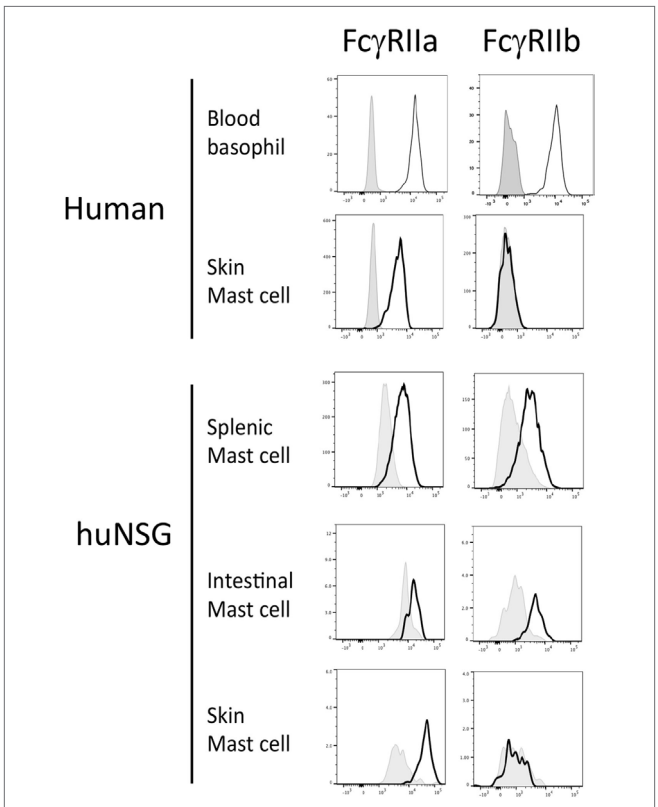

FIGURE 1 | Fcγ receptor (FcγR)IIb expression by mast cells and basophils from humanized mice, human blood and human skin. Cells from human samples or humanized mouse organs were assessed for *FcgRIIa* and *FcgRIIb* expression by flow cytometry. Each experiment was performed at least three times and a representative histogram is presented.

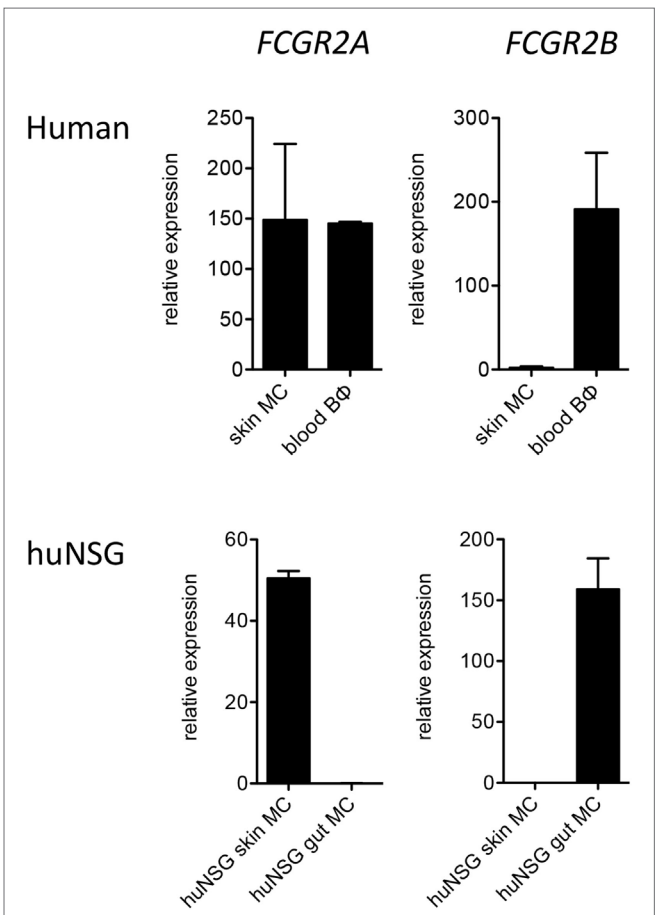

FIGURE 2 | Quantitative PCR analysis of *FCGR2A* and *FCGR2B* transcripts in human FceRI$^+$ cells. Data are from 5 to 7 samples per cell type.

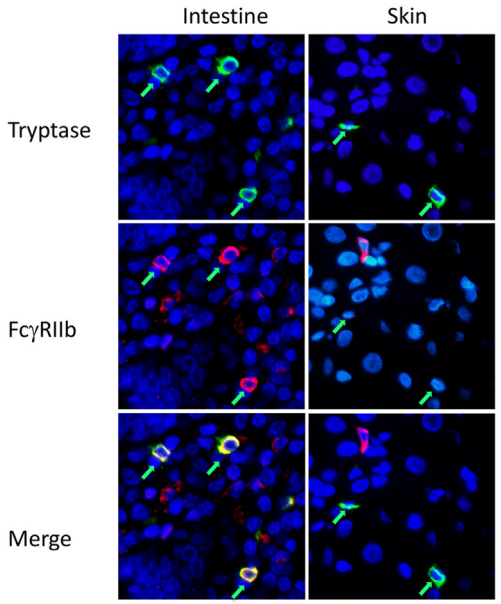

FIGURE 3 | Fcγ receptor (FcγR)IIb expression by mast cells in human intestine and skin. Immunofluorescent staining for mast cell tryptase (green) and FcgRIIb (red) on human intestinal or skin tissue sections in tissue arrays. Mast cells are indicated by green arrows. Larger fields, showing more cells, are presented in Figure S3 in Supplementary Material.

demonstrated in human cord blood cells, but these have an unusual phenotype, expressing only FcγRIIb but not FcγRIIa (25). We therefore sought to test for FcγRII affected IgG-mediated suppression in human mast cells expressing a complement of FcγRs more representative of normal gastrointestinal tissue resident mast cells. Using human mast cells cultivated from the bone marrow of humanized mice, we measured degranulation induced by peanut following sensitization with IgG-depleted sera from peanut allergic patients as a source of peanut-specific IgE. Sensitized mast cells rapidly upregulated LAMP-1, a marker of granule fusion following peanut stimulation (**Figure 4A**). The addition of IgG containing high titer anti-peanut antibodies partially reduced degranulation in a dose-responsive manner. This inhibition was fully reversed by the addition of anti-CD32 antibodies, consistent with an FcγRIIb-mediated effect. In contrast, primary human skin mast cells, which lack FcγRIIb, were not inhibited by the same concentrations of IgG (**Figure 4B**). In the presence of high levels of IgG, skin mast cells actually showed trends toward increased activation, with reversion by anti-CD32, suggesting that activating signals delivered *via* FcγRIIa might enhance degranulation in the absence of FcγRIIb.

As humanized mast cell FcγRIIb expression patterns reflect those in native human tissues with presence of the receptor in gastrointestinal mast cells and absence in the skin, we reasoned that this would be a useful model system in which to assess functional consequences of FcγRIIb ligation *in vivo*. We therefore used these mice to test the impact of IgG and FcγRIIb on IgE-mediated anaphylactic shock driven by human immune cells. Passive immunization of humanized mice with IgE from peanut-allergic sera sensitized them for anaphylactic shock when subsequently injected with peanut (**Figure 4C**). Shock severity was monitored by loss of core body temperature (loss of approximately 2.5°C) and corroborated by serum tryptase levels (**Figures 4C,D**). Administration of IgG containing high levels of anti-PN antibodies reduced the maximal temperature loss to around 1°C, with a non-significant trend for reduced tryptase levels. Neutralization of CD32 (FcγRIIa and FcγRIIb) partially abrogated the inhibitory effect of IgG on anaphylaxis, consistent with functional blockade of FcγRIIb-mediated suppression (**Figure 4C**). This suggests that the *in vivo* inhibitory effect of IgG might be mediated in part by steric blockade of antigen:IgE interaction which would not be susceptible to modulation by CD32. It is also possible that CD32 antibodies are not as effective *in vivo* because of competition for their binding by the many other $CD32^+$ cells in circulation.

DISCUSSION

Mast cells play diverse roles in the pathophysiology of allergy, serving not only to produce the mediators of immediate hypersensitivity reactions but also as sources of cytokines that shape emerging adaptive immune responses in mucosal tissues and skin (34, 42, 43). The role of IgE antibodies in triggering their activation is well established and a number of recent observations suggest that signals delivered by IgG antibodies serve to restrain this activation by IgE. The inhibitory FcγR, FcγRIIb, plays a key role in this process. Here, we demonstrate that its expression by mast cells varies in relation to their tissue location. Our findings reveal that the inhibitory receptor is strongly expressed in tissues of the gastrointestinal tract but absent in the skin. Using a humanized mouse model, we demonstrate that allergen-specific IgG antibodies potently block IgE-mediated anaphylaxis. These findings provide important insights into the contributions of inhibitory IgG signaling in human mast cells as well as the tissue specificity of expression of the responsible receptor.

Our observations provide a potential explanation for uncertainty that has emerged in the literature regarding the relative contributions of FcγRIIb in suppressing hypersensitivity in mice versus humans. The reported absence of the receptor on primary mast cells obtained from human skin raised questions regarding its physiologic relevance in regulating their activation in humans (26). In this report, we confirm not only the absence of FcγRIIb on dermal mast cells but also clearly demonstrate its presence on human mast cells residing in the gastrointestinal tract. Several independent lines of evidence are provided to support this conclusion, including flow cytometric analysis of mast cells obtained from the tissues of humanized mice, RNA quantification of FcγRIIb mRNA in the same cells and immunofluorescence staining of FcγRIIb in tissue arrays representing a range of human organs. The fully consistent demonstration of FcγRIIb expression on gut mast cells by these three approaches lends strong support to the concept that gastrointestinal mast cells preferentially express the receptor.

The absence of FcγRIIb on dermal mast cells may additionally provide an explanation for several common clinical observations in subjects with food allergy. Patients who successfully complete OIT protocols consistently exhibit sharp increases in

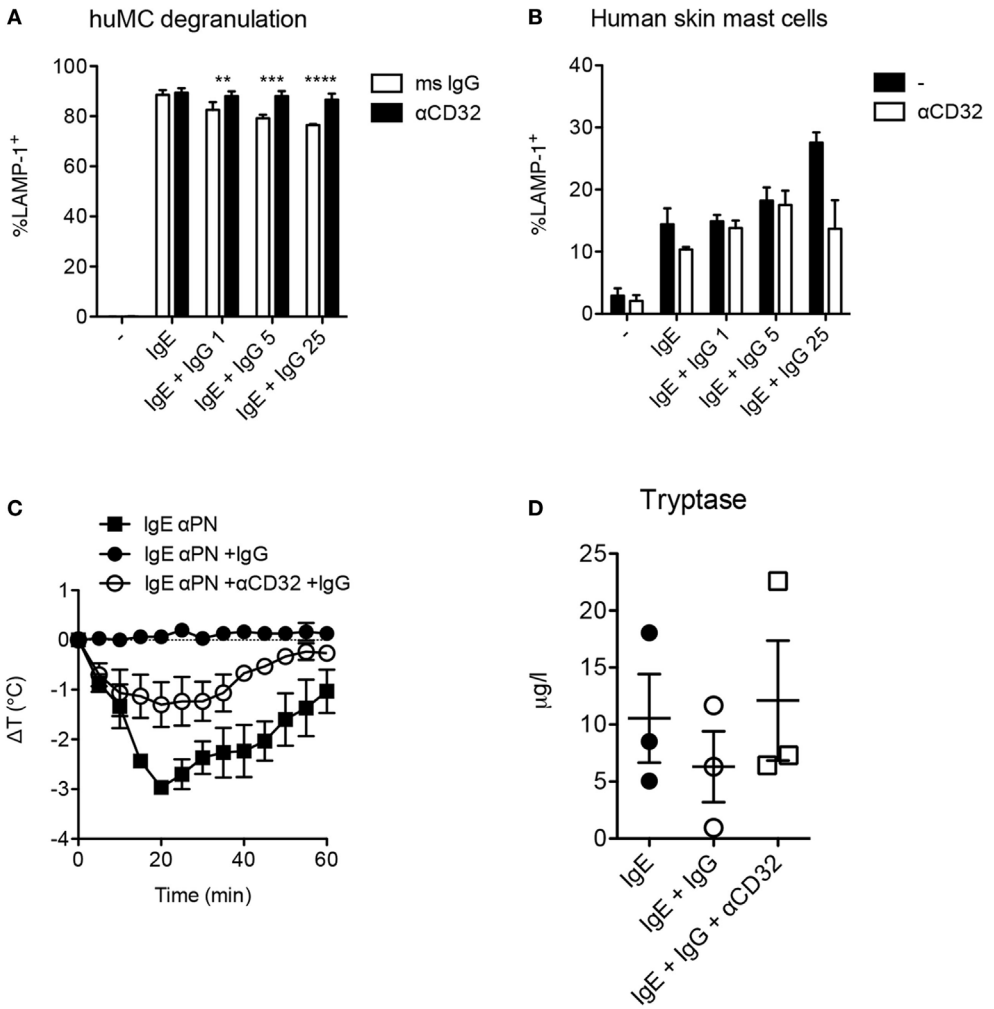

FIGURE 4 | Fcγ receptor II-dependent inhibition of human mast cells and anaphylaxis. **(A)** *In vitro* degranulation of anti-PN IgE-sensitized mast cells from humanized mice and inhibition by IgG anti-PN. Anti-CD32 or an isotype control mouse IgG was added 2 h prior to IgG anti-PN. **(B)** *In vitro* degranulation of human skin mast cells under the conditions used in **(A)**. **(C)** Core temperature loss (anaphylaxis) in humanized mice passively sensitized with IgE anti-PN and challenged by PN injection. **(D)** Serum tryptase levels 1 h post-challenge in humanized mice.

food allergen-specific IgG titers with only modest declines in IgE levels (44, 45). While able to tolerate enteral challenge, they often retain positive allergen skin test reactivity consistent with a scenario in which IgE-mediated activation of gastrointestinal but not dermal mast cells is suppressed by IgG:FcγRIIb signaling. We also note that in oral food challenge of allergic subjects cutaneous reactions including hives are considerably more common than gastrointestinal reactions (2).

As the role of allergen-specific IgG antibodies in respiratory and food allergy comes into sharper focus, greater understanding of the distribution and function of the receptors mediating their effects is needed. Our report provides a contribution to the field, describing the tissue-specific and cytokine-regulated heterogeneity of FcγR expression and the relationship of receptor expression patterns with observations regarding IgG-mediated suppression of allergic responses. Future studies will be needed to determine the tissue-specific factors that determine local mast cell phenotypes as well as the mast cell intrinsic mechanisms for sensing these factors. All these pathways might ultimately act as checkpoints for the allergic response and as potential targets for therapeutic intervention.

ETHICS STATEMENT

All animal work was conducted under protocols approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital.

AUTHOR CONTRIBUTIONS

OB, AE, MF, SM, AS, and HO designed experiments, interpreted the results, and wrote the paper. JT and RC provided fresh human skin samples and participated in the data interpretation and reporting of the results from these samples. MR provided human

tissue arrays and participated in the design and interpretation of the immunofluorescence studies.

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SUPPLEMENTARY MATERIAL

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Antigen Uptake by Langerhans Cells Is Required for the Induction of Regulatory T Cells and the Acquisition of Tolerance During Epicutaneous Immunotherapy in OVA-Sensitized Mice

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The skin is a major immunologic organ that may induce protection, sensitization or tolerance. Epicutaneous immunotherapy (EPIT) has been proposed as an attractive strategy to actively treat food allergy and has been shown to induce tolerance in sensitized mice through the induction of Foxp3$^+$ regulatory T cells (Tregs), especially CD62L$^+$ Tregs. Among immune cells in the skin, dendritic cells are key players in antigen-specific immune activation or regulation. The role of different populations of skin DCs in tolerance induction remains to be elucidated. Using OVA-sensitized BALB/c mice, we demonstrated that the application of a patch containing OVA-A647 to the skin resulted in allergen uptake by Langerhans cells (LCs) and CD11b$^+$ dermal cDC2 and subsequent migration into skin draining lymph nodes. These 2 populations induced Foxp3 expression in CD4$^+$ cells *in vitro*. Only LCs induced LAP$^+$ cells and CD62L$^+$ Tregs. Using Langerin-eGFP-DTR mice, we analyzed the role of LCs in the mechanisms of tolerance induction by EPIT *in vivo*. Following complete depletion of LCs, a dramatic decrease in the number of OVA$^+$ DCs and OVA$^+$ CD11b$^+$ dermal cDC2 was observed in skin draining lymph nodes 48 h after epicutaneous application. Likewise, 2 weeks of EPIT in non-depleted mice induced Foxp3$^+$ Tregs, especially CD62L$^+$, and LAP$^+$ Tregs in skin draining lymph nodes and spleen, whereas no induction of Tregs was observed in LC-depleted mice. Following 8 weeks of treatment, EPIT-treated mice showed significant protection against anaphylaxis accompanied by a significant increase of Foxp3$^+$ Tregs, especially CD62L$^+$ Tregs, which was not seen in the absence of LCs. In summary, although both LCs and CD11b$^+$ dermal cDC2s could induce regulatory T cells, the absence of LCs during EPIT impaired treatment efficacy, indicating their crucial role in skin-induced tolerance.

Keywords: food allergy, Epicutaneous immunotherapy, mechanisms, skin dendritic cells, Langerhans cells, regulatory T cells

INTRODUCTION

Food allergy is a growing public health concern and manifestations can be severe even life-threatening. Its prevalence increased dramatically during the last decades up to 10% in 2018 (1). There is no approved cure other than strict avoidance of identified foods and availability of self-injectable epinephrine. Allergen-specific immunotherapy aiming at reduction of the sensitivity to an allergen is an attractive strategy to actively treat food allergy. Proposed imunological mechanisms include decreased allergen-specific IgE and increased IgG4 levels, reduced responses of effector $CD4^+$ T cells, and induction of regulatory T cells (Tregs). Epicutaneous immunotherapy (EPIT) is a novel therapy that is currently under investigation. Safety and efficacy of Viaskin® Peanut in allergic patients has been studied in a recent phase 3 clinical program after positive results in a phase IIb trial (2).

The mechanisms of EPIT have been investigated in animal models of food allergy. In sensitized mice, EPIT with the corresponding allergen induced desensitization (3, 4), protected from allergic gut inflammation (5) and prevented food-induced anaphylaxis (6) and sensitization to new allergens (7). Tregs induced by EPIT are key: the protective effect of EPIT is abrogated by depletion of $CD25^+$ cells and is transferred to sensitized mice by the transfer of Tregs (8). EPIT increased $CD62L^-$ and $CD62L^+$ $Foxp3^+$ Tregs whereas other immunotherapies induced mainly $CD62L^-$ Tregs in peanut sensitized mice (9). In a recent study, the sustained protection and bystander effect of EPIT was related to epigenetic modifications in this $CD62L^+$ population of Tregs (10).

The unique immunologic features of the skin keratinocytes and antigen-presenting cells might explain the specific induction of this particular population of Tregs by EPIT. Among immune cells in the skin, dendritic cells (DCs) are key players in antigen-specific immune activation or regulation. Skin DCs can be divided into Langerhans cells (LCs) in the epidermis, the superficial layer of the skin, and a different subset of dermal DCs closely related to conventional DCs, which may themselves be divided into cDC1s and cDC2s, identified based on XCR1 and CD11b expression, respectively. It has been shown that cDC1s are more prone to induce a Th1 response and cross-present Ag to $CD8^+$ cells, whereas cDC2s are more likely to induce a Th2 response. LCs activate and induce Th17 or Tregs depending on the environmental stimuli (11). In previous studies, we demonstrated rapid allergen uptake by dendritic cells through intact skin leading to down-regulation of allergen-specific responses in sensitized mice treated by EPIT (12). However, the role of the different populations of skin DCs in tolerance induction remains to be elucidated.

Using OVA-sensitized mice, we demonstrated that epicutaneous application of allergen resulted in uptake by LCs and $CD11b^+$ dermal cDC2s. After migration into skin draining lymph nodes (sdLNs), both populations could induce Foxp3 expression in $CD4^+$ T cells *in vitro*. However, only LCs induced LAP^+ cells and $CD62L^+$ Tregs, and the depletion of LCs significantly decreased the induction of $Foxp3^+$ Tregs, especially $CD62L^+$ and LAP^+ Tregs in skin draining lymph nodes and spleen, compared to non-depleted mice. Moreover, the EPIT-induced protection against anaphylaxis was not seen in the absence of LCs. Thus, although both LCs and $CD11b^+$ dermal cDC2 can induce regulatory T cells, the absence of LCs during EPIT reduced the effectiveness of the treatment, indicating their crucial role in skin-induced tolerance.

METHODS

Mice

BALB/c mice were obtained from Charles River (Charles Rivers, Lyon, France). Langerin-DTR mice, obtained from Pr Malissen (Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, INSERM, CNRS UMR, Marseille, France), were back-crossed on a BALB/c background and maintained as breeding colonies at DBV-Technologies. All procedures were performed according to the European Community rules on animal care with permission and ethical approval # 7811 from the French Authorities. Mice were acclimated in the animal facility for 1 week prior to initiating any procedures. Each experiment was reproduced twice independently.

Sensitization

Mice were sensitized on days 0 and 7 with 200 µl of a homogenous suspension of 10 µg of OVA (grade V, Sigma-Aldrich, France) and 2 mg of aluminum hydroxide (Imject® Alum, Thermo scientific, France) by subcutaneous administration on the back of the neck. Mice were then boosted intranasally on day 14 with OVA (10 µg).

Measurement of Allergen Capture by Skin DCs

For epicutaneous application, the skin of sensitized mice was shaved with electric clippers and then depilatory cream was applied 24 h before applying the epicutaneous patch. For studies of allergen capture and skin DCs migration, VIASKIN® with 100 µg OVA-alexa647 (V-OVA) were applied for indicated times on the back of mice and maintained with a non-occlusive linen. Mice were then sacrificed and skin and skin-draining lymph nodes (sdLN) were harvested.

Skin and sdLN Cell Isolation

Using an insulin microneedle, 8 mm skin biopsies were injected 5–10 times with Liberase TL (250 µg/mL)-DNase (500 µg/mL) solution, placed in 1 ml of Liberase TL-DNase solution in a 24-well plate (dermal side down), and then incubated for 2 h at 37°C in a cell culture incubator (5% CO_2). To stop the enzymatic action at the end of the incubation period, 500 µL of DNase working solution and 75 µl of 0.1 M EDTA were added. Tissues were placed in a Medicon tissue grinder (BD Biosciences) and mixed for 8 min in a Medimachine (BD Biosciences). Cell suspensions were filtered using 50 µm syringe filter (Filcon, BD Biosciences), washed and counted.

Skin draining lymph nodes were harvested in Petri Dishes containing 1 mL RPMI 1640. Then, 1 mL of Liberase TM (0.52 U/mL)/DNase I (50 µg/mL) in RPMI 1640 were added to each Petri Dish. Each LN was flushed using an insulin microneedle

and incubated for 20 min at 37°C. Two hundred fifty microliter of solution of EDTA at 100 mM were added to each Petri Dish to stop the reaction. Lymph node cell suspensions were then obtained by subsequent tissue dissociation and filtration through a cell strainer (100 μm), washed and counted.

In vitro Tregs Induction Measurement

OVA^+ migrating LCs and $CD11b^+$ DCs were isolated from sdLN cells by magnetic sorting using a CD11c isolation kit (Miltenyi Biotec, Paris, France) followed by flow cytometry sorting of MHC-II^+ $CD11c^+$ OVA^+ $CD11b^+$ $EPCAM^+$ and MHC-II^+ $CD11c^+$ OVA^+ $CD11b^+$ $EPCAM^-$, respectively. As control DCs, MHC-II^+ $CD11c^+$ OVA^- $CD11b^-$ $EPCAM^-$ cells were also sorted from the CD11c positive fraction. $CD4^+$ cells were sorted from the CD11c negative fraction using CD4 microbeads (Miltenyi Biotec, Paris, France). Each DC subset was then co-culture with $CD4^+$ cells at ratio 1:5 in 200 μL RPMI 1640 supplemented with FCS, penicillin, streptomycin, and β-mercaptoethanol in 96 wells plate. After 6 days, cells were stained for analysis of Foxp3 or LAP Tregs and expression of CD62L.

Epicutaneous Immunotherapy

Sensitized mice were treated weekly with VIASKIN® containing 100 μg OVA for 48 h. Tregs induction was measured after 2 weeks of treatment in sdLN and spleen. For measurement of allergen-specific oral reactivity, sensitized mice treated for 8 weeks or not treated were boosted by oral gavage with 20 mg OVA every 3 days X's 6 before receiving an oral challenge with 50 mg OVA. Animal temperatures were measured before and during challenge using microchips (Plexx IPTT). Single cell suspensions of mouse spleen were obtained by tissue dissociation and filtration through a cell strainer for *in vitro* re-stimulation and cytokine production measurements previously described (ref).

Langerhans Cells Depletion

Langerin-DTR mice were injected i.p. with 0.1 μg of diphteria toxin 5 days and 1 day before the first application of VIASKIN® and every 2 weeks 24 h before VIASKIN® application. This resulted in complete depletion of skin Langerhans cells throughout the treatment period, as verified by flow cytometry (data not shown).

Flow Cytometry

For DC analysis in the skin and sdLNs, cells were stained with different combinations of the following antibodies: MHC-II-Vioblue, CD11c-PerCPVio700, CD11b-VioGreen, CD11b-PerCPVio700, XCR1-Viobright-FITC, EPCAM-PE, CD86-PE-Vio770, CD86-APC-Vio770, PD-L2-PE-Vio770 (all from Miltenyi Biotec), CD11c-APC-Cy7 (BD Biosciences), XCR1-BV510 (Biolegend). Dead cells were excluded from analysis using Zombie dye (Biolegend) of appropriate color depending of the antibodies used.

For Tregs analysis, cells were stained with combinations of the following antibodies: anti-mouse CD4-FITC (Miltenyi), CD25-APC-Cy7, CD62L-PE-Cy7 (all from BD Biosciences, Le Pont de Claix, France), Latency-associated peptide (LAP)-PE and Foxp3-APC (from e-Bioscience, Paris, France), or control isotypes. Intracellular staining was performed after fixation and permeabilization, using Foxp3 Perm Kit (e-Bioscience, Paris, France). Dead cells were excluded from analysis using Zombie dye aqua (Biolegend).

Flow cytometry was performed on a MACSquant cytometer and analyzed using FlowJo software.

Statistical Analysis

The GraphPad Prism Software 5.0 (San Diego, CA, USA) was used for statistical analyses. Results are expressed as median with range. Statistical significance comparing different sets of mice were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

RESULTS

Characterization of Dendritic Cells Implicated in Allergen Uptake in OVA Sensitized Mice

To determine which skin DCs take up allergen following epicutaneous application, sensitized mice were treated with

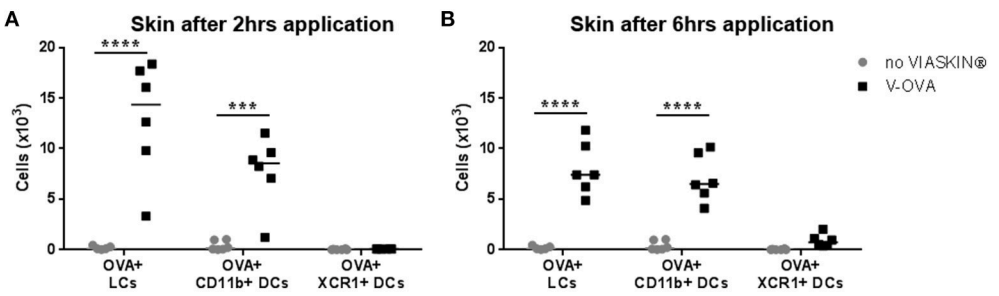

FIGURE 1 | Allergen capture by skin dendritic cells after epicutaneous application. VIASKIN®-OVA-AF647 (V-OVA) was applied on the back of sensitized mice for 2 h **(A)** or 6 h **(B)**. Sensitized not treated mice were used as control (no VIASKIN®). Skin were then harvested and cells isolated and counted before immunostaining for flow cytometry analysis. In live single cells gate, migrating DCs were gated as MHC-IIhigh CD11c+ and Langerhans cells (LCs), $CD11b^+$ DCs and $XCR1^+$ DCs were identified as $CD11b^+EPCAM^+XCR1^-$, $CD11b^+$EPCAM-XCR1–, and CD11b-EPCAM-$XCR1^+$, respectively. Proportion of OVA+ cells inside each population were measured and reported to number of cells. Each symbol represents a mice and bar represent median. Experiment was reproduced twice independently. $^{***}p < 0.001$; $^{****}p < 0.0001$ by 2-way ANOVA followed by Sidak's multiple comparisons test.

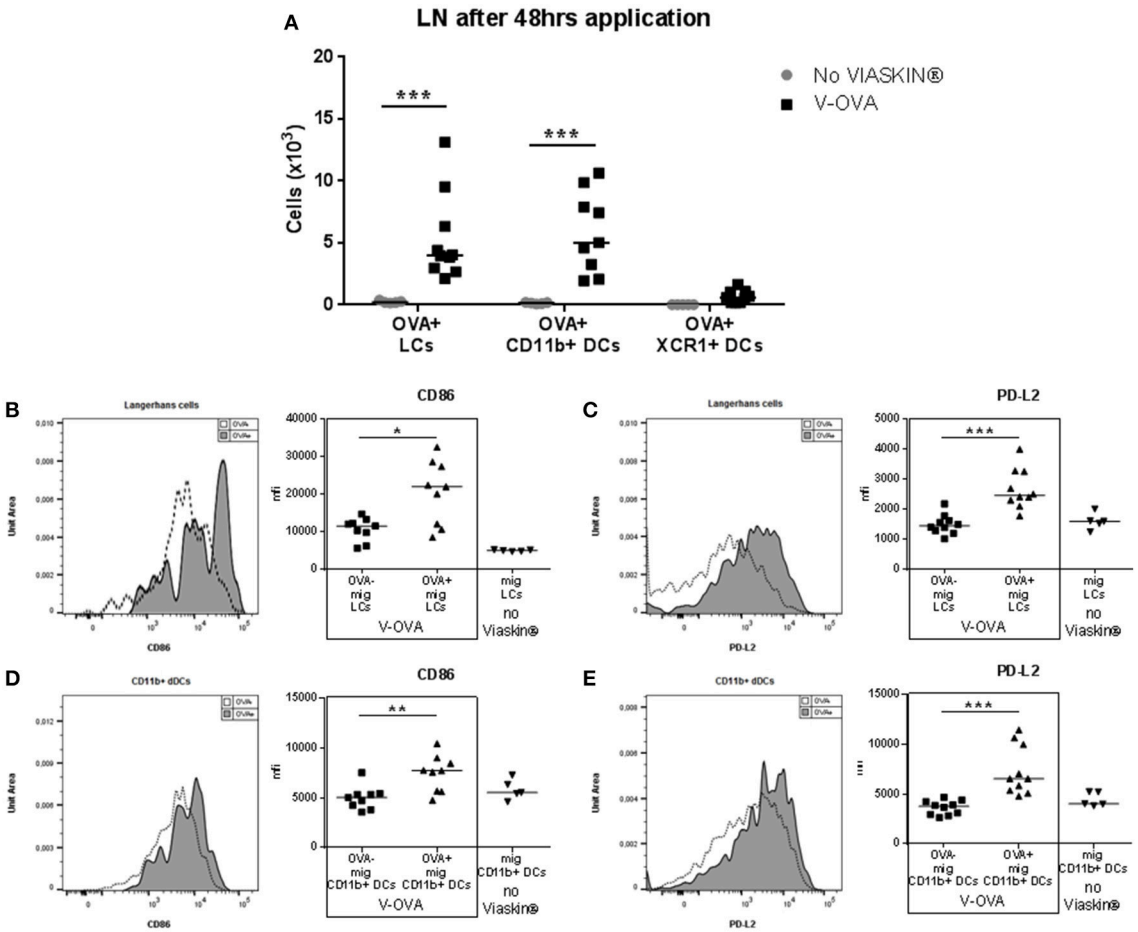

FIGURE 2 | Migration toward draining lymph nodes of skin dendritic cells that capture allergen after epicutaneous application. VIASKIN®-OVA-AF647 (V-OVA) was applied on the back of sensitized mice for 48 h. Sensitized not treated mice were used as control (No VIASKIN®). sdLN were then harvested and cells isolated and counted before immunostaining for flow cytometry analysis. In live single cells gate, migrating DCs were gated as MHC-II^{high} CD11c^{+} and Langerhans cells (LCs), CD11b^{+} DCs and XCR1^{+} DCs were identified as CD11b^{+}EPCAM^{+}XCR1^{-}, CD11b^{+}EPCAM^{-}XCR1^{-}, CD11b^{-}EPCAM^{-}XCR1^{+}, respectively. Proportion of OVA^{+} cells inside each population were measured and reported to number of cells **(A)**. Expression of activation marker CD86 **(B,D)** and PD-L2 **(C,E)** were analyzed in OVA^{-} and OVA^{+} LCs or in total migrating LCs in not treated mice **(B,C)** and in OVA^{-} and OVA^{+} CD11b^{+} DCs or in total migrating CD11b^{+} DCs in not treated mice **(D,E)**. Each symbol represents a mice and bar represent median. Experiment was reproduced twice independently. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

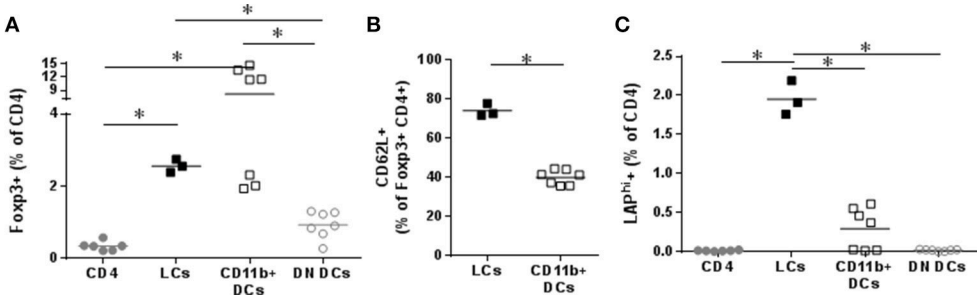

FIGURE 3 | Capacity of skin migrating Dendritic Cells to induce Tregs. VIASKIN®-OVA-AF647 (V-OVA) was applied on the back of sensitized mice for 48 h. sdLN were then harvested and cells isolated and OVA^{+} Langerhans cells (LCs), OVA^{+} CD11b^{+} DCs and OVA^{-} EpCAM^{-} control DCs (DN DCs) were FACS sorted and cultured with CD4+ cells for 6 days. Proportion of CD25^{+}Foxp3^{+} among CD4^{+} cells **(A)**, of CD62L^{+} among CD25^{+}Foxp3^{+} **(B)** and of LAP^{hi} **(C)** among CD4 cells were analyzed. Each symbol represents a well and bar represent median from two independent experiments. *$p < 0.05$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

VIASKIN®-OVA-AF647 for 2 or 6 h. Evaluating OVA$^+$ DCs from each subset showed that both LCs and dermal CD11b$^+$ DCs (DC2s) captured allergen efficiently after 2 h whereas XCR1$^+$ DCs (DC1s) did not (**Figure 1A**). After 6 h of application, OVA$^+$ LCs and OVA$^+$ CD11b$^+$ DCs were still present in the skin, but to a lesser extent suggesting that some cells had already migrated from skin. Extremely low levels of OVA$^+$XCR1$^+$ cells were detectable even after 6 h of application (**Figure 1B**).

In previous experiments, after application of VIASKIN®-OVA on intact skin of OVA-sensitized mice, skin dendritic cell migration toward sdLN peaked after 48 h (12). We then characterized the migration of skin dendritic cells into sdLNs after 48 h. Skin migrating DCs were gated as CD11c$^+$ cells expressing high levels of MHC-II. Among skin migrating cells, the 3 subsets were gated using XCR1, CD11b, and EpCAM expression. As expected from results in the skin, OVA was detected in LCs and CD11b$^+$ DCs, and to a lesser extent in XCR1$^+$ DCs (**Figure 2A**). Skin and sdLN results strongly suggest that allergen uptake following epicutaneous application in sensitized mice is mediated by LCs and CD11b$^+$ DCs. We then further analyzed their activation status by evaluating the expression of CD86 and PD-L2. OVA$^+$ LCS and OVA$^+$ CD11b$^+$ DCs had significantly increased expression of CD86, indicating a higher activation status compared to OVA- migrating cells (**Figures 2B,D**, respectively). Both populations of OVA$^+$ cells also expressed significantly higher levels of PD-L2 suggesting a potential tolerogenic capacity (**Figures 2C,E**). As a control for activation, skin DCs migrating to sdLN after application of VIASKIN®-OVA on tape-stripped skin expressed higher levels of CD86, but lower levels of PD-L2 (data not shown).

To analyze their tolerogenic potential, we measured LC and DC capacity to induce CD4$^+$ cells into regulatory T cells *in vitro*. OVA$^+$ LCs and OVA$^+$ CD11b$^+$ DCs were FACS sorted and co-cultured with CD4 T cells. Both populations significantly increased Foxp3 in CD4 cells *in vitro* compared to CD4 cultured alone or in presence of control DCs (**Figure 3A**). We had previously shown the capacity of EPIT to induce CD62L$^+$ Tregs in comparison to other forms of immunotherapy, e.g., oral and sublingual immunotherapy, and checked the proportion of CD62L in *in vitro* induced Tregs. OVA$^+$ LCs induced higher levels of CD62L on Foxp3$^+$CD4 cells compared to CD11b$^+$ DCs (**Figure 3B**). Furthermore, only OVA$^+$ LCs significantly increased LAP$^+$ CD4 cells *in vitro* (**Figure 3C**) suggesting that LCs are the key players in Tregs induction during EPIT.

The generation of peripheral Tregs requires TGF-β (13). The ability of DCs to activate TGF-β from its "latent" precursor has been recently linked to the expression of specific integrin by DCs in gut, i.e., alphaV beta8 (14, 15). In sdLN, OVA$^+$

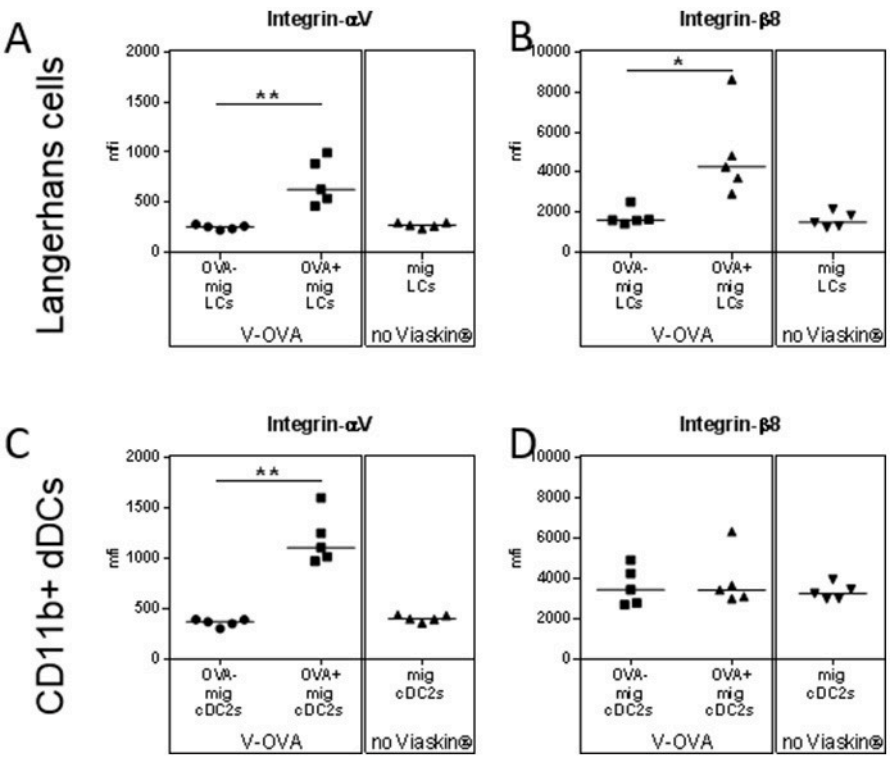

FIGURE 4 | Capacity of skin migrating Dendritic Cells to induce Tregs is mediated by TGF-β. **(A–D)** VIASKIN®-OVA-AF647 was applied on the back of sensitized mice for 48 h. Sensitized not treated mice were used as control (No VIASKIN®). sdLN were then harvested and cells isolated and counted before immunostaining for flow cytometry analysis. Expression of integrin alphaV **(A,C)** and beta8 **(B,D)** were analyzed in OVA$^-$ and OVA$^+$ LCs or in total migrating LCs in not treated mice **(A,B)** and in OVA$^-$ and OVA$^+$ CD11b$^+$ DCs or in total migrating CD11b$^+$ DCs in not treated mice **(C,D)**. *$p < 0.05$; **$p < 0.01$; by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

skin migrating LCs and CD11b$^+$ dDCs expressed higher level of integrin alphaV than OVA$^-$ or control DCs (**Figures 4A,C**). OVA$^+$ skin migrating LCs also expressed higher level of integrin beta8 compared to OVA$^-$ or control LCs whereas CD11b$^+$ dDCs did not (**Figures 4B,D**), suggesting a possible role of integrin beta8, and TGF-β in the capacity of migrating LCs to induce Tregs.

Characterization of the Allergen Uptake in Absence of LCs

To validate the possible role of LCs in the induction of tolerance by EPIT, we compared allergen uptake in sensitized LANG-DTR mice after depletion of LCs. After DT injection, depletion of LCs was confirmed by flow cytometry. As expected, no OVA$^+$ LCs was detected following application of VIASKIN-OVA-AF647 in the skin of LC-depleted mice (**Figure 5A**). DT injection did not alter the CD11b$^+$ DCs subset and allergen capture by those cells was no different in the skin of mice not LC-depleted after 2 or 6 h (**Figure 5B**).

In sdLN, no OVA$^+$ LCs were detected, as expected (**Figure 5C**). Interestingly, LC depletion significantly decreased OVA$^+$ CD11b$^+$ DCs migration into sdLNs after 48 h (**Figure 5D**) even though antigen capture in the skin was not altered. This suggested a possible interaction between LCs and dermal DCs in the skin, that would be needed for CD11b$^+$ DCs migration.

Langerhans Cells Are Required for the Induction of Regulatory T Cells *in vivo*

Two weeks' treatment of sensitized mice with EPIT induced a local increase in regulatory T cells. The amount of CD25$^+$Foxp3$^+$ Tregs significantly increased in sdLNs of EPIT treated mice compared to untreated animals (**Figure 6A**). More specifically, the CD62L$^+$ subset of Foxp3 Tregs was induced (**Figure 6B**). EPIT also induced greater numbers of LAP$^+$ Tregs compared to untreated mice (**Figure 6C**). This induction of Tregs was not limited to local LNs but was also observed in spleen of treated mice compared no untreated animals (**Figures 6D–F**). In the absence of LCs, no induction of Tregs was observed either in sdLNs or in the spleen (**Figure 6**) after 2 weeks of treatment.

Since the usual treatment period for EPIT in sensitized mice is 8 weeks, we checked the induction of Tregs in the spleen after 8 weeks of EPIT. We confirmed that DT alone did not alter normal responses as the Control + DT group showed similar levels of CD25$^+$ Foxp3$^+$ Tregs and LAP$^+$ Tregs, and similar expression of CD62L (**Figure 7**). As expected, EPIT-treated mice had significantly higher levels of CD25$^+$ Foxp3$^+$ Tregs, especially

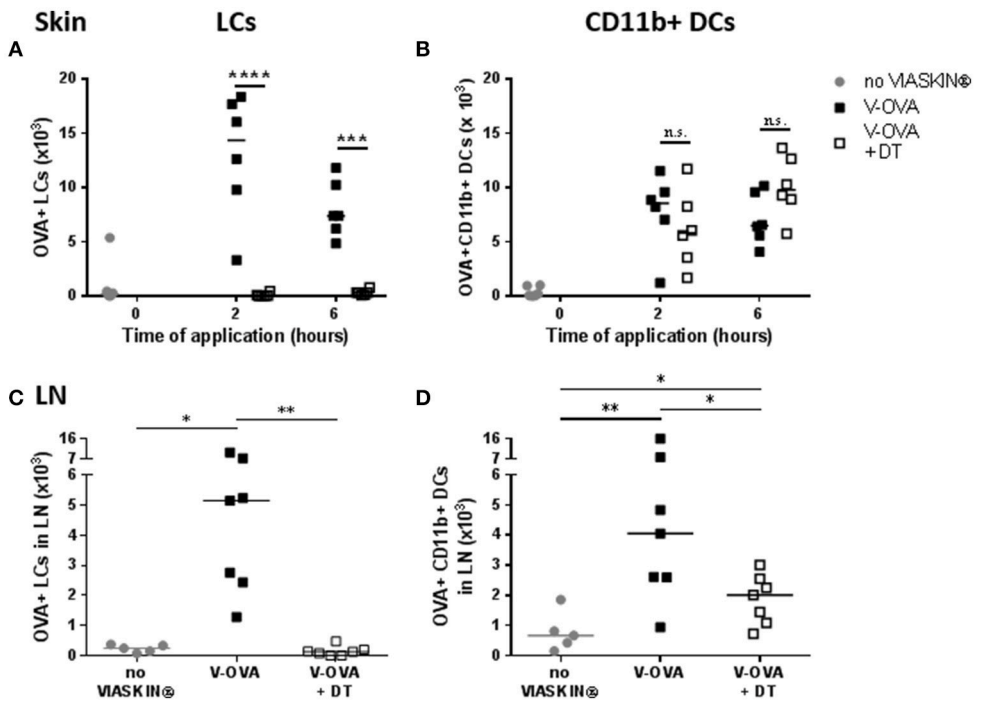

FIGURE 5 | Effect of LCs depletion on allergen uptake by skin Dendritic Cells. VIASKIN®-OVA-AF647 (V-OVA) was applied on the back of sensitized mice (V-OVA) or sensitized mice after depletion of Langerhans cells (V-OVA + DT). Sensitized not treated mice were used as control (no VIASKIN®). Skin **(A,B)** were harvested after 2 or 6 h and sdLNs were harvested after 48 h. Cells were isolated and counted before immunostaining for flow cytometry analysis. In live single cells gate, migrating DCs were gated as MHC-II^{high} CD11c$^+$ and LCs **(A,C)** and CD11b$^+$ DCs **(B,D)** were identified as CD11b$^+$EPCAM$^+$XCR1$^-$ and CD11b$^+$EPCAM$^-$XCR1$^-$, respectively. Proportion of OVA$^+$ cells inside each population were measured and reported to number of cells. Each symbol represents a mice and bar represent median. Experiment was reproduced twice independently. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

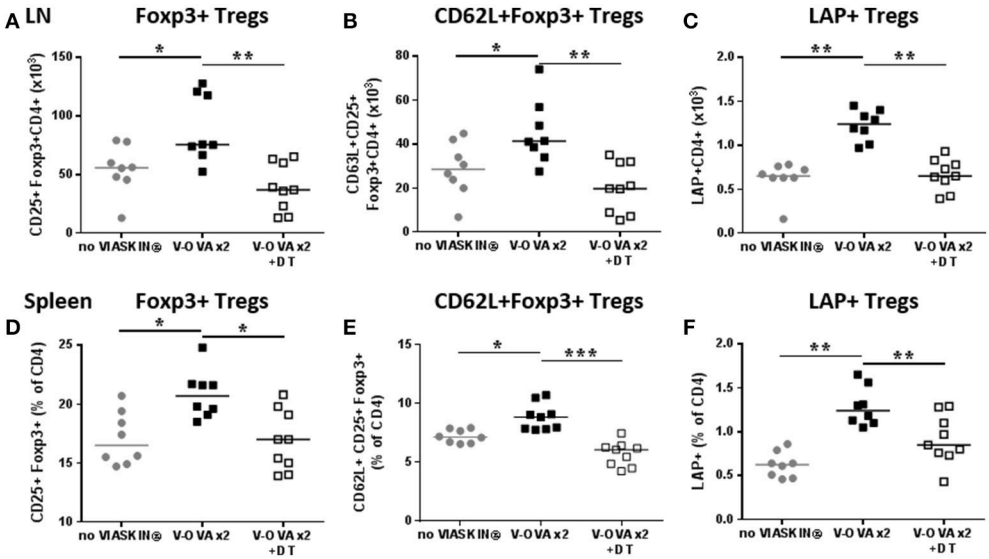

FIGURE 6 | Short term induction of Tregs by EPIT requires LCs. VIASKIN®-OVA (V-OVA) was applied on the back of sensitized mice (V-OVA) or sensitized mice after depletion of Langerhans cells (V-OVA + DT) for 48 h once a week for 2 weeks and level of Tregs was evaluated the week after the 2nd application in sdLN **(A–C)** and spleen **(D–F)**. Sensitized not treated mice were used as control (no VIASKIN®). Cells were isolated and counted before immunostaining for flow cytometry analysis. Number of $CD4^+CD25^+Foxp3^+$ **(A)**, $CD4^+CD62L^+CD25^+Foxp3^+$ **(B)** and $CD4^+LAP^+$ **(C)** cells in sdLN and proportion of $CD25^+Foxp3^+$ **(D)**, $CD62L^+CD25^+Foxp3^+$ **(E)** and LAP^+ **(F)** among CD4 cells were analyzed. Each symbol represents a mice and bar represent median. Experiment was reproduced twice independently. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

$CD62L^+$ Tregs compared to the Control group (**Figures 7A,B**). In contrast to mice treated for 2 weeks, no induction of LAP^+ Tregs was observed after 8 weeks of treatment suggesting that the induction of LAP^+ cells is transient. In the absence of LCs, no induction of Tregs was observed even after 8 weeks of EPIT treatment, confirming the crucial role of LCs in induction of Tregs by EPIT.

Role of LCs in the Induction of Tolerance by EPIT

To confirm that the induction of Tregs by EPIT is associated with desensitization, the anaphylactic response following oral challenge was evaluated in sensitized mice treated by EPIT and compared to untreated animals. Untreated mice experienced a significant decrease in body temperature (median $= -3°C$) following oral challenge with OVA compared to naïve mice. Injection of DT did not alter this response. EPIT treated mice showed a $-0.85°C$ drop in body temperature, significantly less than that in untreated mice (**Figure 8A**). This protection from anaphylaxis was associated with a significant decrease of OVA-specific IgE levels (**Figure 8B**) and a decrease in cytokine response by *in vitro* re-stimulated splenocytes (**Figures 8C–E**) from EPIT-treated mice compared to untreated controls.

In mice treated in absence of LCs, oral challenge induced anaphylactic reactions with a significant drop in body temperature of $-2.2°C$ compared to EPIT-treated mice (**Figure 8A**). EPIT did not induce any decrease of sIgE nor OVA specific cytokine responses when LCS were depleted (**Figures 8B–E**). These results support the crucial role of LCs in mechanisms of induction of tolerance by EPIT in sensitized mice.

DISCUSSION

The different populations of skin DCs are key players in antigen-specific immune activation or regulation. Indeed, skin has been proposed as route of sensitization, but perhaps more importantly, is also a way to induce tolerance, as in the case of EPIT. The role of the different population of skin DCs in this tolerance induction requires further clarification. In this study, we demonstrated that both LCs and $CD11b^+$ dermal cDC2s can take up allergen in the skin and migrate to draining LN to induce regulatory T cells. However, the absence of LCs during EPIT impaired allergen uptake and $Foxp3^+$ Treg induction, especially $CD62L^+$ Tregs, resulting in absence of desensitization and protection from oral allergen exposure, indicating their crucial role in skin-induced tolerance.

The role of LCs in inducing allergic responses or suppressing immunity is still controversial. In the steady state, LCs have been shown to induce tolerance to skin antigen (16–18). LCs have also been implicated in Treg expansion in response to antibody-targeted antigen or irradiation (19, 20). Initiation of epicutaneous sensitization with protein antigens depended on LCs (21) whereas skin sensitization to house dust mite depended on dermal cDC2 and was even increased in absence of LCs (22). In our model, we demonstrated that LCs are critical for the induction of Tregs and subsequent desensitization of

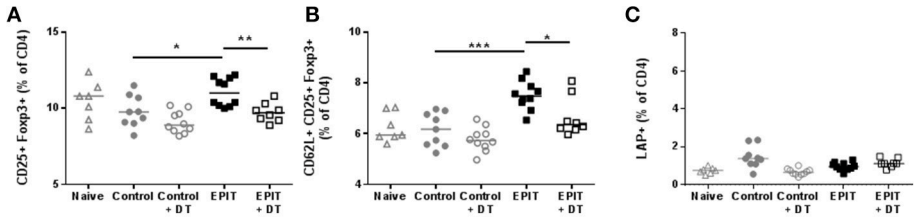

FIGURE 7 | LCs are required for the induction of regulatory T cells. Sensitized mice were treated with VIASKIN® containing 100 μg OVA for 48 h once a week in presence (EPIT) or absence (EPIT + DT) of LCs. Not treated sensitized mice were used as control and effect of DT was also checked in a control group (Control + DT). After 8 weeks of treatment, induction of Tregs was measured by the proportion of $CD25^+Foxp3^+$ **(A)** and $CD62L^+CD25^+Foxp3^+$ **(B)** and LAP^+ **(C)** cells among $CD4^+$ splenocytes Each symbol represents a mice and bar represent median. Experiment was reproduced twice independently. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test.

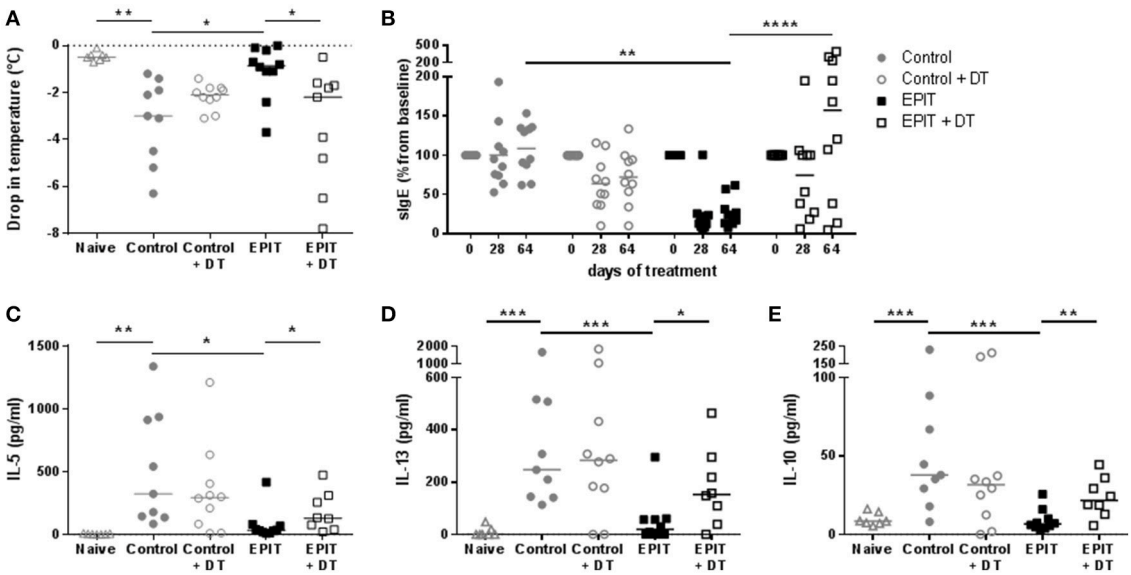

FIGURE 8 | Desensitization induced by EPIT is not observed in absence of LCs. Sensitized mice were treated with VIASKIN® containing 100 μg OVA for 48 h once a week in presence (EPIT) or absence (EPIT + DT) of LCs. Not treated sensitized mice were used as control and effect of DT was also checked in a control group (Control + DT). After 8 weeks, mice were challenged orally and anaphylactic reaction was measured by the drop in temperatures **(A)**. Percentage of OVA-specific IgE compared to baseline was measured after 28 and 64 days of treatment **(B)**. Production of IL-5 **(C)**, IL-13 **(D)**, and IL-10 **(E)** by *in vitro* restimulated splenocytes isolated at the end of treatment were analyzed. Each symbol represents a mice and bar represent median. Experiment was reproduced twice independently. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test **(A,C–E)**; and $^{**}p < 0.01$; $^{****}p < 0.0001$ by 2-way ANOVA followed by Tukey's multiple comparison test **(B)**.

mice by EPIT, suggesting of tolerogenic capacities of LCs in the context of previously established sensitization. Differences of immunological status and/or keratinocyte activation could explain the divergence concerning the role of LCs between the different models, as we previously observed their impact on allergen uptake (12, 23).

Dermal cDC2s have been previously implicated in IL-17-mediated psoriasis-like response (24). These cells appear critical for the induction of Th2 responses and epicutaneous sensitization to house dust mite (22, 25). On the other hand, cDC2s can suppress antibody production by blocking the development of follicular helper T cells, and inducing $Foxp3^+$ Tregs (26, 27). In our model, dermal $CD11b^+$ cDC2s were as efficient as LCs in taking up allergen following epicutaneous application and migrating into sdLNs. Moreover, migrating cDC2s could induce $Foxp3^+$ Tregs, suggesting that they may play a role in tolerance induction during EPIT. However, these cells are not sufficient since depletion of LCs without alteration of the $CD11b^+$ DC compartment failed to induce Tregs *in vivo*. We can postulate that cDC2s play a role in the mechanisms of induction of Tregs in sensitized mice in which they need interaction of LCs to be fully activated and to migrate. Indeed, depletion of LCs did not alter allergen capture by skin cDC2s, but did impair their migration into sdLNs. Interaction between LCs and dermal DCs may be needed for optimal migration of dermal DCs. Cooperation of LCs and dermal DCs has

been observed previously in the induction of Th2 responses (28), and migrating LCs can also interact with DCs to induce DC maturation and antigen transfer (29). Further studies are required to decipher the possible interactions between these populations and by which mechanisms LCs cooperate with DCs.

In addition to the possible interactions between LCs and dermal DCs, cDC2s were not sufficient to induce Tregs and provide protection *in vivo* because particular Treg populations implicated in EPIT were not induced by cDC2s. *In vitro*, migrating cDC2s induced Foxp3$^+$ Tregs, but low levels of LAP$^+$ Tregs. The induction of LAP$^+$ Tregs has been observed during EPIT in different models (6, 9). The absence of induction of these LAP$^+$ Tregs by CD11b$^+$ DCs could explained the absence of protection observed after depletion of LCs. Noteworthy, the induction of LAP$^+$ Tregs seems transient in this model with a significant increase following 2 weeks of treatment, which is no longer observed after 8 weeks of treatment either in the sdLN or in the spleen. The LAP$^+$ Tregs may participate in the first mechanistic steps of EPIT to induced CD25$^+$Foxp3$^+$ Tregs that are crucial for efficacy of EPIT (8).

One unique feature of EPIT is its ability to induce both CD62L$^+$ and CD62L$^-$ Foxp3$^+$ Tregs, whereas only CD62L$^-$ Tregs are induced by other immunotherapies (oral and sublingual) in our models (9). This population of CD62L$^+$ Tregs seems central to the mechanism of EPIT given the epigenetic modification, i.e., foxp3 demethylation, the sustained protection and the bystander effect, which were recently shown to be features of the CD62L$^+$ population of Tregs (10). Migrating LCs induced significantly more CD62L$^+$ Tregs than cDC2s *in vitro* and the depletion of LCs abrogated any induction of CD62L$^+$ Tregs *in vivo*. Given the importance of this particular population of Tregs in EPIT, the capacity of LCs to induced them may explain their crucial role in efficacy of EPIT.

As previously shown for gut DCs (14, 15), skin migrating LCs, but not cDC2s, expressed higher level of integrin beta8, suggesting that requirement of LCs to induce particular population of Tregs could be due to their capacity to activate TGF-β. However, further experiments are needed to decipher the exact role of this pathway in mechanism of induction of Tregs during EPIT.

This unique property of cutaneous LCs is also distinguished from the induction of Tregs with sublingual immunotherapy (SLIT). Although Langerhans-like cells are present in the oral mucosa, studies of allergen uptake during SLIT in an animal model demonstrated an important role for both CD11b$^+$ DCs and CD11b$^+$ macrophage-like cells (30, 31). These populations of APCs support the differentiation of Tr1 cells. Thus, the difference of Tregs induced by EPIT compared to SLIT may be explained by the intrinsic difference of APC implicated in these different routes of treatment. Similarly, subcutaneous immunotherapy, which "bypasses" the epidermidis and resident LCs, induces Tr1 cells, suggesting the importance of LCs, and epicutaneous route for induction of the CD62L$^+$ population of Tregs.

In conclusion, we demonstrated that during EPIT of sensitized mice, allergen is taken up by LCs and dermal cDC2s that migrate to the sdLNs to induce Tregs. However, the capacity to induce both CD62L$^+$ Foxp3$^+$ Tregs and LAP+ Tregs reside mainly in LCs. Indeed, the absence of LCs during EPIT decreased treatment efficacy indicating their crucial role in skin-induced tolerance.

ETHICS STATEMENT

Project license from French authorities #7811.

Comité d'Ethique en Expérimentation Animale de DBV Technologies N°127 (CEEA127).

AUTHOR CONTRIBUTIONS

ViD wrote the article and is corresponding author. LM had valuable contribution to the underlying concept and the design of the research. LL, VéD, CP, and AB performed the experiments and some analysis. CD and HS (senior author) had valuable contribution to the underlying concept and the design of the research.

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Eliciting Dose and Safety Outcomes From a Large Dataset of Standardized Multiple Food Challenges

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Background: Food allergy prevalence has continued to rise over the past decade. While studies have reported threshold doses for multiple foods, large-scale multi-food allergen studies are lacking. Our goal was to identify threshold dose distributions and predictors of severe reactions during blinded oral food challenges (OFCs) in multi-food allergic patients.

Methods: A retrospective chart review was performed on all Stanford-initiated clinical protocols involving standardized screening OFCs to any of 11 food allergens at 7 sites. Interval-censoring survival analysis was used to calculate eliciting dose (ED) curves for each food. Changes in severity and ED were also analyzed among participants who had repeated challenges to the same food.

Results: Of 428 participants, 410 (96%) had at least one positive challenge (1445 standardized OFCs with 1054 total positive challenges). Participants undergoing peanut challenges had the highest ED_{50} (29.9 mg), while those challenged with egg or pistachio had the lowest (7.07 or 1.7 mg, respectively). The most common adverse event was skin related (54%), followed by gastrointestinal (GI) events (33%). A history of asthma was associated with a significantly higher risk of a severe reaction (hazard ratio [HR]: 2.37, 95% confidence interval [CI]: 1.36, 4.13). Higher values of allergen-specific IgE (sIgE) and sIgE to total IgE ratio (sIgEr) were also associated with higher risk of a severe reaction (1.49 [1.19, 1.85] and 1.84 [1.30, 2.59], respectively). Participants undergoing cashew, peanut, pecan, sesame, and walnut challenges had

more severe reactions as ED increased. In participants who underwent repeat challenges, the ED did not change ($p = 0.66$), but reactions were more severe ($p = 0.02$).

Conclusions: Participants with a history of asthma, high sIgEr, and/or high values of sIgE were found to be at higher risk for severe reactions during food challenges. These findings may help to optimize food challenge dosing schemes in multi-food allergic, atopic patients, specifically at lower doses where the majority of reactions occur.

Trials Registration Number: ClinicalTrials. gov number NCT03539692; https://clinicaltrials.gov/ct2/show/NCT03539692.

Keywords: oral food challenge, adverse events, dose curves, food allergy, safety outcome

INTRODUCTION

The prevalence of food allergies has continued to rise over the past decade and has become a significant health issue (1). Food allergies have become more common, and now affect 6–11% of the population in the United States, Canada, Australia, and Europe (2–8). Among children, 40% are affected by two or more food allergies (9). The diagnosis of food allergies imposes a significant burden on patients and their families and leads to a decreased quality of life due to dietary restrictions, increased anxiety, and social limitations (10). In recent years, in the US, the number of emergency room visits for food-induced anaphylaxis has risen to ∼200,000/year and continues to rise (11, 12).

The double-blind placebo-controlled food challenge (DBPCFC) is the gold standard method to diagnose food allergies. Recent studies have focused on comparing the utility of other clinical factors to be able to predict food challenge outcomes (13) and to understand the role of allergen-specific IgE (sIgE) and skin prick tests (SPTs) (14). However, there have been few comparisons of multiple DBPCFCs performed across a large population in which the challenges were done with the same standardized method. In a prior publication from our group (15), we demonstrated the presence of multiple food allergies in many individuals. Our sites perform clinical trials in food allergy and as such, a large number of DBPCFCs are conducted in a medical facility with trained personnel using the same doses and time intervals in a food challenge. Sometimes participants undergo repeat food challenges (without interim intervention) to the same allergen for qualification into clinical trials. Therefore, the objective of this research was to test whether food challenge reactions, if repeated over time, differed by severity, by eliciting dose (ED), or by organ system involvement. This was determined according to the type or dose of food allergen (16, 17). Another objective was to assess whether certain food allergens were associated with a certain type of reaction (i.e. a gastrointestinal (GI) allergic reaction vs. a skin allergic reaction).

MATERIALS AND METHODS

Oral Food Challenges (OFCs)

From September 2010 to March 2016, participants with suspected food allergy were recruited to undergo standardized food challenges to at least 500 mg of cumulative food protein to each of their allergens as part of screening for clinical trial enrollment. The low cutoff of 500 mg of food protein was chosen as these subjects had a high likelihood of exhibiting an allergic reaction. The precise amounts of commercially available, FDA standardized and validated GMP-grade protein were quantified based on protein gels, prepared and weighed out in our GMP facility, and distributed to other sites under a clinical trial agreement that ensured consistency in challenge material from batch to batch and between sites. Patients with a prior history of food-allergy reaction requiring intubation or eliciting hypotension were excluded, while patients with previous reactions to food requiring epinephrine for other severe symptoms were eligible. During the initial screening visit before multiple studies, SPT and IgE testing were performed at the Center for some trials, whereas, for others, results from prior testing at a physician's office were included. SPT consisted of a positive histamine control, a negative saline control (both from Hollister-Stier) and allergen extracts from Greer. SPTs were performed on the volar surface of the forearm or back after application of the respective allergen solution. Mean wheal diameter was measured after 20 min. Allergen-specific IgE levels were measured by ImmunoCAP fluorescence enzyme immunoassay.

One thousand four hundred and forty-five DBPCFCs were performed using standardized methodology according to validated guidelines (18–20). The same DBPCFC methods and doses were used across the Sean N. Parker Center for Allergy and Asthma Research at Stanford University, Cincinnati Children's Medical Center, Robert H. Lurie Children's Hospital of Chicago, Children's Hospital of Philadelphia, Virginia Mason Medical Center, Seattle Children's Hospital, Icahn School of Medicine at Mount Sinai, and Children's Hospital Los Angeles. All personnel were trained using procedures as per the protocol. Each challenge consisted of several escalating doses of the food protein in flour form concealed in an appropriate vehicle, such as applesauce or pudding, ingested by the participant every 15 min as tolerated. Challenges to almond, cashew, egg, hazelnut, milk, peanut, pecan, pistachio, sesame, walnut, and wheat were included in the analyses. Typically challenges started with as small as 1 mg (for pistachio), then 2, 5, 20, 50, 100, 100, 100, 123 (for pistachio), or 124 mg. Patients challenged with pistachio were individuals

with a known cashew allergy, and, as such, pistachio challenges were started at 1 mg due to concerns for safety. All allergen doses indicate mg of food protein. Those participants with positive DBPCFCs to placebo (oat) were excluded. A subset of patients performed repeat challenges to the same food in the course of screening for multiple trials. Vital signs and pertinent physical examinations were repeated every 15 min, or more frequently during the challenge, at the discretion of the clinician. Reaction types and severities were determined according to modified Bock criteria (18) and Common Terminology Criteria for Adverse Events (CTCAE v 4.03). Some studies recorded symptoms in CTCAE criteria and some with modified Bock. Our ranking system was based on Bock and the CTCAE was converted to Bock grading by allergists on our team. All objective and subjective symptoms were recorded and ranked against one another in order of severity by onsite physicians based on their clinical judgment. Subjective symptoms included abdominal pain, oropharyngeal itching, nausea, or pruritus. Objective adverse symptoms were regarded as more severe than subjective symptoms of the same grade and this was taken into consideration when ranking symptoms in **Table 1**. Participants tolerating at least 500 mg cumulative dose during the challenge were considered to be negative responders for the purposes of this analysis. All aspects of the studies from which data was obtained were authorized by the IRB.

TABLE 1 | Ranked adverse events by severity.

Symptom	Rank
Mild pruritus	1
Moderate pruritus	2
Mild nasal itching	3
Moderate nasal itching	4
Severe nasal itching	5
Mild nausea	6
Moderate nausea	7
Severe nausea	8
Mild Ab pain	9
Moderate Ab pain	10
Mild rhinorrhea	11
Mild nasal congestion	12
Moderate rhinorrhea	13
Mild sneezing	14
Moderate nasal congestion	15
Mild rash	16
Mild urticaria	17
Moderate sneezing	18
Mild angioedema	19
Severe rhinorrhea	20
Severe nasal congestion	21
Mild cough	22
Severe sneezing	23
Mild emesis	24
Severe Ab pain	25
Severe pruritus	26
Moderate rash	27
Moderate emesis	28
Moderate angioedema	29
Moderate cough	30
Moderate urticaria	31
severe rash	32
Severe urticaria	33
severe emesis	34
Severe angioedema	35
Severe cough	36
Mild airway obstruction	37
Moderate airway obstruction	38
Severe airway obstruction	39
Mild wheezing	40
Moderate wheezing	41
Severe wheezing	42
Mild cardio	43
Moderate cardio	44
Severe cardio	45

Higher ranking indicates more severe symptoms.

Data Management

Any value of sIgE greater than 100 IU/L was truncated to 101 for statistical analysis. Only SPT and/or sIgE that were collected within 12 months of the OFC were included in the analysis. If a subject had more than one value for SPT or sIgE, then the value obtained closest to the challenge was used (14). Negative control SPTs were subtracted from the raw food SPTs prior to analysis. If the newly derived SPT was negative, it was set to zero. Any SPT that was collected after the food challenge or collected more than 12 months before the

TABLE 2 | Baseline demographics.

Characteristic*	Total (n = 410)
Age in years, median (range)	9 (1–52)
Male	250 (61%)
Non-hispanic	390 (97%)
RACE	
Caucasian	250 (62%)
Black	6 (1%)
Asian	106 (26%)
Multiracial	37 (9%)
Other	5 (1%)
ATOPIC HISTORY	
Asthma	232 (62%)
Allergic rhinitis	284 (77%)
Atopic dermatitis	272 (74%)
Number of food allergens, median (range)	5 (1–16)
Mono-food allergic	8 (2%)
Total IgE (IU/L), median (range)	498.5 (18–3366)

**Count and percent of total subjects unless otherwise noted.*

challenge was excluded. If a subject had more than one value for either SPT or sIgE, then the value obtained most recently was used.

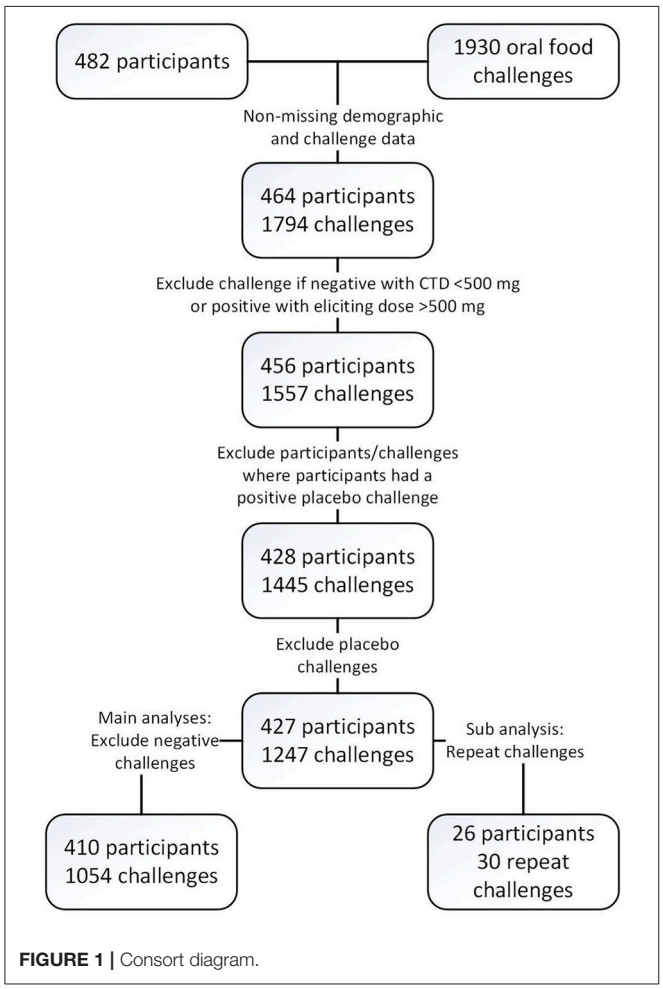

FIGURE 1 | Consort diagram.

In an effort to standardize OFCs across studies, challenges that were considered positive in their original studies based on thresholds higher than 500 mg but had cumulative tolerated doses (CTDs) of 500 mg or higher were re-classified as having negative challenges with no eliciting dose (ED) to a cumulative of 500 mg of protein. Subjects who had unknown or non-reported ethnicity were coded as missing ethnicity. Subjects with race of Native Hawaiian, other, or not reported were coded as other. Only positive challenge data were analyzed.

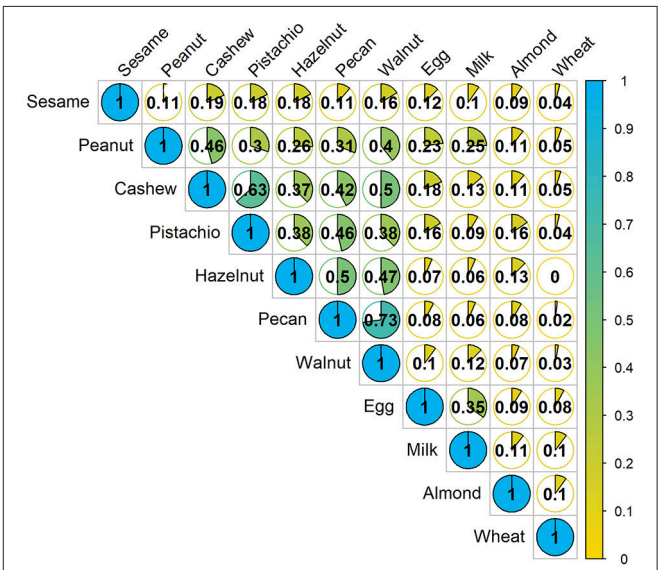

FIGURE 2 | Concurrent occurrences of food allergy based on food challenge outcomes: The fraction in each cell represents the Jaccard similarity coefficient, which is the amount of co-allergy accounting for the number of positive challenges for each allergen separately. Higher values indicate more similarity between the two allergens. Denominator only includes participants who were screened for multi-food allergy studies.

TABLE 3 | Eliciting dose (ED) thresholds by food.

Challenge Food	N	Number of subjects (% of total)	Eliciting dose (mg) median (range)	Eliciting dose curves (ED) (mg) (95% CI)		
				ED_5	ED_{10}	ED_{50}
Almond✠	30	29 (7)	25.0 (5–500)	0.86 (0, 1.92)	1.73 (0, 3.60)	20.77 (5.76, 35.78)
Cashew	151	150 (35)	25.0 (0.1–500)	0.07 (0, 0.13)	0.25 (0.05, 0.46)	8.78 (5.40, 12.16)
Egg	63	60 (14)	8.1 (0.1–500)	0.04 (0, 0.12)	0.18 (0, 0.42)	7.07 (2.61, 11.54)
Hazelnut	68	65 (15)	25.0 (1.6–500)	0.07 (0, 0.17)	0.29 (0, 0.68)	14.38 (5.36, 23.39)
Milk	67	66 (15)	32.7 (1.7–500)	0.21 (0, 0.49)	0.74 (0, 1.55)	20.41 (9.73, 31.09)
Peanut	347	330 (77)	75.0 (0.1–500)	0.49 (0.24, 0.73)	1.52 (0.89, 2.15)	29.90 (23.81, 35.98)
Pecan✠	88	88 (21)	25.0 (1.7–500)	0.38 (0.04, 0.71)	0.79 (0.19, 1.39)	10.68 (5.71, 15.64)
Pistachio	60	59 (14)	5.0 (5–275)	0 (0, 0.1)	0.01 (0, 0.04)	1.71 (0, 3.61)
Sesame	30	30 (7)	25.0 (5–500)	0.26 (0, 0.75)	0.88 (0, 2.24)	21.19 (5.28, 37.10)
Walnut	121	120 (28)	25.0 (1.7–500)	0.15 (0, 0.31)	0.56 (0.07, 1.05)	18.01 (10.54, 25.47)
Wheat	13	13 (3)	32.7 (5–500)	0.03 (0, 0.17)	0.16 (0, 0.75)	12.64 (0, 33.20)

All models fit to Weibull distribution unless otherwise noted by✠ (Log-normal).

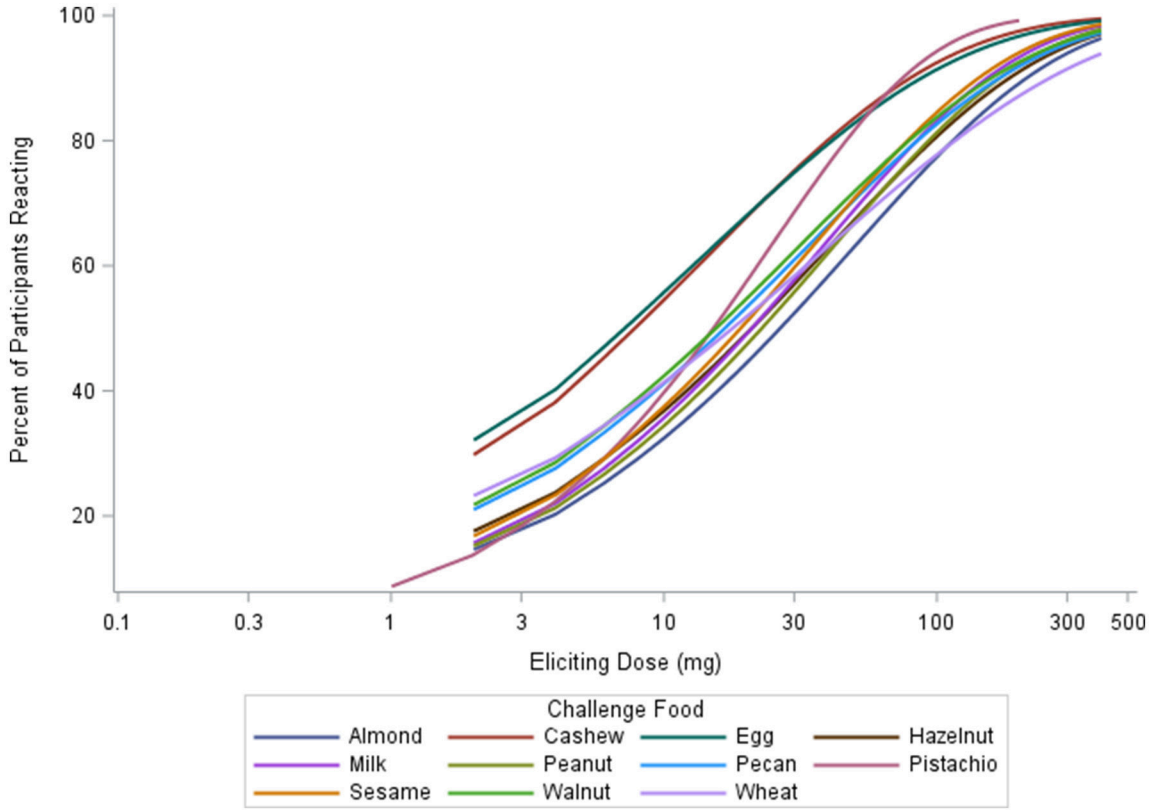

FIGURE 3 | Eliciting dose (ED) thresholds by allergen.

Statistical Analysis

To determine how often participants were allergic to multiple foods, pairwise comparisons of all major foods were conducted. The Jaccard similarity coefficient was implemented, accounting for the different number of participants allergic to each food (21). A detailed description of this method and its implementation in food studies has been previously published (22). Only participants who conducted food challenges for multi-food studies were included in this analysis.

To determine ED curves for each challenge food, data were analyzed using interval-censoring survival analysis fitted to three different probability distributions (Log-Normal, Log-Logistic, and Weibull) to estimate the ED for 5, 10, and 50% of patients (23).The three distributions were compared for each food, and the one with the lowest Akaike information criteria (AIC) was chosen. Interval-censoring analysis uses the lowest- and no-observed adverse effect levels (LOAELs and NOAELs) based on challenge information (23). If a participant reacted at the first challenge dose, the NOAEL was set to zero and the LOAEL was set to the first challenge dose. Turnbill intervals were implemented due to overlapping dose steps from various studies. The estimated ED and 95% confidence intervals were reported at each ED level. SAS's PROC LIFEREG was used to implement the analysis (24).

Multiple symptoms could have been reported during each challenge based on participant symptoms. Based on clinical reasoning, all 45 possible symptoms (3 grades for each of the 15 symptoms) were ranked in order of severity (**Table 1**). This list was then used to select the most "severe" symptom reported from each challenge. Therefore, only the most severe symptom reported [grade and SOC (system organ class)] was analyzed per challenge. Frailty models were fit to "time" (i.e., eliciting dose)

TABLE 4 | Adverse events by allergen and organ system.

	Number of AEs (% Total)				Total
Allergen	**Gastrointestinal**	**Respiratory**	**Skin**	**Other**	
Almond	9 (20.5)	3 (6.8)	32 (72.7)	0 (0.0)	44
Cashew	116 (37.2)	40 (12.8)	150 (48.1)	6 (1.9)	312
Egg	42 (36.8)	14 (12.3)	57 (50.0)	1 (0.9)	114
Hazelnut	22 (23.2)	10 (10.5)	63 (66.3)	0 (0.0)	95
Milk	23 (21.1)	14 (12.8)	71 (65.1)	1 (0.9)	109
Peanut	292 (36.7)	108 (13.6)	389 (48.9)	6 (0.8)	795
Pecan	49 (29.7)	20 (12.1)	95 (57.6)	1 (0.6)	165
Pistachio	26 (28.0)	6 (6.5)	61 (65.6)	0 (0.0)	93
Sesame	18 (39.1)	3 (6.5)	25 (54.3)	0 (0.0)	46
Walnut	61 (31.3)	23 (11.8)	110 (56.4)	1 (0.5)	195
Wheat	1 (7.1)	2 (14.3)	10 (71.4)	1 (7.1)	14
Total	666 (33.1)	247 (12.3)	1084 (53.8)	17 (0.8)	2014

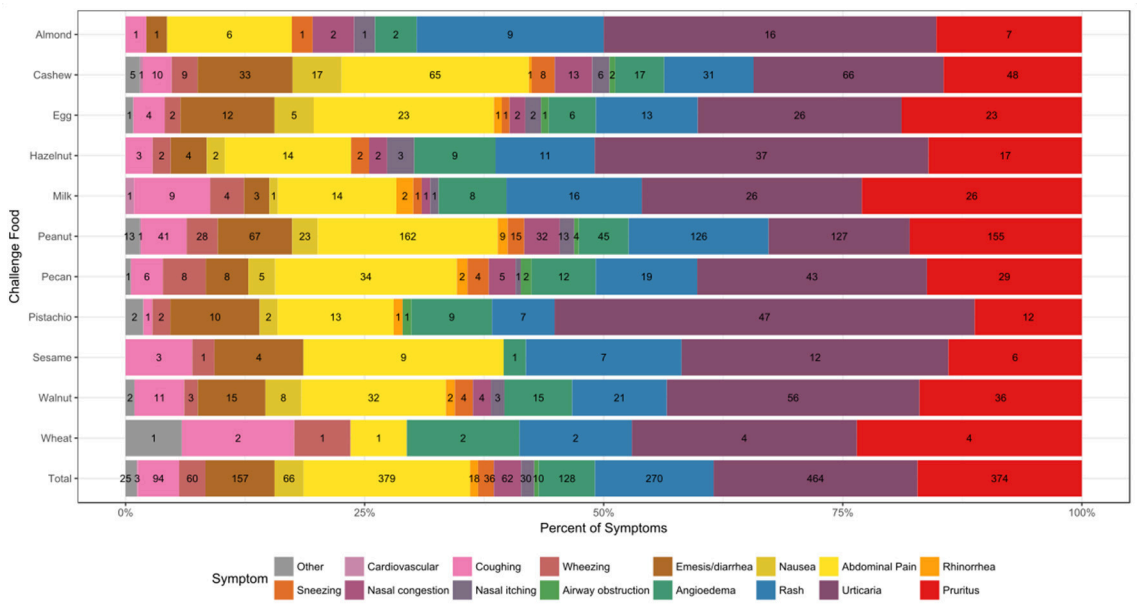

FIGURE 4 | Symptom type by allergen. Reaction counts are reported within the figure.

until the most severe symptom as a function of each clinical and demographic feature. An event was defined by whether or not the most severe symptom observed was a Bock grade 3. For each model, each participant contributed multiple observations corresponding to the number of food challenges. Due to possible correlations within participant or within food, random effects for participant and food were included in each model. Hazard ratios and 95% CIs were reported. Further, the correlation between ED and the severity ranking was measured by challenge food using the Spearman rank correlation test.

A subset of participants was challenged to the same food twice. The Kruskal-Wallis rank sum test was used to test whether ED changed from the first to second challenge. Spearman's rank order correlation was used to assess the association between change in ED and number of months between repeat challenges. These two tests were also used to assess changes in the symptom severity ranking. Lastly, Spearman's rank order correlation was also used to determine if change in ED was associated with change in symptom rank. *P*-values were reported.

All analyses were conducted at the 0.05 alpha level. No adjustments for multiple comparisons were made. Analyses were conducted using R v.3.4.3 (25) and SAS Software (24). Data are available and can be found on a secure REDcap database that is part 11 compliant.

RESULTS

Baseline Demographics

Age of participants ($n = 410$) ranged from 1 to 52, with a median age of 9 years old, and the cohort was comprised of mostly non-Hispanic (97%), Caucasian (62%), and males (61%). The majority of participants also had an atopic history, including asthma (62%), allergic rhinitis (77%), and atopic dermatitis (74%). The average number of doctor-diagnosed food allergies was 5, with only 2% of the cohort being mono-food allergic. The median total IgE (tIgE) was 499 kU/L (**Table 2**).

Challenge Overview

Four hundred and twenty-seven participants across multiple studies contributed 1,445 baseline challenges to the database (**Figure 1** and **Table 3**) of which 410 had 1,054 positive challenge outcomes. The most common positive challenge was for peanut ($n = 347$) followed by cashew ($n = 151$) and walnut ($n = 121$; **Table 3**). Seventy-seven percent of participants had a peanut allergy.

A Jaccard analysis assessing the similarity of co-allergy among the foods which were challenged in our cohort is illustrated in **Figure 2**. A higher similarity index corresponds to a higher degree of overlap of results obtained between two foods. Overall, higher similarity was observed within peanut and tree nut allergies compared to milk, egg, wheat or sesame. Allergies to pecan and walnut were 73% similar, followed by cashew and pistachio, which were 63% similar.

Eliciting Dose

The median ED was <35 mg of food protein for all foods, except for peanut, with the highest median ED at 75 mg, and pistachio, having the lowest at 5 mg (**Table 3**). Participants undergoing peanut challenges had the highest ED_{50} dose (i.e., the dose which elicits a reaction in 50% of subjects in those that ultimately react) of all foods (29.9 mg), followed by sesame (21.2 mg) and almond (20.7 mg). Pistachio had the lowest dose to elicit a reaction in 50% of subjects at 1.7 mg, however, only the participants with a positive reaction to cashew were

challenged with pistachio. Participants challenged with egg had the second lowest ED_{50} dose (7.07 mg). Across each of the three ED thresholds, almond and peanut consistently had the highest dose values. A higher percentage of participants challenged with egg and cashew reacted at lower EDs compared to other foods (**Figure 3**). Participants undergoing pistachio challenges had the largest increase in reactions over EDs than any other food, while participants with wheat had the lowest increase in percentage of participants reacting.

Adverse Events

A total of 2014 adverse events occurred during the 1,054 positive challenges (**Table 4**). The majority of adverse events occurred during peanut challenges ($n = 795$) followed by cashew ($n = 312$), which were also the most frequent challenges conducted. Within each food, adverse events related to skin were the most prevalent (54%), followed by GI events (33%). More specifically, urticaria and pruritus were the most common skin reactions, while abdominal pain was the most common GI reaction (**Figure 4**). The distribution of symptom type was similar across foods.

Table 1 lists the ordered rank of the potential adverse events that could occur during each participant's challenge, with lower ranked adverse events corresponding to more concerning symptoms. For example, severe cardiac symptoms, with a severity grade of 3, was ranked as number 45, compared to pruritus, with a severity grade of grade 1, which was ranked as number 1. Among the lower ranked adverse events (based on modified Bock criteria) (18), 673 (74%) were graded as mild, 134 (15%) as moderate, and 98 (11%) as severe (data not shown).

Participants with a history of asthma were more than twice as likely to have their most severe AE be a Bock grade of 3 at any point in their challenge compared to those without a history of asthma (hazard ratio [HR]: 2.37, 95% confidence interval [CI]: 1.36, 4.13; **Table 5**). Higher values of sIgE and sIgEr were significantly associated with higher risk of experiencing a severe reaction [HR: 1.49 [1.19, 1.85] and 1.84 [1.30, 2.59], respectively]. Participants who were challenged with cashew, peanut, pecan, sesame, and walnut had a higher severity ranking that was significantly associated with higher ED and, as ED increased, so did the severity (**Figure 5**).

Repeat Challenges

Of the 1445 total challenges (positive and negative), 30 were repeated by 26 participants. Only one participant had two repeat challenges to the same allergen (peanut), while all others only repeated a challenge to the same food once. Out of the 1054 positive baseline challenges, 21 were repeats with positive challenge outcomes, corresponding to 18 participants. Sixteen repeat challenges were to peanut, two to egg, and one each to almond, milk, and walnut (**Figure 6**). One participant had a repeat negative challenge to peanut and another had a repeat negative challenge to almond. The delta change in severity ranking from first to second challenge was significantly different from zero ($p = 0.04$; Wilcoxon signed rank test).

TABLE 5 | Univariate associations of severity.

Characteristic	Not Severe	Severe	Hazard ratio (95% CI)	Challenges included
Female	40%	39%	0.96 (0.6, 1.53)	905
Hispanic	98%	99%	1.11 (0.14, 8.74)	887
Race (ref = Caucasian)				895
Black	1%	1%	0.93 (0.11, 7.88)	
Asian	29%	37%	1.56* (0.95, 2.59)	
Multiracial	11%	5%	0.62 (0.23, 1.69)	
ATOPIC HISTORY				
Asthma	60%	76%	2.37** (1.36, 4.13)	825
Allergic rhinitis	77%	82%	1.1 (0.59, 2.04)	812
Atopic dermatitis	77%	75%	1.05 (0.59, 1.86)	813
Age	8	8	0.99 (0.96, 1.03)	905
FEV_1	99	99	1 (0.98, 1.03)	494
FEV_1/FVC	0.85	0.86	4.23 (0.04, 457.57)	492
Mono-Allergic	2%	2%	0.51 (0.09, 3.02)	905
Number of diagnosed food allergies	6	5	1 (0.93, 1.09)	905
sIgE (log-scale)	17	43	1.49*** (1.19, 1.85)	575
tIgE (log-scale)	439	583	1.2 (0.81, 1.78)	385
sIgEr (log-scale)	0.04	0.06	1.84*** (1.3, 2.59)	385
SPT	12	13.5	1.04* (1, 1.08)	600

*Each column corresponds to a single frailty model. SPT, skin prick test; sIgE, allergen-specific Immunoglobulin E; sIgEr, ratio of sIgE to total IgE (tIgE). Values in the "Not Severe" and "Severe" columns are the percentages, means, and medians for each characteristic on the raw scale. Median values are presented for age and each biomarker. *$p < 0.10$; **$p < 0.05$; ***$p < 0.01$.*

Additionally, the median time between repeat challenges was 735 days (range 2–982). While there was no difference in ED from the first to second challenge ($p = 0.66$), the severity rank significantly increased in the second challenge, corresponding to more severe symptoms experienced ($p = 0.02$, **Figure 6A**). By contrast, there was no significant association between change in ED and change in severity rank from the first to second challenge ($p = 0.14$, **Figure 6B**). Change in either ED or severity rank was not associated with time between repeat challenges ($p = 0.94$ and $p = 0.56$, respectively, **Figure 6C**).

DISCUSSION

The diagnosis of food allergy is highly complex (20, 26). Currently, SPT and sIgE are commonly used; however, these tests have a high false-positive rate, particularly in children, and lack specificity. Individuals who have a positive test but who do not have an allergic reaction to the allergen on ingestion are said to be sensitized to the allergen. Research on more reliable tests for diagnosing allergy such as the Basophil Activation Test (BAT), CRD, sIgE, IgG4, and total IgE (27) is ongoing. Currently, the gold standard for confirming food allergy (rather than food sensitization) is the DBPCFC (20, 26). However,

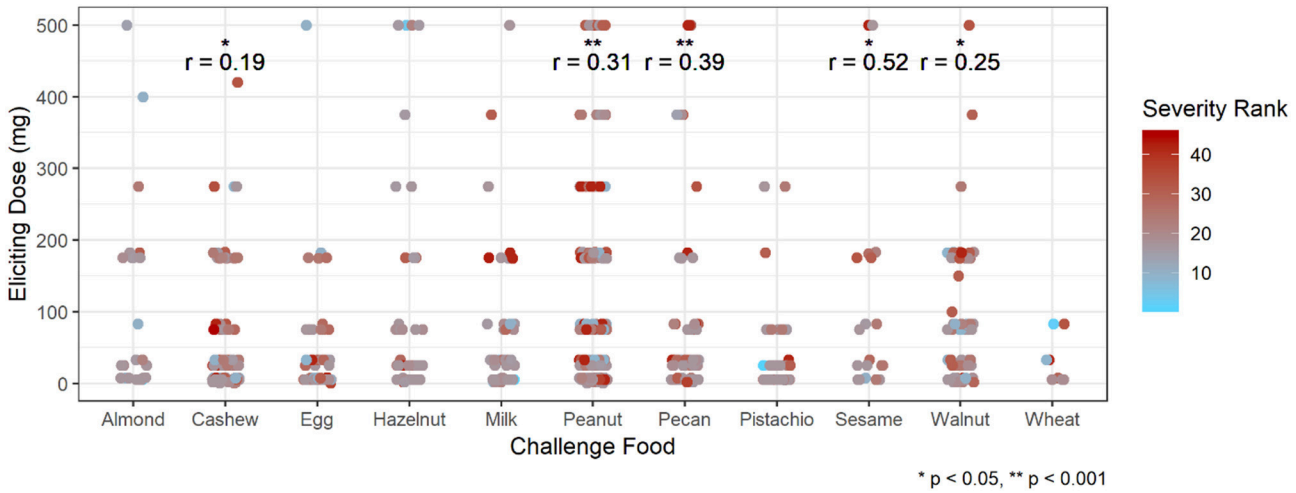

FIGURE 5 | Correlation of eliciting dose and adverse event severity ranking by challenge food. *$p < 0.05$, **$p < 0.001$ by Spearman rank correlation test. Red ranking corresponds to more severe symptoms, while blue corresponds to more mild symptoms.

there are several drawbacks in performing DBPCFCs. Presently, standardized dosing strategies for DBPCFCs are not widely practiced, and the optimal dosing schemes across allergens are unknown. DBPCFCs require multiple days of challenges which can significantly increase the cost. The most significant limitation is that food challenges carry the risk of potentially inducing severe anaphylaxis, which may require hospitalization or care in the intensive care unit (28), therefore DBPCFCs are typically performed under clinical supervision by trained staff who are able to recognize and treat any severe food reaction.

Our data show that the ED_{50} across all allergens is below 30 mg of protein; therefore safety in challenges may be increased by including additional steps at lower doses of the challenge. Compared to previously published thresholds by Blom et al. for cashew, egg, peanut, milk, and hazelnut (23), our findings of ED_5, ED_{10}, and ED_{50} were lower. One potential reason for this might be that the majority of our cohort was multi-food allergic (98%), and highly atopic with over 50% of the cohort with concurrent asthma, allergic rhinitis, and or atopic dermatitis. Additionally, the majority of our challenges had a dosing interval of 15 vs. 30 min reported by Blom et al. Participants undergoing peanut challenges had the highest ED_{50} dose (29.9 mg). Although pistachio had the lowest ED_{50} of 1.7 mg, it represented a small group of participants who had a previous reaction to a cashew challenge. The challenge of such subjects therefore was initiated at a lower dose (of 1 mg) due to safety concerns. Few studies have evaluated prognostic indicators for predicting OFC outcomes (29) and this is an area of ongoing research. In this study we attempted to identify potential prognostic indicators that may be associated with outcomes during OFC to a variety of foods, which could aid in risk stratification for allergists who may be considering a challenge. Our data suggest that food challenges with peanut, sesame, cashew, egg and walnut were more likely to be associated with GI-related symptoms, whereas hazelnut and milk were more likely to be associated with hives. The severity of the reacting symptom is also of concern when conducting a food challenge. Similar to what we and others have shown, a concomitant history of asthma increases the risk of having a severe reaction (29, 30). Not surprisingly, elevated specific IgEs and specific to total IgE ratios were associated with more severe symptoms. However, a severe reaction is possible even at low sIgE values (31). Often, the DBPCFCs conducted for inclusion of clinical trials have more stringent stopping rules and it is felt that more severe symptoms are elicited because of a higher ingested cumulative protein dose. When we assessed the severity of symptoms across doses, we found that severe symptoms were indeed modestly correlated with increasing doses for particular allergens (cashew, peanut, pecan, sesame, and walnut challenges). Perhaps we did not see this for all allergens due to insufficient sample size for those allergens.

In our data set, we also had the unique opportunity to assess ED and the severity of adverse events across repeat food challenges in a small subset of participants. We found that individuals had similar eliciting doses on the first and second challenge, with increasing severity on repeat challenges but with no association with time between challenges, which is consistent with prior findings of repeat challenges (32, 33). However, these results should be interpreted with caution as it is based on a small sample size, limited to 40 repeat challenges, constituting <4% of the total challenges in this cohort. Additionally, the analysis was not adjusted for allergen. Larger cohorts are needed to validate these preliminary findings. CRD was not done and this is a weakness of the paper and will be done in the future.

As food challenges and oral immunotherapy become more popular in outpatient clinics, our findings could

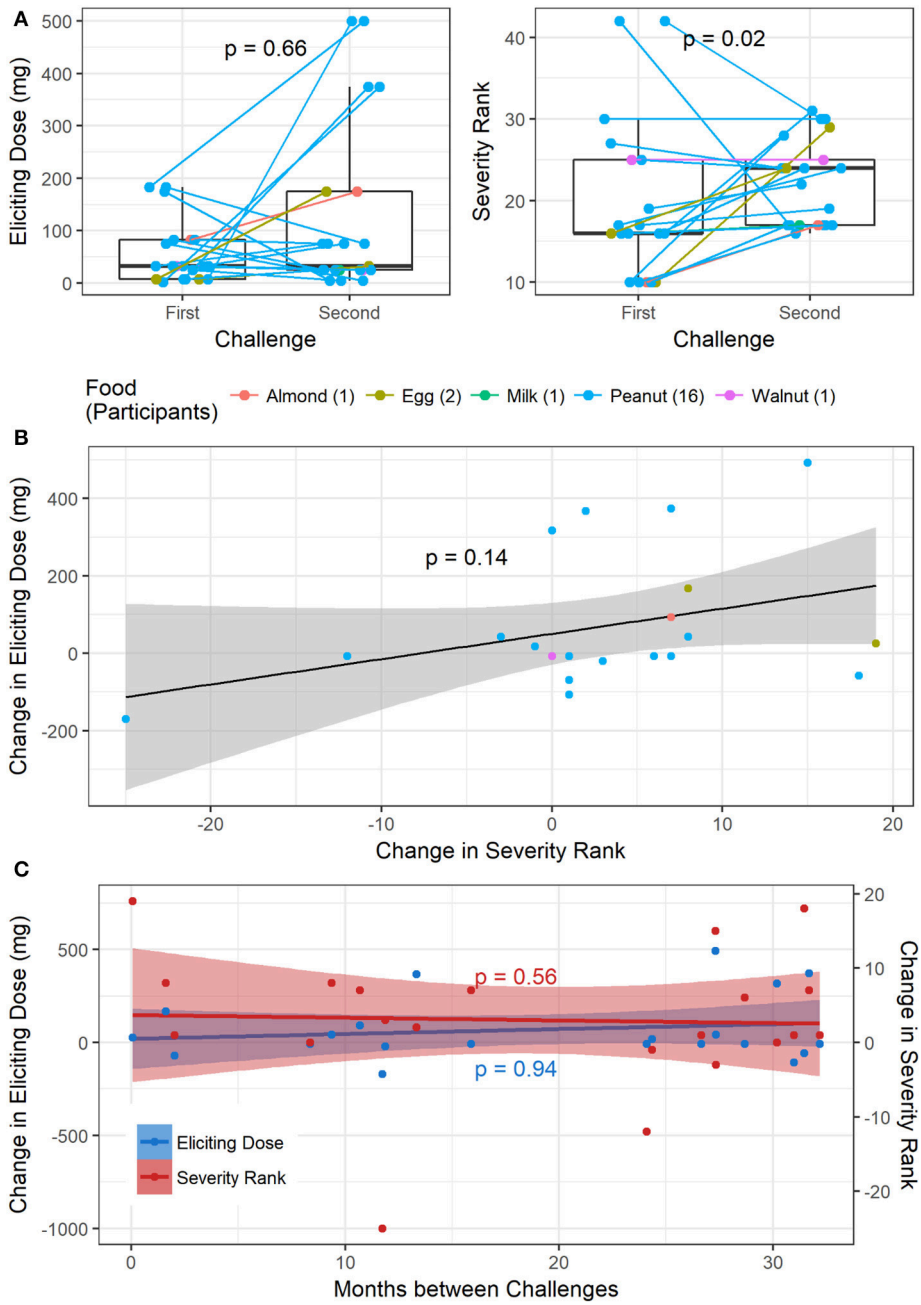

FIGURE 6 | Repeat challenges: **(A)** boxplot of change in ED and severity ranking from first to second challenge. **(B)** Association between change in ED and change in severity ranking. **(C)** Association between change in ED and time between challenges, and change in severity and time between challenges.

provide guidance and better insight into what to expect in performing food challenges in the outpatient clinic setting.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ICH/GCP/CFR guidelines by the Stanford IRB with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Stanford IRB.

AUTHOR CONTRIBUTIONS

Study was designed by RC, AL, AS, SS, and KN. Study was conducted by RC, AL, AS, SS, AA, JP, JS, JT, ST, JW, and KN. Data analysis was conducted by NP, SA, MD, KN, KL, and MW.

Manuscript was written by RC, NP, SA, AL, AS, SS, SG, MD, and KN.

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Adjuvanted Immunotherapy Approaches for Peanut Allergy

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Food allergies are a growing public health concern with an estimated 8% of US children affected. Peanut allergies are also on the rise and often do not spontaneously resolve, leaving individuals at-risk for potentially life-threatening anaphylaxis throughout their lifetime. Currently, two forms of peanut immunotherapy, oral immunotherapy (OIT) and epicutaneous immunotherapy (EPIT), are in Phase III clinical trials and have shown promise to induce desensitization in many subjects. However, there are several limitations with OIT and EPIT, such as allergic side effects, daily dosing requirements, and the infrequent outcome of long-term tolerance. Next-generation therapies for peanut allergy should aim to overcome these limitations, which may be achievable with adjuvanted immunotherapy. An adjuvant can be defined as anything that enhances, accelerates, or modifies an immune response to a particular antigen. Adjuvants may allow for lower doses of antigen to be given leading to decreased side effects; may only need to be administered every few weeks or months rather than daily exposures; and may induce a long-lasting protective effect. In this review article, we highlight examples of adjuvants and formulations that have shown pre-clinical efficacy in treating peanut allergy.

Keywords: food allergy, peanut allergy, immunotherapy, adjuvants, vaccine

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INTRODUCTION

Food allergies are common, now estimated to affect 8% of children and 3–6% of the general population in the US (1, 2). The prevalence of food allergies has increased over the past 20 years, although reasons for this increase remain unclear. Peanut allergy is of particular interest because it is typically a life-long allergy (3); in contrast to milk and egg allergies that often spontaneously resolve in childhood. Additionally, peanuts, along with tree nuts, cause the highest number of fatal and near-fatal reactions to foods (4). The prevalence of peanut allergy in the US is estimated at 1–2%, with one ongoing survey finding an increase from 0.4% in 1997 to 1.4% in 2008 (5). There are now 17 named peanut allergens with Ara h 1, 2, 3, and 6 being the major allergens implicated in patients with peanut-induced anaphylaxis (6). The biochemical nature and protein sequences of these allergens have been determined and the genes cloned for recombinant production making development of therapeutics more readily achievable (7, 8).

There are currently no FDA-approved therapies to treat food allergies, limiting allergic individuals to carry injectable epinephrine to treat reactions from accidental exposures. Many types of IgE-mediated allergies are successfully treated with allergen immunotherapy, which has been practiced for over a century, typically administered as subcutaneous injections of soluble allergen extracts (9). Although rather effective for treating environmental and insect venom allergies, subcutaneous injections with peanut extract were tested over 20 years ago and were determined to be unsafe due to high rates of anaphylaxis following injections leading to abandonment of this

approach (10, 11). Over the past 10 years, investigators have altered the route of administering allergen immunotherapy to treat food allergy with the intention of minimizing side effects while continually exposing allergic individuals to the offending allergen. These routes of antigen administration include oral ingestion of the allergen (Oral Immunotherapy; OIT), sublingual administration of a soluble protein extract (Sublingual Immunotherapy; SLIT), and epicutaneous administration of dried allergens to the skin (Epicutaneous Immunotherapy; EPIT). Each of these treatment modalities has advantages and disadvantages and each are extensively reviewed by Feuille and Nowak-Wegrzyn (12).

THE PROMISE OF ADJUVANTS IN FOOD ALLERGY

Undoubtedly, while this is an exciting time in food allergy research as there are two forms of immunotherapy, OIT and EPIT, in Phase III clinical trials, it is equally important to realize the limitations of these approaches. The major limitations are allergic side effects, daily dosing requirements, and ultimately only inducing desensitization and not long-term tolerance. There is room to improve upon these current approaches and one of these ways may be to include immunologic adjuvants. An adjuvant can be defined as anything that enhances, accelerates or modifies an immune response to a particular antigen (13). Adjuvants may allow for lower doses of antigen to be given leading to decreased side effects; may only need to be administered every few weeks or months rather than daily exposures; and may induce a long-lasting protective effect. Aluminum salts (alum) are the most widely used adjuvants for vaccines and have been historically used in subcutaneous immunotherapy formulations to treat respiratory and venom allergies (14). However, alum may not be suitable for peanut therapy due to the severe adverse events associated with subcutaneous peanut immunotherapy and the requirement of prolonged alum exposure to decrease pro-allergic responses (14). Alum may initially exacerbate immunotherapy adverse effects by enhancing allergen-specific IgE antibodies until allergen-specific IgG antibodies that block IgE responses are increased with repeated alum exposures (15). Although alum adjuvants may not be ideal for peanut immunotherapy, there are other encouraging examples of adjuvant utility in peanut immunotherapy. One small OIT trial demonstrated an advantage of a probiotic administered simultaneously in subjects receiving peanut OIT (16). It appears that this method induces a longer-lived state of desensitization, although much larger studies are needed to confirm these initial findings. However, the use of adjuvanted peanut immunotherapy is in its infancy possibly due to the low number of adjuvants approved for use in humans. Prophylactic and therapeutic peanut allergy animal models are useful tools that can be used to identify potential adjuvants that modify host peanut-specific immune responses toward desensitization and/or sustained unresponsiveness to improve the current limitations of peanut allergy immunotherapy.

PRE-CLINICAL STUDIES OF ADJUVANTED PEANUT IMMUNOTHERAPY

Animal models of peanut allergy represent invaluable tools to evaluate novel therapeutics or vaccines that may increase the safety and efficacy of peanut immunotherapy. Several types of vaccine adjuvants, including microbial by-products, nanoparticles and nanoemulsions have been evaluated to treat peanut hypersensitivities in animals (**Figure 1**). However, additional adjuvants that modify mast cell immunity, enhance Th1 and/or T regulatory cell responses, including interferon (IFN)-γ and/or interleukin (IL)-10, respectively or utilize host machinery to down-regulate pro-allergy phenotypes, including Th2-associated IL-4, IL-5, and IL-13, may be beneficial for treating peanut allergy. In the following sections, we will discuss animal studies that describe beneficial effects of adjuvanted immunotherapy to treat peanut hypersensitivity. Additionally, we will discuss adjuvants used in vaccine or non-peanut allergy studies that induce immune responses that may be beneficial for peanut allergy immunotherapy.

Microbial Adjuvants

The magnitude of microbial exposure is thought to modulate allergic disease. Early studies of peanut allergy demonstrated that reduced microbial exposure through antibiotic use or lack of toll-like receptor (TLR) 4 signaling enhanced peanut-induced anaphylactic reactions in mice (17). It is possible that reduced microbial exposure or inability to respond to microbes increases responses associated with allergic disease. For example, mice that lack bacterial colonization (germ-free) have enhanced T helper cell type 2 (Th2) responses and more severe allergic disease than conventional mice (18). While microbial stimulation may be important for proper immune development and allergy regulation, not all bacteria have the same function. A consortium of *Clostridia* from the XIVa, XIVb, and IV clusters isolated from conventional mice but not *Bacteroides uniformis*, enhances Tregs and reduces allergic disease severity (18), which suggests that specific bacteria are important for allergy modulation. Since allergy is mediated by Th2 responses, immune modulators that reduce Th2 responses and increase Treg responses, such as specific commensal bacteria, may be effective immunotherapy adjuvants.

Routine probiotic consumption may be an acceptable method to acquire immune modulating microbes that enhance the efficacy of peanut allergy immunotherapy. Probiotics are considered "good bacteria" and are frequently used to treat intestinal irregularities, including diarrhea. Lactobacillus and Bifidobacterium species are common bacteria used for probiotics (19) and may improve peanut allergy. Oral delivery of a proprietary cocktail of probiotics containing strains of both Lactobacillus and Bifidobacterium successfully reduced allergic disease symptoms in peanut-hypersensitive mice (20). Reductions in allergy symptoms were accompanied by an increase in IL-10 and transforming growth factor (TGF)-β, which are cytokines associated with Treg cells and a decrease in the Th2 cytokine IL-13 (20). Similarly, gastric exposure to Lactobacillus before and during peanut-sensitization reduces

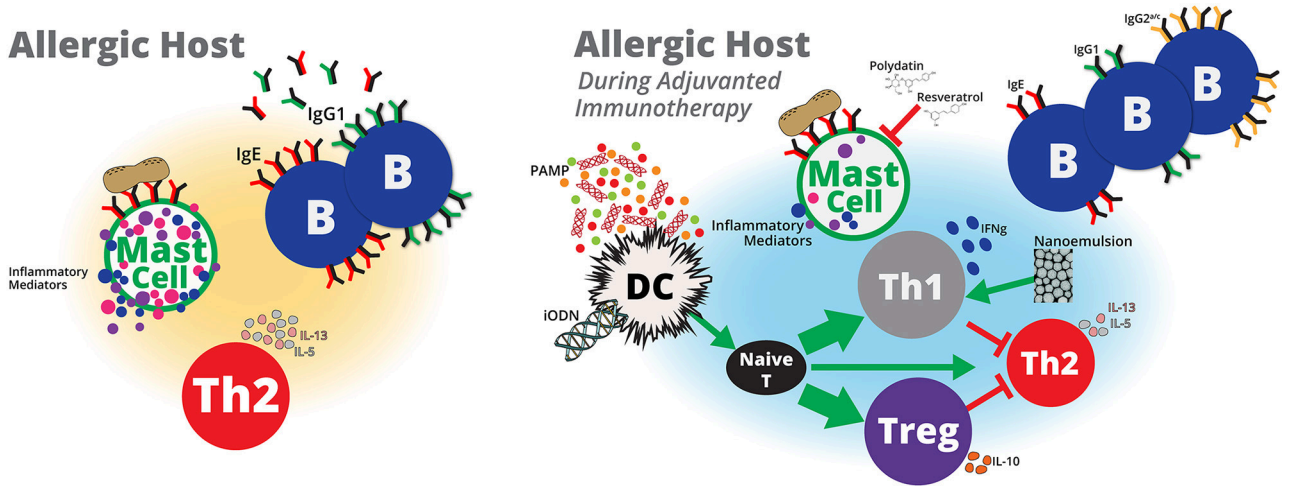

FIGURE 1 | Adjuvanted immunotherapy formulations are potential next-generation therapies that may modulate host immunity to treat peanut allergy. Pathogen-associated molecular patterns (PAMP) derived from pathogens, such as *E. coli* or *L. monocytogenes*, or probiotics, such as *Lactobacillus* and *Bifidobacterium*, or microbial components, such as CpG ODN may activate dendritic cells to induce naïve T cells to differentiate into more Th1 and/or Treg cells and less toward Th2 cells. Inhibitory ODNs that lack CpG and nanoemulsions may also lead to enhanced Treg and Th1 responses, respectively. Increased Th1 and/or Treg cells may suppress or balance peanut-specific Th2 responses to induce protective immunity against peanut. A reduction in peanut-specific Th2 immunity will also reduce the number of peanut-specific IgG1 and IgE antibodies. IgE bound to mast cells cross-linked by peanut antigens induces mast cell activation, which contributes to allergy symptoms. Adjuvants that reduce peanut-specific IgE or stabilize mast cells may prevent therapy-related adverse events and allow for higher doses of peanut to generate protective peanut-specific immune responses. Although immunotherapy with peanut alone appears to be a promising therapy to treat peanut hypersensitivity, safety and the lack of sustained unresponsiveness are potential limitations that may be addressed with adjuvants. Inclusion of vaccine adjuvants to peanut immunotherapy formulations offer potential benefits to peanut immunotherapy that that include, induction of durable protective immune responses in shorter dosing regimens with minimal adverse events.

peanut-specific IgE, Th2-associated cytokines and mast cell degranulation in mice (21). Bacteria can also be engineered to express peanut allergens and serve as "peanut-vaccines" that utilize self-components to modulate host immunity. *Lactococcus lactis* that produce the peanut protein, Ara h 2, decrease peanut-specific IL-4 and IL-10 responses and increase IFN-γ in mice immunized prophylactically before sensitization compared to mock-immunized mice (22). Probiotics may exert their allergy protective effects by inducing and sustaining Treg responses through their natural components that activate host toll-like receptors (TLR). The TLR ligands present in probiotic bacteria may activate host cells to secrete immunosuppressive cytokines, including TGF-β, which supports Treg differentiation and binds receptors on dendritic cells (DCs), specifically DC-SIGN to enhance IL-10 producing Tregs (23). Probiotic metabolism may generate metabolites that also activate Tregs through G protein-coupled receptors (23). Tregs have been associated with positive outcomes of peanut immunotherapy (24) and probiotics, such as *Lactobacillus acidophilus*, *L. casei* and *Bifidobacterium bifidium* increase Treg cell numbers and their suppressive functions (25). Enhanced probiotic use should be carefully monitored since probiotics are live cultures that may also influence host microbiota and potentially lead to off-target effects including, excessive immune stimulation, alternative metabolic activities and potential infections in susceptible populations (26, 27). However, probiotics are often used as nutritional supplements and are generally well tolerated (27); therefore, they may be a safe and noninvasive method to favorably modulate the protective immune responses induced by peanut immunotherapy.

Vaccine vectors generated from common pathogens that infect the gastrointestinal (GI) tract have been engineered to express antigens from different sources, including peanut. Similar to probiotic bacteria, these vectors contain pathogen-associated molecular patterns (PAMPs), such as unmethylated CpG DNA, lipoproteins and lipopolysaccharides that can activate the host immune system (28). Since these pathogens have developed mechanisms to evade host immunity to cause infections, their use as attenuated or inactivated vaccine vectors may be beneficial for treating peanut allergy. Peanut-hypersensitive mice treated with three weekly rectal immunotherapy doses of heat-killed *E. coli* (HKE) expressing Ara h 1, 2 and 3 developed decreased peanut-induced IL-4,−5,−13, and−10, increased TGF-β and IFN-γ and less severe allergic symptoms in compared to sham-treated animals (29). While creating genetically modified bacteria may be time-consuming, a more simple approach to treating allergy may combine inactivated pathogens with a known allergen dose in an immunotherapy formulation. Immunotherapy with heat-killed *Listeria monocytogenes* (HKLM) combined with Ara h 1, 2, and 3 administered subcutaneously three times a week for 4 weeks to peanut-hypersensitive mice reduced peanut-induced hypothermia and allergy symptoms (30). Interestingly, the protective effects of HKLM for peanut allergy have also been observed in a dog model. HKLM combined with peanut required higher doses of peanut to induce an allergic reaction

in animals with a known history of peanut-induced atopy (31), suggesting that the presence of the bacteria increases the activation threshold required for peanut to induce an allergic response. Although animal models support the use of inactivated pathogenic bacteria as adjuvants to improve peanut allergy, it is possible that host inflammatory responses to these bacteria will induce adverse events while modifying pro-allergic Th2 responses. Human studies demonstrated severe adverse reactions, such as throat discomfort, severe abdominal pain and anaphylaxis, which required subjects to discontinue to study after rectal administration of heat-killed *E. coli*-producing peanut proteins (32). While both *E. coli* and *Listeria* are potent inducers of Th1-immunity, they may not generate effective T regulatory responses. Peanut immunotherapy may benefit more from Treg-inducing adjuvants than strong Th1-inducing adjuvants that only dilute Th2 responses and potentially induce adverse reactions themselves. Therefore, vectors derived from bacteria that may cause gastroenteritis, such as *E. coli* and *L. monocytogenes,* may enhance immunity that prevent allergic development but may not be safe for treating established allergy due to the potential for inflammatory adverse events.

Microbial By-Product Adjuvants

Purified microbial macromolecules, such as DNA, lipopeptides and proteins, may induce similar beneficial immune responses for peanut allergy as whole-cell bacteria without the associated risks. Bacteria and viruses express toll-like receptor ligands that are PAMPS, which activate the host immune system to provide adjuvant activity and may modulate pre-existing immune responses. TLR9 ligands, such as unmethylated CpG oligodeoxynucleotides (ODN), are potent inducers of Th1 (33) and Treg (34) immunity and have been evaluated in mouse models of peanut immunotherapy. Peanut combined with CpG prophylactically and therapeutically reduced peanut-induced anaphylaxis in hypersensitive mice when injected or administered orally (35, 36). Exposure to peanut in the presence of CpG decreased peanut-specific Th2 responses, including IgE, IgG1, IL-5, and IL-13 and increased peanut-specific IFN-γ (36). The Th1-immunity induced by CpG may be beneficial for reducing allergy development but CpG may be more beneficial for peanut immunotherapy clinical studies if they induce Treg responses. CpG activates human dendritic cells to increase costimulatory molecules and naïve T cells stimulated by CpG-activated dendritic cells differentiate into Treg cells that suppress effector T cell responses (34). Although TLR ligands (TLRL) can induce similar Th1-associated immune responses as whole-cell bacteria, such as *E. coli* and *L. monocytogenes*, it is possible that TLRL adjuvants only direct immune responses to the co-administered peanut allergen. TLRL adjuvants that only enhance inflammatory responses to the peanut allergen may induce milder inflammation compared to whole-bacterium adjuvants that induce inflammation to peanut allergens and the bacteria itself. Thereby, TLRL adjuvants may enhance immunotherapy safety since it is possible that increased inflammation will contribute to potential off-target adverse effects. TLR ligands, like CpG, may improve OIT, SLIT, and EPIT by suppressing peanut-specific Th2 responses through an increase in Tregs and inducing *de novo* peanut-specific Th1-associated immune responses that balance the pre-existing peanut-specific Th2 cells, both that may improve the likelihood of sustained unresponsiveness.

Although TLRL appear to be promising adjuvants for peanut immunotherapy, selection of age-appropriate adjuvants should be considered when treating peanut-hypersensitive subjects. Host immunity vary with age in response to TLR stimulation (37, 38). Neonates and infants are often less responsive to CpG than adults (39). While CpG may be a more effective adjuvant in older children and adults, infant peanut immunotherapy may benefit from other PAMP adjuvants such as, R848 combined with trehalose-6,6-dibehenate (TBD), that enhance antigen-specific Th1 responses in young populations (37). If PAMP adjuvants are to be used in peanut immunotherapy, then future research must respect the age of the patient during treatment.

Vaccine Formulations as Prospective Immunotherapy Adjuvants

Proper physical formulation that combines peanut allergens with structures designed to improve mucosal allergen delivery and modify host immunity may enhance peanut immunotherapy safety and efficacy. Nanoparticles are structures smaller than 1,000 nm that can transport vaccine formulations and may exert immunomodulatory activity by reducing antigen degradation, increasing antigen uptake by antigen presenting cells (APC) and prolonging antigen retention (40). Nanoparticle immunomodulatory activity may generate immune responses beneficial for the treatment of peanut allergy. Oral vaccination with peanut-containing nanoparticles in naïve mice induces a balanced peanut-specific Th1:Th2 response with enhanced IL-10 responses (41). Nanoparticle-formulated peanut improved hypersensitive responses in mice that received CpG-adjuvanted peanut OIT in nanoparticles (42). OIT with peanut alone, nanoparticles alone, or CpG-nanoparticles in the absence of peanut did not improve allergic disease in peanut-hypersensitive mice but the combination of peanut, CpG and the nanoparticles reduced peanut-induced anaphylaxis (42). Improved allergic disease accompanied a shift from peanut-specific Th2 responses toward peanut-specific Th1 responses, which supports nanoparticles' immune modulating properties.

Nanoemulsions exert immune modulation and allow for lower vaccine component doses to induce comparable immunity to vaccines in comparison with high doses in saline (43). Thus, immunotherapy may benefit from nanoemulsions that allow for lower doses of peanut to modify peanut-specific immunity and reduce the risk of adverse reactions. Nasal vaccination with peanut formulated in a nanoemulsion reduced the severity of allergy in hypersensitive mice whereas the same peanut dose in saline was ineffective (44). Therefore, the physical formulation of peanut immunotherapy is something that should be considered when designing next-generation immunotherapies.

Nanoformulations, including nanoparticles and nanoemulsions are classified by size but can be synthesized from a vast array of materials, including biodegradable chitosan and poly-D-L-lactide-co-glycolide (PLGA) and non-biodegradable

ceramic materials (45). Common nanoformulation components such as, oils and surfactants, may have toxic effects and cause cell death (46). *In vitro* and *in vivo* studies that determine the bio-distribution of nanoformulations will also be important since nanoformulations may accumulate within cellular compartments (40) and alter cellular function (47). The toxicity and safety of materials in nanoformulations must be considered when incorporated in a peanut immunotherapy formulation to reduce the risk of additional adverse events.

Physical and immunomodulation properties of nanoformulations may contribute to the protective activity of immunotherapy. Mucosal routes may be preferred for immunotherapy since mucosal routes are naturally tolerogenic and injection immunotherapy may increase systemic anaphylaxis risks (10). Nanoformulations may protect the immunotherapy vaccine components from degradation or clearance to improve allergic disease after mucosal delivery. Additionally, nanoparticles can be designed to control the time and location of antigens released after administration (48). The frequent clinic visits required during therapy may decrease and improve subject compliance through the use of time-release nanoparticles. Controlling the amount of peanut released during immunotherapy by formulation in nanoparticles may also reduce adverse events by limiting the amount of peanut available to activate mast cells.

Inhibitory Ligand Adjuvants Preventing Peanut Sensitization

Host immune machinery could potentially have intrinsic activity that can be manipulated to serve as adjuvants to enhance peanut immunotherapy and inhibit allergic disease. Siglec-engaging tolerance-inducing antigenic liposomes (STALs) reduces peanut hypersensitivity in mice when combined with Ara h 2 (49). STALs are liposomes that bind immunomodulatory receptors, including sialic acid-binding Ig-like lectins found on the surface of many cells, such as monocytes, NK cells and B cells (50). Antigen-containing STALs directed against the Siglec on B cells, CD22, can induce apoptosis in antigen-specific B cells and lead to tolerogenic immune responses (51). Pre-treatment with Ara h 2 STALs that target CD22 significantly reduced Ara h2-specifc IgG1 and IgE and prevented peanut-induced hypothermia. Similarly, fusion molecules that contain Ara h 2 and the Fc region of IgG1 (AHG2) reduced peanut-induced allergic responses in hypersensitive mice (52). Peanut-hypersensitive mice displayed a reduction in hypothermia and clinical symptoms with AHG2 therapy compared to placebo (52). Mast cells contain Fc receptors that can activate or inhibit degranulation in response to antigen crosslinking immunoglobulins on the cell surface (53). It is possible the AHG2 molecule inhibits mast cell degranulation through binding inhibitory FcγRs on mast cells in the presence of peanut proteins that would normally activate mast cells through the FcεRI (52). Pre-treatment of peanut-allergic subjects with synthetic molecules that activate host inhibitory receptors in effector cells, similar to those described above, before beginning peanut immunotherapy regimens could potentially reduce adverse reactions by decreasing the number of IgE producing B cells and inhibiting mast cell degranulation. Practical application may require a mixture of multiple inhibitory molecules that are specific for the various peanut proteins since allergic subjects often have IgE responses to several peanut proteins. However, these molecules could be combined with current immunotherapy regimens and allow higher allergen doses to redirect host allergic immune responses. Pre-clinical studies are required to evaluate these therapies in models of established peanut allergy to advance safety and efficacy.

LESSONS LEARNED FROM ALTERNATIVE ADJUVANTS THAT MAY BENEFIT PEANUT IMMUNOTHERAPY

Peanut immunotherapy could be improved by adding immune modulating molecules that reduce allergy effector cell functions. Inhibition of mast cells and basophil activity during immunotherapy may allow for delivery of higher doses of peanut that can more effectively induce regulatory T cell responses without the complication of allergic symptoms. Inclusion of molecules that directly modulate T cell responses in the immunotherapy formulation may act directly on the T cells to induce sustained unresponsiveness at a more rapid rate than current peanut immunotherapy protocols. Below we will discuss findings from pre-clinical studies that have evaluated adjuvants that modulate mast cells, basophils and T cells in other allergic disease models. The information gained from these studies may be applicable to improve immunotherapy for peanut allergy.

Mast cells are allergy effector cells that directly cause symptoms subsequent to allergen cross-linking IgE on the cell surface (54), therefore mast cell inhibition may be a powerful tool to suppress allergic responses. Resveratrol is a chemical found within plants that has an anti-inflammatory impact on mast cells (55) and attenuates antigen-IgE mediated mast cell activation (56). Mouse diets supplemented with resveratrol reduced allergic sensitization in a mouse model of ovalbumin (OVA) allergy (57). A reduction in OVA-induced hypothermia and OVA-specific IgE, IL-13 and IFN-γ was observed in mice on a resveratrol-supplemented diet. Polydatin is a glucoside of resveratrol and it stabilizes mast cells and decreases the severity of mast-cell dependent passive cutaneous anaphylaxis in mice (58). Cromolyn is another mast cell stabilizer that suppresses allergic airway inflammation in a mouse model of house dust mite (HDM) inflammation (59). Systemic exposure of cromolyn prior to HDM exposure reduced total inflammatory cell numbers and IL-5 in the serum and BAL of sensitized mice (59). Cromolyn is commonly used in asthmatics and provides relief from allergic rhinitis symptoms (60). Importantly, resveratrol and polydatin are components of dietary supplements and are routinely used in humans, which may support the safety of these molecules to be included in peanut immunotherapy formulations.

Rapamycin is another chemical that is used as an immunosuppressive agent clinically and inhibits mast cell cytokine production in response to FcεRI activation (61). OVA-induced mast cell activation and allergic disease

severity, including diarrhea score, clinical symptom score and hypothermia, is reduced with oral administration of rapamycin (62). A dose-dependent exposure to rapamycin prevented mast cell growth in the presence of IL-9, decreased serum antibody and splenic cytokine responses (62), suggesting a global immunosuppressive effect. Thus, inclusion of mast cell stabilizers in a peanut immunotherapy formulation may enhance the safety in peanut immunotherapy clinical trials by suppressing mast cell activity that contributes to adverse reactions, however, precaution using global immune-suppressive therapeutics should be considered to reduce the risk of infections.

Adjuvants that enhance Treg responses may contribute to enhance the safety and efficacy of therapies to treat peanut allergy. Inhibitory oligodeoxynucleotides (iODN), which do not contain CpG sequences, activate plasmacytoid dendritic cells to induce Tregs from naïve T cells (63). Since both Th1 and Th2-associated cytokine responses are reduced after treatment with iODNs (64), additional studies are required to evaluate the specificity of suppressive responses induced by iODNs. While global immune suppression may increase the risk of infections, allergen-specific suppression may improve allergic disease. A mouse model of atopic dermatitis described a reduction in clinical skin score, ear thickness and total IgE after oral therapy with encapsulated iODN (65). Although CpG ODN can enhance Tregs, they are also potent inducers of Th1 immunity. Therefore, replacing CpG ODNs with iODNs may further contribute to allergen immunotherapy protective immune responses without the associated Th1-responses that may predispose to other pathologies, including autoimmunity.

The combination of mast cell stabilizers and molecules that induce Tregs may have an additive effect to improve immunotherapy. While it is unclear how mast cell stabilizers modulate allergen-specific immunity, it is possible that the inhibition of mast cell activation reduces the amount of Th2 cytokines available to contribute to disease since IL-4,−5, and−13 are secreted by mast cells (66). It would also be interesting to evaluate the influence of mast cell stabilizers on Treg cells since resveratrol-exposed mice demonstrated suppressed Th1 and Th2 responses (57). The adjuvant combination of mast cell stabilizers and iODNs that enhance Tregs should be used with careful consideration not to create an immune-suppressive environment but if properly formulated may improve current immunotherapy. The potential benefit of combination therapies including, reduced mast cell degranulation and induction of potent allergen-specific Tregs, may decrease adverse events and lead to sustained unresponsiveness to increase efficacy.

CONCLUSION

Peanut OIT, SLIT, and EPIT are showing promise as the first therapies to induce protective immune responses in allergic individuals. While some level of desensitization is often achieved through these forms of immunotherapy, the need for sustained unresponsivess, a reduction in adverse events, and more favorable immunotherapy regimens require the development of additional immunotherapy formulations. Adjuvanted allergen immunotherapy approaches may address the current limitations of OIT, SLIT, and EPIT. Further pre-clinical studies are required to investigate the safety and efficacy of various adjuvanted-peanut immunotherapy prior to clinical testing in peanut allergic subjects.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Gut Mucosal Antibody Responses and Implications for Food Allergy

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The gastrointestinal mucosa is a critical environmental interface where plasma cells and B cells are exposed to orally-ingested antigens such as food allergen proteins. It is unclear how the development of B cells and plasma cells in the gastrointestinal mucosa differs between healthy humans and those with food allergy, and how B cells contribute to, or are affected by, the breakdown of oral tolerance. In particular, the antibody gene repertoires associated with symptomatic allergy have only begun to be characterized in full molecular detail. Here, we review literature concerning B cells and plasma cells in the gastrointestinal system in the context of food allergy, with a focus on human studies.

Keywords: gastrointestinal mucosa, B cell, plasma cell, food allergy, plasma cell, antibody repertoire, IgE, memory

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FOOD ALLERGY

The healthy physiological response to dietary antigen exposure in the gastrointestinal tract is a state of immunological non-responsiveness called oral tolerance. The development of IgE-mediated sensitization to allergens, and symptomatic food allergy, can be considered as evidence of a breakdown in oral tolerance, but the mechanisms leading to these pathological outcomes in humans are not fully understood (1–3). In this review, we focus on IgE-mediated food allergies, in contrast to other pathological responses to dietary antigens such as celiac disease responses to gluten. Food allergy affects 3–8% of the US population, and its prevalence is increasing (4). Allergy to peanuts is especially common, affecting up to 1% of the US population and up to 3% of children, and is distinct due to its severity and persistence into adulthood to a greater extent than egg, milk, wheat, and soy allergies (5). Avoidance is the current standard of care (6), and no diagnostic tests can accurately predict an individual's risk for anaphylaxis or the threshold allergen dose. Clinical trials have demonstrated the efficacy of allergen-specific immunotherapy to desensitize against food allergy, as evaluated in a recent meta-analysis of 25 randomized and 6 non-randomized studies, with a total of 1,259 patients treated via oral, sublingual, and epicutaneous routes, and assessment of clinical outcomes of desensitization in 27 studies and sustained unresponsiveness in 8 studies (7). Almost all studies in this meta-analysis showed increased efficacy of immunotherapy compared to controls, albeit with increased risk of adverse events during the trials. Whether the mechanisms leading to desensitization overlap with those of normal oral tolerance is less clear. There have been significant methodological challenges to progress in understanding the mechanisms of normal oral tolerance, the development of food allergy, and therapeutic responses to allergy immunotherapies, as well as extrapolating the results from mouse experiments to humans.

In humans, gastrointestinal (GI) tissues are usually procured during diagnostic testing in the context of suspected GI illness, or during autopsy, and are not routinely collected for patients with food allergy, limiting the availability of both healthy, and allergic patient tissues. Mouse models for allergy, although enabling well-controlled perturbation of immune function, and study of all tissues, have significant limitations. Different models vary according to strain in their propensity to develop IgE responses, and can require non-physiological methods of sensitizing mice to allergens, such as damaging the mouse GI tract with toxins (8, 9). More recently, improved humanized mouse models supporting human immune cell populations have addressed some of these limitations (10).

Despite these challenges, a combination of human and mouse studies has revealed some important themes in the loss of tolerance to food allergens, and the development of symptomatic food allergy. The "barrier regulation" hypothesis (11) posits that allergic sensitization begins with the impairment of the epithelial barrier in the skin, GI tract, and potentially other sites. Reasons for impaired barrier function can include mutation of genes encoding the skin and mucosal epithelium barrier protein filaggrin (12) or the SERPINB (serine protease inhibitor B) gene cluster (13). Individuals with food allergies have increased barrier permeability (14–16). In the context of a damaged epithelial barrier, exposure to food allergens through a non-oral route, or in increased quantities, are hypothesized to contribute to allergic sensitization. Indeed, human epidemiological studies indicate that non-oral contact with food allergens (such as exposure to peanut-containing dust in the home) is correlated with a child's risk of developing food allergy (17, 18). Further, symptomatic food allergy is often observed when a child first ingests the allergenic food, consistent with prior sensitization by non-oral routes (2). Allergic sensitization is thought to require the activity of T cells expressing Th2 cytokines such as IL-4 and IL-13, although the exact nature of the T cell help, if any, required to cause allergen-specific B cells to class switch to IgE *in vivo* in humans have not yet been described. Similarly, the extent to which switching to IgE occurs in various tissues of the body is unclear. Additionally, other cell types such as mast cells, which are commonly found in the tissue, secrete IL-4, IL-13, and other cytokines that may influence B cell development (19). Increased titers of high-affinity allergen-specific IgE antibodies are frequently detected in patients with symptomatic allergy. These antibodies bind FcεRI on tissue-resident mast cells and circulating basophils, where they participate in early/immediate hypersensitivity responses when crosslinked by allergens. Allergen-specific IgE has also been reported to contribute to allergy pathogenesis through facilitated antigen presentation and epitope spreading via uptake of antigen-IgE complexes by the low-affinity IgE receptor, CD23, present on dendritic cells, B cells, and other antigen-presenting cells (APCs) (20–24). IgE can also assist in the transport of antigen from the lumen across the epithelium via CD23 on the surface of epithelial cells, as has been demonstrated in human gut (25), cultured human respiratory epithelial cells (26) and a mouse model for allergy (27).

ANATOMICAL LOCALIZATION OF B CELLS/PLASMA CELLS IN THE GUT

The GI tract is the primary interface with dietary antigens, and is composed of immunologically active tissues. It has been estimated that up to 80% of all plasma cells in humans are in the gut, although lower estimates have also been proposed (28, 29). Most B cells in the GI tract are in the gut-associated lymphoid tissue (GALT), which includes the tonsils, adenoids, Peyer's patches of the small intestine, appendix, and lymphoid follicles of the large intestine and rectum. Plasma cells are found in the submucosa of GI tissues, particularly in the layer of loose connective tissue called the lamina propria, as well as the GALT (30, 31). The GALT is separated from the lumen by epithelial cells, which in addition to forming a protective barrier against the gut microbiota and ingested pathogens, also play an important role in transporting secretory IgA antibodies and secretory IgM into the lumen. Much of the gut epithelium is villous, but regions of the epithelium are associated with lymphoid follicles and are called the follicle-associated epithelium (FAE). Lymphatic circulation through the lamina propria of the intestine passes to the mesenteric lymph nodes and lymphoid follicles within the GALT, where antigen presentation and interaction with T helper cells can induce B cell class-switching and affinity maturation to generate an antibody response.

In human and mouse, the majority of antibody-secreting cells (ASCs; plasmablasts and plasma cells) in the GI tract express IgA, with estimates of 75–80% in the gastric mucosa, duodenum and jejunum, and 90% in the colon (32). IgG-expressing ASCs have been reported to represent 13% of ASCs in the gastric mucosa, and 3–4% in the small intestine and large bowel (32). IgM+ ASCs are also detected: 11, 18, and 6% of total ASCs in the gastric mucosa, small intestine and colon are IgM (32). An important knowledge gap in the context of food allergy is the frequency of the more rare IgE+ ASC or B cells in the human GI tract, as this has not been studied systematically and comprehensively using modern methods in either healthy subjects or allergic individuals.

DEVELOPMENT OF GUT B CELLS

What is the anatomical origin of the B cells and plasma cells detected in the gut? Most B-lineage cells in lymph nodes and other secondary lymphoid tissues are thought to be derived from precursors that develop in the bone marrow, where they are exposed to self-antigens, and where autoreactive B cells are deleted from the repertoire (33). Do B cells and plasma cells detected in the GI tract share this origin? B cell development outside of the bone marrow has been demonstrated in the rabbit, chicken, sheep, and mouse (34, 35). Wesemann et al. recently determined that rare RAG2-expressing pre-B-cells exist in the mouse intestinal lamina propria, but are absent from Peyer's patches (35). These pre-B-cell populations are upregulated in response to colonization with gut bacteria compared to germ-free mice, but rapidly decrease in frequency after weaning (35). Deep

sequencing of BCR repertoires showed that immunoglobulin heavy chain V_H gene segment repertoires were similar between bone marrow and gut, but the immunoglobulin kappa light chain V_K gene segment repertoires were distinct, suggesting that repertoire development can be influenced by microbial factors, and that this occurs a critical time window in early life (35). The possibility of B cells originating in human GI tissues early in life is particularly interesting in the context of food allergy, where early feeding with allergenic foods correlates with reduced risk of allergy development (11, 36, 37). Pre-B cells have been detected in the human fetal intestine and other tissues including liver, lung, kidney, and spleen, in addition to bone marrow, as early as 18–20 weeks of gestation (6, 38). Aggregates of B cells have been seen in intestinal tissues by 11 weeks of gestation, but germinal centers in Peyer's patches do not appear until after birth and exposure to the environment (32). Even in adults, transitional B cells can be detected in gut tissue (39), and there is some evidence for lambda light chain revision in mature intestinal B cells (40). More evidence is needed to establish the extent of these developmental pathways in humans, and whether they influence disease states such as food allergy.

GUT MUCOSAL B CELL RESPONSES

B cell responses in gut tissues require introduction of antigen from the lumen into the underlying tissue. In healthy mice and humans, oral antigen can be sampled from the lumen by microfold (M) cells that overlay Peyer's patches, by dendritic cells and macrophages, and by intestinal goblet cells and epithelial cells that express antibody receptors that can transport antibody-antigen complexes (41). Antigen transcytosed by M cells and dendritic cells in the FAE is delivered to the Peyer's patches, while antigen that is sampled at the villous epithelium enters the lamina propria, where it is transported through the lymph to the mesenteric lymph nodes (41). Germinal center responses can occur at either of these sites; however, the immune responses may differ qualitatively, as data from mouse indicate that mesenteric lymph nodes are required for oral tolerance, while Peyer's patches are dispensable (42–45). As noted above, prior studies have identified disrupted epithelial cell junctions in allergic patients and those with asthma, suggesting that the routes of antigen exposure in these individuals may differ significantly from healthy subjects (46).

In germinal center reactions B cells can adopt a memory B cell fate or differentiate into plasmablasts that may further differentiate into short- or long-lived plasma cells. Antibody-secreting cells (plasmablasts and plasma cells) are guided to their effector sites in the lamina propria of the GI tract, or through the efferent lymph to the blood and other tissues through expression of homing receptors. The mixtures of homing receptors expressed on the B cells depend on such factors as stimulation from dendritic cells, the site where the ASC was generated, and the isotype of the ASC (47–49). As reviewed in (47), early adoptive transfer experiments in mice found that IgA-expressing ASCs arising from Peyer's patches, mesenteric lymph nodes, and other sites in the GALT traffic to a subset of mucosal sites (intestine, urogenital tract, mammary glands, salivary glands, and respiratory tract), whereas IgA-expressing ASCs from mediastinal and tracheal-bronchial lymph nodes trafficked to the salivary glands and respiratory tract, and rarely to the intestine. In contrast, IgG ASCs preferentially migrate to the bone marrow. Interestingly, Carbon-14 dating experiments indicate that plasma cells can persist for decades in humans, suggesting that, like bone-marrow-resident plasma cells, gut-localized plasma cells could be long-lived, and possibly even provide protective antibody responses for life (50). The combinatorial expression of homing molecules provides one mechanism for the dispersal of locally generated ASCs and B cells to distal humoral and mucosal sites (47), a feature that has appeal for mucosal vaccination strategies (51) and also for food allergy immunotherapy.

ISOTYPE-SWITCHING IN THE GUT

IgE, the causative antibody isotype for human type I hypersensitivity reactions, is generated by isotype switching in B cells expressing constant region genes located upstream of the IgE locus in the genome. Because class switch recombination deletes genomic DNA between the starting isotype and the resultant isotype, the process is unidirectional and irreversible, but it should be noted that within a B cell clone there may be members expressing a variety of different upstream isotypes, including IgM (52, 53). Two major pathways of class-switching to IgE have been described in mouse: direct switching, from IgM to IgE, and sequential switching, from IgM to IgG and then to IgE. Sequential class-switching through an IgG intermediate is clearly not an absolute requirement for IgE generation, as mice carrying deletions of the γ1 intron or γ1 switch regions do not class-switch to IgG1 and are able to generate wild-type levels of IgE (54, 55). However, the extent to which direct class-switching contributes to the generation of IgE under normal conditions is unclear. It has been proposed that high affinity IgE is generated through class-switch from IgG1 memory B cells, which act as the storage for IgE memory (56). However, evidence for memory IgE+ B cells in humans has recently been obtained by Sicherer et al. (4) and Heeringa et al. (57). Employing a negative gating fluorescence-activated cell-sorting strategy, the authors identified a population of IgE+ memory B cells that was higher in frequency in young individuals with food allergy, atopic dermatitis and/or asthma relative to non-allergic controls. IgE+ plasmablasts had fewer somatic hypermutations and less evidence for antigen selection than the IgE+ plasmablasts from healthy controls, potentially indicating that these cells may not give rise to high-affinity IgE+ antibodies. Correlation of allergen-specific binding and affinity of IgE derived from plasmablast populations and putative memory B cell populations, if these can be obtained, may help to clarify the relevance of these populations to disease phenotypes, in future research.

Most of what we know about IgE production in humans derives from studies of primary or cultured cells from

secondary lymphoid sites such as lymph nodes, adenoids and tonsils, and rare circulating IgE+ B cells that appear in the peripheral blood (4, 53, 58–63). In humans, data from these tissues suggests that the precursors of IgE are often IgG-expressing B cells, with smaller contributions from IgM-expressing B cells (4, 58, 64). Importantly, there is also some published evidence for local production of IgE class-switched B cells in non-lymphoid tissues. Data from measurements of IgE constant region germline transcripts (εGT), mature IgE transcripts, class-switch recombination (CSR) excised DNA circles and switch circle transcripts, and the expression of factors required for CSR such as IL-4, IL-13 and AID, have indicated that local class-switching to IgE can occur in a variety of non-lymphoid tissues (52, 64–66). IgE class switching has been reported in human bronchial mucosa of patients with atopic and non-atopic allergy (65), in the sinus mucosa of individuals with chronic sinusitis (66), and in nasal polyps of individuals with chronic rhinosinusitis (64). More recently, high-throughput sequencing has been used to identify the nasal mucosa as a tissue site that harbors reservoirs of allergen-specific IgE and clonally-related B cells expressing other non-IgE isotypes in individuals with seasonal allergies (52). Additional perspectives on the evidence for pathways of IgE development in human and mouse, including controversies regarding IgE memory in mice, have recently been published (67–72).

What is known about class-switching in the GI tract in mice and humans? The regulation and localization of class-switching to IgA in the gut, and its importance in maintaining homeostasis with gut commensals, has been studied extensively [reviewed in (73)]. Recent mouse research has demonstrated that class-switching to IgA predominantly occurs in the epithelium-proximal region of the Peyer's patches termed the sub-epithelial dome (74). The authors found that IgA class switching was TGFß-dependent and required interaction of activated B cells with dendritic cells; migration to the subepithelial dome was promoted by CCR6 upregulated in Peyer's patch B cells in response to CD40 signaling. Pre-GC and memory B cells were both found to have high expression levels of CCR6, suggesting "privileged access" of some B cell subtypes to the SED, where microfold (M) cells in the FAE transcytose luminal antigens.

Much less is known about the development of IgE in the gut. Secreted IgE antibodies are detectable in intestinal juices (75, 76) and in the stools (77) of adults with food allergy, suggesting antibody production by local IgE+ plasma cells. Milk-specific IgE has also been detected in the stools of children with milk allergy (78). The numbers, cellular phenotype, and origin of IgE-expressing B cells in the human gut, however, are poorly described. Early studies employing immunofluorescence staining approaches detected IgE+ cells in human stomach, small intestine, colonic, and rectal mucosa, but due to limitations in immunofluorescence staining for other markers to prove cell lineage identity, it was not determined whether these IgE+ cells were B cells, plasma cells, or instead were mast cells or basophils bearing soluble IgE antibodies (79–81). Analysis of intestinal biopsies from 25 patients with food allergy and 14 healthy controls has identified εGT in gut intestinal mucosa (82). This study found that εGT and IL-4 levels were higher in biopsies from food allergic individuals compared to controls, but that εGT could also be detected in a subset of healthy individuals. A recent study of 11 pediatric patients with the allergy-associated condition eosinophilic esophagitis (EOE), and 8 healthy controls, found that total B cells were present at elevated densities in samples from the EOE patients, and that IgE constant region germline transcripts were elevated in EOE esophageal biopsies (83). This study further detected mature IgE mRNA transcript levels in esophageal biopsies by PCR amplification and gel electrophoresis, but did not see statistically significant differences in the proportion of positive biopsies in EOE and control groups (83).

INTESTINAL MICROFLORA AND DIVERSIFICATION OF THE ANTIBODY REPERTOIRE

Constant exposure to microbial contents of the GI tract is likely to provide much of the antigenic stimulus experienced by B cells in these tissue sites. Several studies in humans have linked dysbiosis of the microbiota with the development of allergy [reviewed in (11)]. How do the components of the microbiota shape the antibody repertoire? Initial reports are beginning to address this large topic. Analysis of human colonic IgA sequences from 6 individuals before antibiotic treatment, after 4 days of treatment, and 46 days after the end of the treatment revealed persistent clones whose frequencies were not altered by antibiotic treatment, suggesting that the IgA repertoire is stable under these conditions of altering the microbiome, at least within the short timeframe of the study (84). Data from a large-scale analysis of recombinant natively-paired antibodies produced from mouse intestinal plasma cells and B cells indicate that most IgA are polyreactive with broad specificity for intestinal microbiota, and although they arose from B cells selected into germinal center reactions in Peyer's patches, this process was not dependent upon microbial colonization or upon food antigens, nor was their affinity dependent on the acquisition of somatic hypermutation (85). The conclusions of this study were that polyreactive anti-microbial IgA antibodies are natural antibodies that arise without instruction by the intestinal microbiome. In contrast, a study of human IgA and IgG antibodies cloned from single plasmablasts sorted from ileum lamina propria found that only 26% of IgA antibodies and 26% of IgG antibodies were polyreactive, defined as binding to at least 2 antigens in a panel of antigens including double-stranded DNA, LPS, and insulin (86).

What evidence is there for GI microbiota affecting IgE production in mice or humans? It has been reported that germ-free mice develop high IgE serum levels and undergo CD4 T-cell-dependent class-switching to IgE in Peyer's patches, suggesting that normal microbiota can help to suppress the levels of IgE-expressing B cells or plasma cells (87). Although not directly addressing the question of allergen-specific IgE production, one

study with a mouse model for peanut allergy demonstrated that Clostridia-containing microbiota were associated with class-switching to IgA, which was proposed to have a protective effect against sensitization to allergens. Consistent with this hypothesis, increased rates of peanut allergen protein Ara h 2 and Ara h 6 uptake and appearance in the serum were observed in *Rag–/–* knockout mice, which are unable to generate B cells (88).

ANTIBODY GENE REPERTOIRES AND CLONAL RELATIONSHIPS IN THE GUT AND OTHER TISSUES

Immunological analyses of humans have typically relied on analysis of the peripheral blood, as these specimens are readily obtained with low risk to research participants, but it is an

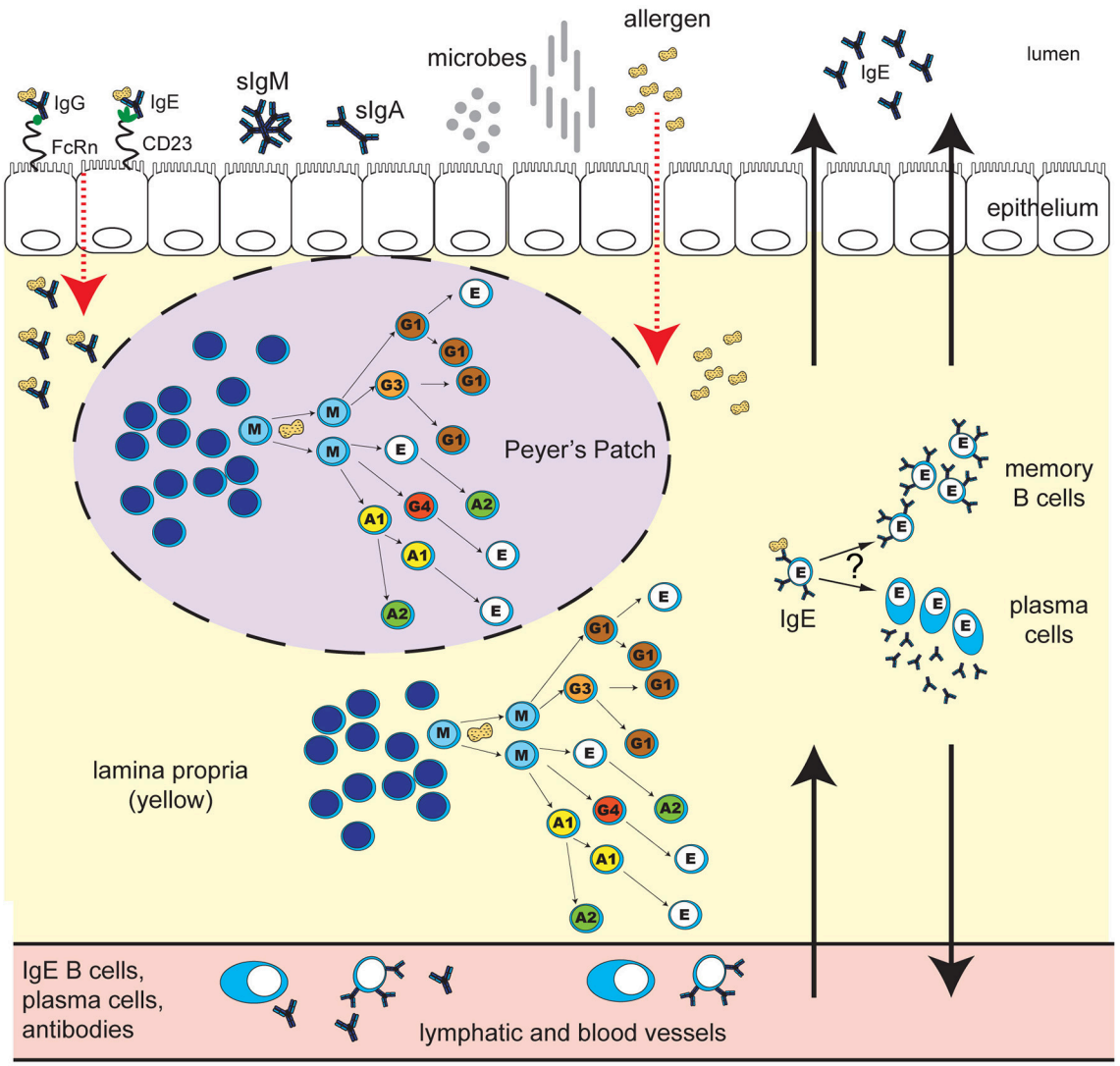

FIGURE 1 | Human IgE development in food allergy: unresolved questions. B cell class-switching to IgE in humans may occur from IgM, IgD, IgG3, IgG1, IgA1, or IgG4, but not from IgA2, which is downstream of IgE in the chromosomal locus. The frequency of class-switching to IgE in the gastrointestinal tract, and the microanatomical localization of these events, is unclear. Potential sites of IgE development include secondary lymphoid structures such as Peyer's Patches (purple oval with dashed line), extrafollicular sites in the lamina propria (yellow), or alternatively, other secondary lymphoid or mucosal sites in the body. What T cell help, if any, is required for IgE switching in different gastrointestinal sites is unclear. B cell exposure to food allergen proteins can occur via introduction of allergen proteins to the GALT, and possibly other tissue sites. The key routes of allergen access from the gut lumen may include epithelial barrier disruption, or transcytosis of immunoglobulin-allergen complexes via Fc receptors expressed on epithelial cells (pictured: allergen-IgE bound to "low-affinity" IgE receptor CD23, and allergen-IgG bound to FcRn). Uptake can also occur via dendritic cells and macrophages (not shown). Key questions for ongoing research include: Do B cells that class-switch to IgE in the gastrointestinal tract differentiate into memory B cells or plasma cells *in situ*? To what extent do daughter cells persist locally in the tissue as opposed to distributing to other sites in the body via the lymph and blood? What are the effector functions of locally-generated vs. systemic food-allergen-specific IgE? How do the microbiota influence these processes? How does the development of IgE in the gastrointestinal tract differ in individuals with food allergy compared to healthy individuals, and how are these pathways altered during immunotherapy for food allergy?

open question whether the B cells in the blood can provide an adequate sampling of those in other tissue sites. A variety of studies have examined the extent to which B cells clonally related to those in the gut appear in other tissues such as the peripheral blood (89), bone marrow (90), and mammary glands (84); other work has addressed the extent of dispersal of members of B cell clones between different sites in the GI tract (91). A recent study using high-throughput DNA sequencing analyzed B cell clonal distribution between blood, spleen, bone marrow and lung, in comparison to mesenteric lymph nodes, jejunum, ileum, and colon. The GI tissues showed high frequencies of shared clones between tissues, while other clones were found shared at high frequencies between blood, marrow, spleen, and lung, suggesting two major networks of B cell clonal populations (92). Whether this pattern of clonal sharing would hold for B cells and plasma cells expressing different isotypes, such as IgA subtypes compared to IgG subtypes, will require further study, as the data were generated from genomic DNA template from which isotype information cannot be obtained. In investigations of celiac disease, analysis of clonal relationships between plasma cells in distinct intestinal biopsy locations identified significant sharing of clone members between biopsy sites, particularly for the clones expressing antibodies specific for the autoantigen tissue transglutaminase-2 (93). Another analysis of highly abundant IgA-expressing clones in the blood of two human adults found that 36.5% of these selected high-frequency IgA lineages could also be detected in jejunal samples from the same individuals (89). It remains to be seen whether smaller clones are similarly shared between different tissue sites, whether categories of tissue-specific clonal B cell lineages exist in humans, and how anatomical clone sharing relates to the biology of IgE-expressing cell lineages.

BCR repertoire sequencing of flow cytometrically sorted B cell subpopulations has been used to link a population of IgM+ memory B cells with IgM+ plasma cells that produce antibodies that coat commensal bacteria (94). The authors found that, compared to peripheral blood and colon, a high percentage of B cells in the small intestine are memory B cells that express IgM. Memory IgM+ B cells shared clonal overlap with IgM+ plasma cells and, to a lesser extent, IgA+ plasma cells (94). This study also showed that IgM+ memory B cell/plasma cell pools in the gut showed decreased usage of the IgH V genes IGHV1-18, IGHV1-69, and IGHV4-34, and increased usage of IGHV3-7 and IGHV3-23, as has been observed in antigen-experienced, somatically hypermutated peripheral blood B cell repertoires (95). Compared to naïve B cells sorted from the gut, gut IgM memory and plasma cell subsets have more mutated IGHV gene regions and shorter CDR-H3 lengths, providing further evidence for antigen experience (96, 97).

SUMMARY AND OUTSTANDING QUESTIONS

Despite significant progress in increasing our understanding of B cell and plasma cell populations in the tissues of the GI tract, and ongoing enthusiasm for human studies motivated by clinical questions, a number of mysteries about the role of these cell populations in healthy immunity and in food allergy remain unanswered (**Figure 1**). Detailed analysis of the molecular and cellular mechanisms that guide mucosal IgE-related B cell and plasma cell development and antibody production offers the prospect of answering many of these key questions, particularly in the context of large, well-controlled clinical trials of allergy immunotherapy interventions. Integration of mechanistic studies in clinical trials could aim to address the following areas: determining whether monitoring of B cells/plasma cells in the gut compared to cells in the blood identifies better correlates of successful clinical outcomes or risk of adverse events; understanding how B cell and plasma cell phenotypes in blood, GI tract or bone marrow contribute to serologic changes that occur during immunotherapy, particularly in allergen-specific IgE, IgG4, and IgA; assessing the effect that microbiome modifications may have on gut B cells and plasma cells, and their relationship to therapeutic outcomes. Inclusion of GI biopsies in immunotherapy trials will be required to provide a starting point for addressing these questions. Insights obtained from such studies may lead to new strategies for the prevention, diagnosis, and treatment of IgE-mediated food allergy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Epigenetic Regulation via Altered Histone Acetylation Results in Suppression of Mast Cell Function and Mast Cell-Mediated Food Allergic Responses

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Mast cells are highly versatile cells that perform a variety of functions depending on the immune trigger, context of activation, and cytokine stimulus. Antigen-mediated mast cell responses are regulated by transcriptional processes that result in the induction of numerous genes contributing to mast cell function. Recently, we also showed that exposure to dietary agents with known epigenetic actions such as curcumin can suppress mast cell-mediated food allergy, suggesting that mast cell responses *in vivo* may be epigenetically regulated. To further assess the effects of epigenetic modifications on mast cell function, we examined the behavior of bone marrow-derived mast cells (BMMCs) in response to trichostatin A (TSA) treatment, a well-studied histone deacetylase inhibitor. IgE-mediated BMMC activation resulted in enhanced expression and secretion of IL-4, IL-6, TNF-α, and IL-13. In contrast, pretreatment with TSA resulted in altered cytokine secretion. This was accompanied by decreased expression of FcεRI and mast cell degranulation. Interestingly, exposure to non-IgE stimuli such as IL-33, was also affected by TSA treatment. Furthermore, continuous TSA exposure contributed to mast cell apoptosis and a decrease in survival. Further examination revealed an increase in I-κBα and a decrease in phospho-relA levels in TSA-treated BMMCs, suggesting that TSA alters transcriptional processes, resulting in enhancement of I-κBα transcription and decreased NF-κB activation. Lastly, treatment of wild-type mice with TSA in a model of ovalbumin-induced food allergy resulted in a significant attenuation in the development of food allergy symptoms including decreases in allergic diarrhea and mast cell activation. These data therefore suggest that the epigenetic regulation of mast cell activation during immune responses may occur *via* altered histone acetylation, and that exposure to dietary substances may induce epigenetic modifications that modulate mast cell function.

Keywords: mast cells, food allergy, trichostatin A, histone deacetylase (HDAC) inhibitors, epigenetics

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INTRODUCTION

IgE-mediated mast cell activation plays a critical role in the development of allergic responses to food antigens (1, 2). Mast cells and their mediators drive acute episodes of food allergy resulting in the development of severe intestinal anaphylaxis, which is often manifested as diarrhea, shock, and painful abdominal cramps. Furthermore, the effects of mast cells are mediated through a complex interplay of cellular interactions involving allergen-specific Th2 cells and other cell types such as eosinophils, epithelial cells, and ILC2s, which together contribute to the development of acute intestinal inflammation underlying the clinical symptoms (2–4).

The incidence of food allergy in the West has been exponentially rising and approximately 3–6% of individuals manifest food allergy symptoms (5, 6). However, not everyone undergoes allergic sensitization to the same allergen and allergic children are often able to outgrow some of the allergies they previously exhibited. Although the cells and molecules mediating allergic reactions have been well-studied, the mechanisms underlying the regulation of allergic sensitization and immune activation are still poorly understood. Accumulating evidence from a number of studies suggests that the development of the allergic response is tightly regulated via a complex network of interactions between immune cells, genes, and the environment that result in the inhibition of tolerance mechanisms and the promotion of allergic sensitization to environmental allergens (2, 7). Both genetic polymorphisms and exposure to various environmental stimuli have been shown to increase the susceptibility of developing allergic disease. With respect to the latter, colonizing microbiota, history of prior infections, dietary components, and exposure to environmental factors such as pollution or antibiotic treatment have all been demonstrated to shape the outcome of the allergic response (2, 6–13)). None of these variables by themselves however can account for differences in allergic sensitization in diverse patient subsets, suggesting that the induction of immune activation may be finely regulated *via* subtle epigenetic interactions involving environmental components and immune genes.

Several types of chromatin epigenetic modifications have been shown to influence gene expression (14). These include methylation of DNA at CpG islands or various post-translational modifications of histone tails, such as acetylation and methylation, resulting in enhanced or decreased access of transcriptional factors to gene promoters or enhancers. The role of epigenetic modifications in driving T cell differentiation and development has been well-established (15–19). Several studies also suggest a role for epigenetic modulation of allergic sensitization and inflammation (18, 20–27). However, the effects of epigenetic modification in modulating the behavior of T cells and particularly mast cells during allergic responses to food antigens has not been extensively examined. We previously demonstrated that frequent ingestion of curcumin, which is an active ingredient of the curry spice turmeric, modulates intestinal mast cell function and suppresses the development of mast cell-mediated food allergic responses, suggesting that exposure to dietary components can regulate the development of food allergy (28). This is especially interesting since a number of people worldwide consume curcumin on a daily basis and it has been shown to have immunomodulatory properties, which influence the activation of immune cells. Recent studies further suggest that the effects of curcumin may be mediated via regulation of epigenetic modifications that enhance or inhibit inflammatory responses (29–31). We therefore hypothesized that mast cell function during food allergy may be epigenetically regulated resulting in the development or suppression of allergic reactions.

In order to examine the effects of epigenetic regulation of mast cells, we used the well-established histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). TSA, a fungal antibiotic, belongs to a class of extensively studied histone deacetylase inhibitors that have been used to examine epigenetic interactions involving histone acetylation (32–36). The addition of acetyl groups at lysine residues in histone molecules by histone acetyl transferases (HATs) is generally thought to increase DNA accessibility and promote gene expression. In contrast, HDACs remove the acetyl groups, thereby increasing chromatin compaction and inhibiting gene transcription. TSA is a pan-HDAC inhibitor (HDACi), inhibiting the enzyme activity of several class I and class II HDACs, including HDAC 1, 2, 3, 4, 6 and 10 isoforms (37). As such, treatment with pan-HDACi's such as TSA can induce hyperacetylation of histone molecules, with the potential to enhance gene expression (38). Furthermore, they can also directly modulate the activity of non-histone proteins including transcription factors and cell cycle proteins (39, 40). However, depending on the type of immune cell and antigen treatment, both pro- and anti-inflammatory effects have been observed, suggesting that HDAC inhibition can affect the activation of multiple genes both upstream and downstream of the target molecule being examined (32, 34, 41–44). This includes immunomodulatory effects involving NF-κB (45–47), as well as the production of pro-inflammatory cytokines by antigen-exposed immune cells such as macrophages and ILC2s (48–51). Similarly, TSA-mediated suppression of both adaptive and innate allergic airway inflammation has also been observed in mouse models (51–58).

Here, we show for the first time, that treatment with TSA attenuates IgE-mediated mast cell activation during food allergy responses. Balb/c mice sensitized and orally challenged with chicken egg ovalbumin (OVA) develop robust allergic responses including allergic diarrhea, intestinal mast cell activation and the induction of Th2 responses. In contrast, the development of intestinal anaphylaxis and mast cell activation was significantly attenuated in TSA-treated mice. Similarly, Th2 cytokine production and gene expression was also affected in TSA-treated animals. *In vitro* examination of TSA treatment on IgE-activated mast cells demonstrated a significant inhibition of the production of proinflammatory cytokines such as IL-6, IL-13, TNF-α, and IL-4. This was accompanied by decreased mast cell degranulation and FcεRI expression. TSA treatment also modulated mast cell responses to IL-33 stimulation,

demonstrating that the effects of TSA are not limited to the IgE signaling pathway. Further examination revealed decreased NF-κB activation in TSA-treated mast cells, suggesting that exposure to TSA alters transcriptional processes regulating NF-κB activation. Taken together, our data elucidate a novel role for TSA in modulating mast cell function during food allergy, suggesting that the activation and function of mast cells is epigenetically regulated.

MATERIALS AND METHODS

Animals

Balb/c mice were purchased from Taconic Farms (Germantown, NY, United States) and bred in house. All research was approved by the Institutional Animal Care and Use Committee (IACUC) of Western New England University and was conducted according to IACUC guidelines. Animals used for research were sacrificed using compressed CO_2 gas.

Food Allergy Sensitization and Challenge Protocol

To induce food allergy, Balb/c mice were intraperitoneally immunized with 50 μg chicken egg ovalbumin (OVA) and 1 mg alum on days 0 and 14 of the experimental protocol as previously described (28, 59). Both OVA and alum were obtained from Sigma Aldrich. Two weeks after the second OVA sensitization, mice were challenged intragastrically with 50 mg OVA every other day for a total of 6 challenges. One hour following the sixth challenge, mice were sacrificed and the development of food allergy was assessed.

TSA Administration

Starting 1 day before the challenge phase of the experiment 75 μg or 2.5 mg/kg by weight of TSA (Sigma Aldrich) in phosphate buffered saline (PBS) was administered intraperitoneally to Balb/c mice which had received OVA sensitization. Administration was continued daily while OVA challenges were performed on alternating days. TSA was also administered to Balb/c mice which had received OVA sensitization but did not receive OVA challenges.

Treatment With Curcumin

The effects of curcumin (Sigma Aldrich) on BMMCs were examined as previously described (28). Briefly, cells were treated with vehicle or 30 μM curcumin in DMSO for varying periods of time (1 and 24 h, respectively) and cells and supernatants were collected for mRNA analysis and assessment of cytokine secretion.

Measurement of Intestinal Anaphylaxis

Intestinal anaphylaxis of challenged mice was assessed by scoring the percentage of mice exhibiting allergic diarrhea as previously described (28, 59). Briefly, mice were observed for the presence of diarrhea for 1 h following the sixth challenge and scored as positive or negative for the presence of diarrhea.

Histological Analysis and Enumeration of Mast Cells

Intestinal mast cells were enumerated, as previously described (28, 59). Tissue sections were stained with chloroacetate esterase (CAE) and mast cells were counted in complete cross sections of jejunum.

Quantitative PCR Analysis and ELISAs

Quantitative real-time PCR was performed using Taqman probes (Life Technologies) as previously described (28, 59). Expression of IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-33, IFN-γ, and NF-κB was calculated relative to GAPDH transcripts. ELISAs for murine mast cell protease (mMCP-1) (Life Technologies) and OVA-specific IgE were performed on serum samples taken immediately after sacrifice as previously described (28, 59). ELISAs for IL-4, IL-5, IL-6, TNF-α, and IFN-γ (Biolegend) as well as IL-13 (Affymetryx) were performed on cell supernatants according to manufacturer's instructions as previously described (28, 59).

Mesenteric Lymph Node Stimulation

Mesenteric lymph node (MLN) cells were harvested from animals after sacrifice and cultured with complete RPMI medium, 200 μg/mL OVA or anti-CD3 (0.2 μg/ml) and anti-CD-28 (0.2 μg/ml) for 4 days. Anti-CD3 and anti-CD-28 were obtained from Biolegend. Cytokines were enumerated in supernatants, as previously described (28, 59).

Bone Marrow-Derived Mast Cell (BMMC) Culture

BMMCs were generated from Balb/c mice as previously described (28, 59). Briefly, bone marrow was obtained from the femurs of naïve mice and cultured with 10 ng/mL rIL-3 and rSCF (Shenandoah Biotechnology) in complete RPMI medium (Life Technologies) for 4 weeks prior to experimentation.

In vitro Studies With TSA

One million BMMCs per mL were cultured in triplicate with IL-3 and SCF or 2 μg/mL DNP-IgE (Sigma Aldrich). To determine the effects of TSA on proliferation and cytokine production TSA in dimethyl sulfoxide (DMSO) was added in concentrations of 10, 30,100, 300, or 500 nM for varying time points. Control wells were treated with vehicle alone. Cells were then stimulated with 200 ng/mL DNP-BSA (Sigma Aldrich) or 20 ng/mL rIL-33 (Biolegend).

β-Hexosaminidase Assay

BMMCs were cultured with rIL-3 and rSCF in the presence or absence of 500 nM TSA for 1 h or overnight. Cells were activated and supernatants and cell lysates were collected 1 h later. β-hexosaminidase (β-hex) activity was assessed, as previously described (28, 59). Briefly, cells were washed and supernatants and pellets were collected. Pellets were lysed with 0.5% Triton X-100. Both supernatants and pellets were then treated with 4-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NAG) substrate (Sigma) for 1 h. Plates were read at 405 nm using a spectrophotometer to determine β-hexosaminidase activity. Data are depicted as percent specific release according

to the following formula: (Stimulated supernatants/(supernatant ± pellet)*100−unstimulated supernatants/(supernatant ± pellet)*100).

Flow Cytometry

BMMCs were incubated with monoclonal antibodies for c-Kit, FcεRI, annexin V and IgE conjugated to either APC, FITC or PE (Biolegend). All antibodies were diluted 1:200 prior to incubation for 20–30 min. Flow cytometry and analysis was performed using an Accuri C6 cytometer and Flowjo software.

Intracellular Cytokine Staining

Peritoneal cells were isolated by peritoneal lavage from naïve Balb/c mice. One million cells/mL were treated with or without DNP-IgE and TSA as described above and activated with DNP-BSA. 3 μg/mL of Brefeldin A (Thermofisher Scientific) was added to all samples according to manufacturer's instructions. Six hours later, cells were surface stained for mast cells using c-Kit APC and FcεRI/IgE-FITC. They were then treated for 10 min with fixation reagent (Thermofisher Scientific) followed by washing with 1X Permeabilization buffer (Thermosfisher Scientific). Intracellular cytokines were assessed by staining with IL-13, IL-6, and TNF-α conjugated to PE (Biolegend).

Western Blot

BMMCs were cultured with rIL-3 and rSCF in the presence or absence of 500 nM TSA and DNP-IgE for 1 h or overnight prior to activation with DNP-BSA. After addition of antigen, cells were incubated for 6–8 h. Whole cell extracts were then obtained using RIPA buffer containing 1% Triton X-100 and quantified with Coomassie Plus (Bradford) Protein Assay (ThermoFisher Scientific). Equal amounts of protein were loaded onto 10% SDS-PAGE gels and transferred to PVDF membrane. Membranes were blocked for 1 h in 5% milk or BSA and incubated overnight with primary antibodies [phospho-relA (1:500) and β-actin (1:5,000)]. Antibodies were obtained from Santa Cruz Biotechnology and Abcam, respectively. Membranes were then washed with PBS tween 20 and incubated with the appropriate secondary antibodies. Membranes were washed once again before the addition of chemiluminescent reagent (Invitrogen). Membranes were imaged using a Biorad Chemidoc.

Statistical Analysis

Data are expressed as mean ± SEM, unless stated otherwise. Statistical significance comparing different sets of mice (between 2 groups) was determined by the Student's *t*-test. $p < 0.05$ were considered significant. Analysis was performed using GraphPad Prism software and/or Microsoft Excel.

RESULTS

TSA Treatment Modulates Cytokine Production in Bone-Marrow Derived Mast Cells

Mast cells are highly versatile cells that perform a variety of functions depending on the immune trigger, context of activation and cytokine stimulus. Antigen-mediated mast cell responses are regulated by transcriptional processes that result in the induction of numerous genes contributing to mast cell function. To examine the effects of TSA treatment on resting mast cell function, BMMCs were treated with TSA for 24 h in the presence of rIL-3 and rSCF and the expression and secretion of mast cell cytokines was examined.

Treatment of resting mast cells with rIL-3 and rSCF for 24 h induced the transcriptional upregulation of TNF-α, IL-6, IL-4, and IL-13 (**Figures 1A–D**). This was also accompanied by the secretion of IL-6 and IL-13 into culture supernatants (**Figures 1E,F**). In contrast, addition of TSA along with the mast cell growth factors suppressed the expression and secretion of these cytokines, suggesting that TSA suppresses mast cell cytokine production in resting mast cells (**Figures 1A–F**). A similar inhibition of cytokine production was observed in curcumin-treated mast cells as we have previously demonstrated (**Figures 1A–F**) (28).

Pretreatment With TSA Inhibits Cytokine Production in IgE-Activated Mast Cells

IgE-induced mast cell activation plays a critical role in the development of anaphylactic symptoms to allergenic stimuli. To further examine the effects of TSA treatment on IgE-mediated activation of mast cells, BMMCs were cultured with rIL-3 and rSCF and activated with DNP-specific IgE and DNP-BSA. Challenge with DNP-BSA in IgE-primed BMMCs resulted in a robust induction of the genes for TNF-α, IL-6, IL-4, and IL-13 compared to unactivated controls (**Figures 2A–D**). Similarly the secretion of TNF-α, IL-6, and IL-13 was also significantly enhanced in IgE-activated BMMCs (**Figure 2E**). In contrast, pre-treatment with TSA for 24 or 1 h resulted in a significant suppression of both the expression and secretion of cytokines (**Figures 2A–F** and data not shown). A similar pattern of cytokine inhibition was also observed in freshly isolated peritoneal mast cells that had been pre-treated with TSA and activated with IgE and antigen, suggesting that the effects of TSA are not limited to BMMCs alone, but can also extend to connective tissue mast cells *in vivo* (**Supplementary Figures 1A,B**). To further determine whether the effect of TSA on BMMCs was dose-dependent, BMMCs were treated with increasing concentrations of TSA overnight, and its effects on IgE-mediated activation were assessed. Treatment with increasing concentrations of TSA demonstrated a dose-dependent suppressive effect on the secretion of IL-6 and IL-13 (**Figures 2G,H**). To further assess the effects of TSA on BMMC cytokine production, we also examined the expression levels of IFN-γ and IL-17 in BMMCs. Interestingly, the expression of both IFN-γ and IL-17 was detected in BMMCs, suggesting that they can produce Th1 and Th17 cytokines (**Figures 2I,J**). Treatment with IgE and antigen resulted in enhanced expression of both IFN-γ and IL-17 in BMMCs compared to unactivated controls. However, in contrast to the expression of TNF-α, IL-6 and IL-13, no changes in IFN-γ and IL-17 expression were observed in TSA-treated and IgE-activated BMMCs (**Figures 2I,J**). These data therefore suggest that TSA treatment can differentially modulate

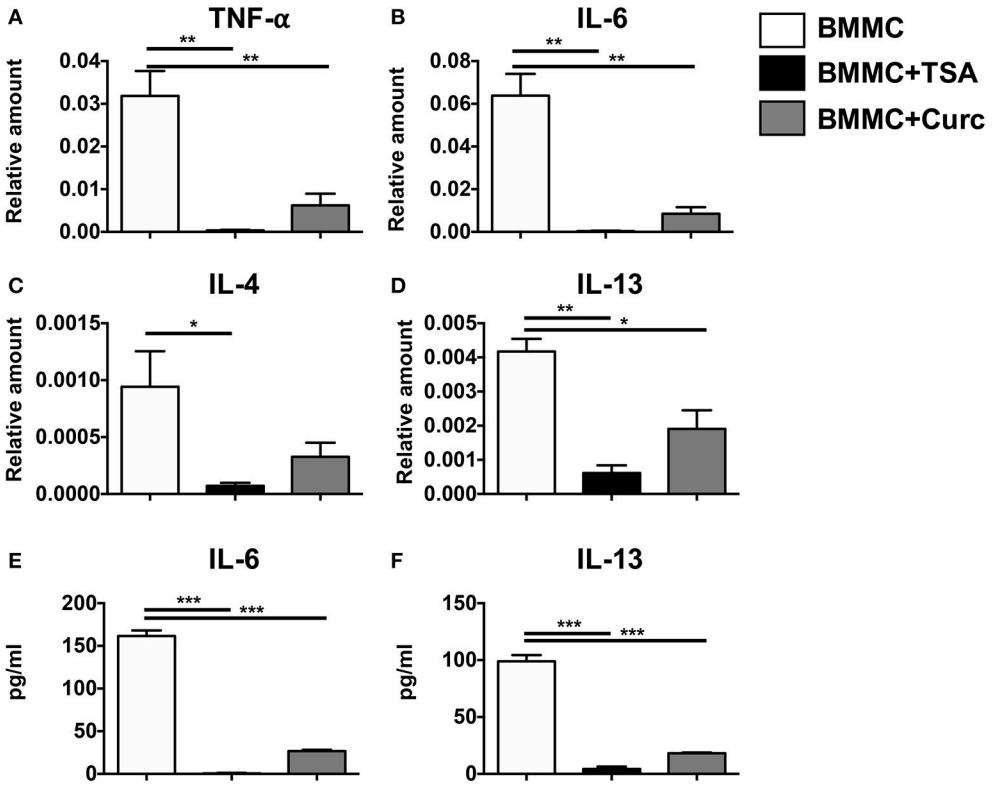

FIGURE 1 | TSA suppresses cytokine gene expression and secretion in resting mast cells. BMMCs were treated with 500 nM TSA, 30 μM curcumin, or vehicle in triplicates. 1 and 24 h later, respectively, cells were collected for mRNA analysis and supernatants were evaluated for cytokine secretion. **(A–D)** mRNA transcripts relative to GAPDH are shown **(E,F)** Levels of IL-6 and IL-13 are shown. Data are representative of 2–3 experiments. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$ by Students *t*-test.

the production of cytokines by mast cells stimulated with IgE and antigen.

TSA Treatment Attenuates Mast Cell Degranulation and FcεRI Expression

Since pretreatment with TSA inhibited the production of proinflammatory cytokines from IgE-activated mast cells, suggesting that TSA can modulate the *de novo* synthesis of mast cell cytokines, we wondered whether it could also similarly regulate mast cell degranulation. Mast cell degranulation was assessed by examining the release of β-hexosaminidase (β-hex) in cell culture supernatants. As we had anticipated, while IgE and antigen-activated BMMCs exhibited increased β-hex release, 24 h pre-treatment with TSA significantly decreased the percent of β-hex release in activated mast cells, suggesting that TSA treatment can inhibit IgE-mediated degranulation of BMMCs (**Figure 3A**). Similar results were also obtained with freshly isolated peritoneal mast cells that had been treated with IgE and antigen (**Supplementary Figure 1C**). Surprisingly, however, 1 h pre-treatment with TSA did not result in any attenuation of BMMC degranulation (**Figure 3A**).

Since the IgE-mediated activation of mast cells is dependent on the expression of the high affinity IgE receptor, FcεRI on mast cells, we examined whether TSA treatment modulates the expression of this receptor in resting mast cells. BMMCs were cultured with rIL-3 and rSCF for up to 24 h in the presence or absence of TSA and the expression of FcεRI was assessed by flow cytometry at several time points. One and 4 h after treatment with TSA, no changes in mean fluorescence expression of FcεRI was observed in TSA-treated cells as compared with untreated controls (**Figures 3B,C**). However, a gradual decrease in FcεRI expression was observed starting at 8 h after treatment with significant suppression observed at 24 h, from an MFI of approximately 31,896–3,829 (**Figures 3B,C**). This suggests that one potential explanation for the decreased IgE-mediated degranulation of TSA-treated mast cells may be related to decreased IgE binding. However, it does not account for inhibition of cytokine production in IgE-activated mast cells, since cytokine suppression was observed in mast cells pretreated with TSA for both 1 and 24 h, suggesting that decreased IgE binding may only partly be responsible for the observed effects. To therefore assess whether TSA treatment prevented the *de novo* synthesis of mast cell cytokines after IgE-mediated activation had already occurred, we treated BMMCs with TSA immediately post-activation with IgE and antigen. To our surprise, TSA treatment after antigen-mediated activation had occurred had no effect on the release of cytokines, suggesting that the effects of TSA pretreatment are mediated by preventing the transcriptional activation of antigen-induced genes (**Figure 3D**).

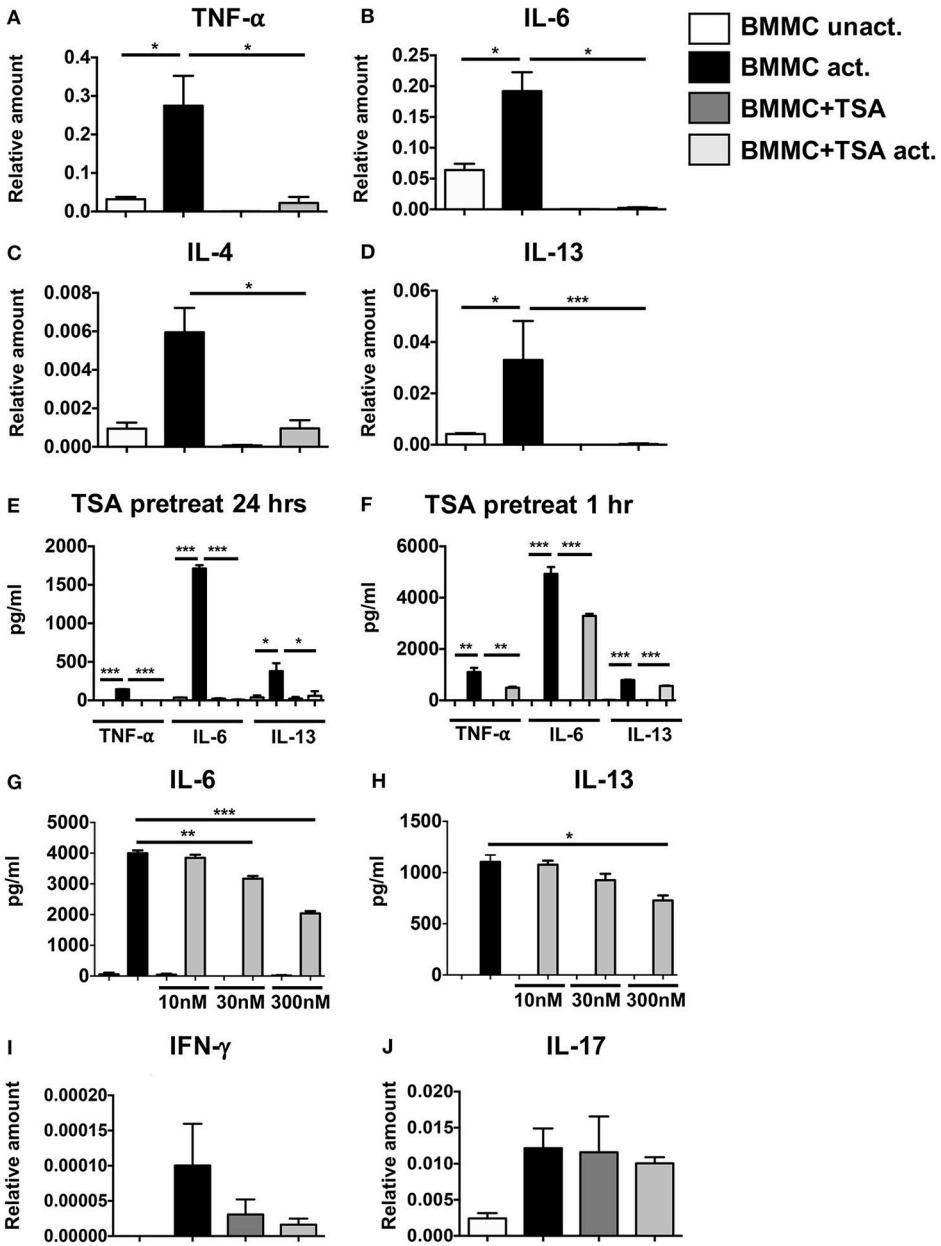

FIGURE 2 | TSA suppresses cytokine gene expression and secretion in antigen-activated cells. BMMCs were pre-treated with 500 nM TSA or vehicle overnight. Some groups of cells were also simultaneously primed with 1μg/ml DNP-IgE. The next day, all cells were activated with 200 ng/ml DNP-BSA. **(A–D)** 1 h later, cells were collected for mRNA analysis. Transcripts relative to GAPDH are shown **(E)** Levels of cytokines secreted 12 h after activation are shown. **(F)** In other experiments, BMMCs were pre-treated with 500 nM TSA for 1 h prior to activation with antigen. Levels of cytokines secreted 12 h later are shown. **(G,H)** BMMCs were pre-treated overnight with varying doses of TSA prior to IgE and antigen activation. Cytokines secreted 12 h later are shown. **(I,J)** mRNA transcripts in BMMCs pre-treated with 500 nM TSA overnight and activated with IgE and antigen. Data are representative of 5–6 independent experiments. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$ by Students *t*-test.

TSA Pretreatment Inhibits IL-33-Mediated Cytokine Production in Mast Cells

Our data suggest that TSA treatment modulates cytokine production in IgE-activated mast cells by regulating FcεRI expression. We were curious if TSA could also regulate cytokine production in mast cells stimulated independently of IgE. We therefore examined the effects of TSA on IL-33-stimulated mast cells, which is a potent inducer of mast cell cytokines. Treatment of mast cells with IL-33 resulted in the production of elevated levels of the cytokines IL-6, IL-13, and TNF-α (**Figures 4A–C**). In contrast, pre-treatment with TSA prior to IL-33 stimulation significantly decreased the levels of mast cell cytokines in a dose-dependent manner (**Figures 4A–C**). This suggests that TSA can also inhibit the production of

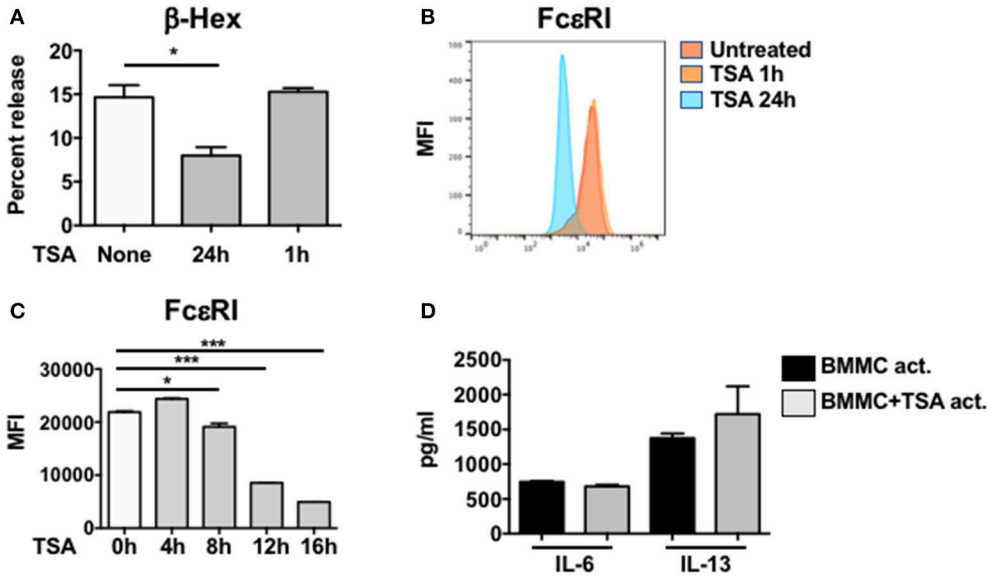

FIGURE 3 | TSA attenuates mast cell degranulation and FcεRI expression. **(A)** BMMCs were treated with vehicle or 500 nM TSA for either 24 or 1 h prior to IgE-induced activation with DNP-BSA. β-hex levels were enumerated in cell supernatants and lysates. Percent release of β-hex is shown **(B)** BMMCs were treated with vehicle or 500 nM TSA for either 24 or 1 h. The expression of FcεRI was evaluated by flow cytometry. Mean fluorescence intensity (MFI) is shown. **(C)** Kinetics of FcεRI expression after TSA treatment is shown. **(D)** BMMCs were activated with DNP-IgE and antigen. One minute after activation, cells were treated with either vehicle or 500 nM TSA. Cytokine levels in supernatants were enumerated 12 h later. Data are representative of 2 independent experiments. $*p < 0.01$; $***p < 0.0001$ by Students *t*-test.

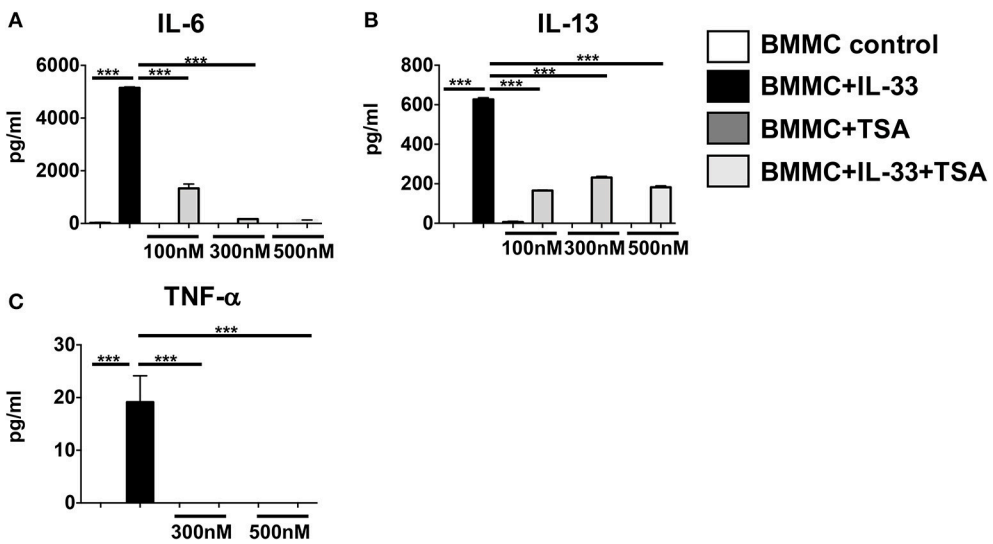

FIGURE 4 | TSA suppresses IL-33-induced mast cell cytokines in a dose-dependent manner. **(A–C)** BMMCs were cultured with rIL-33 and varying doses of TSA or vehicle for 24 h. Levels of cytokines in supernatants were assessed by ELISA. Data are representative of 2 independent experiments. $***p < 0.0001$ by Students *t*-test.

mast cell cytokines stimulated with innate cytokines such as IL-33.

Long-Term TSA Exposure Reduces Survival of BMMCs and Induces Apoptosis

We have previously shown that one of the mechanisms by which curcumin modulates mast cell function is to inhibit mast cell survival via the induction of apoptosis (28). To determine whether a similar mechanism may be involved in TSA-induced regulation of BMMCs, we examined the effects of TSA treatment on BMMC proliferation and survival. Continuous exposure to TSA over a period of 6 days resulted in a progressive decline in mast cell numbers, while BMMCs cultured with rIL-3 and rSCF proliferated normally over the same timeframe (**Figure 5A**).

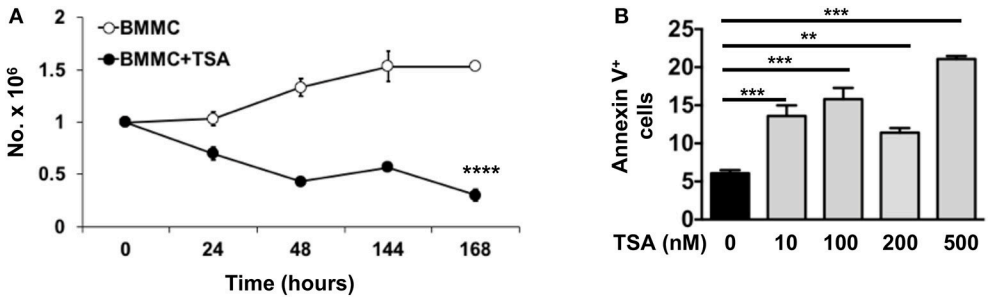

FIGURE 5 | TSA induces apoptosis and inhibits mast cell proliferation. **(A)** BMMCs were cultured with rIL-3 and rSCF for 6 days in the presence or absence of 500 nM TSA. Cells were counted daily and live and dead cells were enumerated. **(B)** BMMCs were cultured with varying doses of TSA for 24 h and the expression of Annexin V was assessed by flow cytometry. Data are representative of 2 independent experiments. $^{**}p < 0.001$; $^{***}p < 0.0001$ by Students *t*-test. $^{****}p < 0.0001$, by ANOVA.

This suggested that TSA may induce apoptosis in BMMCs as has been described in other cell types similarly exposed to TSA (42, 60, 61). Further examination of BMMCs treated with TSA for 24 h demonstrated a dose-dependent increase in Annexin V signal via flow cytometry, suggesting that TSA is able to induce apoptosis in BMMCs after treatment for 24 h (**Figure 5B**).

TSA Treatment Results in Decreased NF-κB Activation in IgE-Activated BMMCs

To further determine the cause of decreased mast cell cytokine production in activated mast cells, we next examined the NF-κB signaling pathway, a key regulatory pathway that is essential for the transcriptional activation of various cytokines. Since I-κBα is the negative regulator of NF-κB, we assessed whether TSA treatment would lead to transcriptional upregulation of I-κBα upon activation in IgE-activated BMMCs. Thirty minutes post activation with IgE and antigen, no changes in I-κBα expression were observed between TSA-treated and control BMMCs activated via IgE and antigen (**Figure 6A**). However, 12 h later, increased I-κBα expression was observed in TSA-treated and IgE-activated BMMCs compared to similarly treated controls (**Figure 6B**). Since I-κBα expression has been shown to fluctuate over time after cellular activation (62) and NF-κB acts as the transcription factor that directly regulates cytokine expression, we examined BMMCs for NF-κB expression after activation with DNP-IgE and antigen. At this time point, NF-κB expression was completely suppressed in TSA-treated BMMCs (**Figure 6C**). To further confirm the effects of TSA on NF-κB induction, we examined the levels of phospho-relA to determine the extent of NF-κB activation. The p65 (RelA) sub-unit of NF-κB plays a crucial role in the activation of NF-κB and its phosphorylation at Ser^{276} (phospho-relA staining) can be assessed by Western blot as we have previously described (28). Western blot analysis demonstrated increased levels of phospho-rel A in IgE-activated BMMCs compared to controls. In contrast, the level of phospho-relA was attenuated in similarly activated TSA-treated BMMCs (**Figures 6D,E**). These data therefore suggest that modulation of BMMC function by TSA may be mediated through altered activation of NF-κB.

TSA Treatment During Oral Allergen Challenge Inhibits Diarrhea and Mast Cell Activation During Food Allergy Induction

Since TSA treatment had a profound suppressive effect on mast cells in cell culture, we sought to determine whether it could similarly modulate mast cell function *in vivo* during the development of food allergy. Balb/c mice were sensitized and orally challenged with OVA as described in Materials and Methods and the development of food allergy was assessed. One hour after the final oral challenge with OVA, allergen-sensitized and challenged mice exhibited the development of profuse diarrhea as previously demonstrated (59). In contrast, similarly challenged TSA-treated animals exhibited a lower incidence of diarrhea overall in comparison with untreated animals (**Figure 7A**). Examination of sera for mast cell markers revealed elevated levels of murine mast cell protease-1 (mMCP-1) in OVA-challenged animals, suggesting increased mast cell activation (**Figure 7B**). Similarly, elevated levels of OVA-specific IgE were also present in the sera of allergic animals (**Figure 7C**). In contrast, the levels of both mMCP-1 and OVA-IgE were decreased in TSA-treated allergic animals (**Figures 7B,C**). Furthermore, the numbers of intestinal mast cells in TSA-treated mice were also decreased compared to untreated mice (**Figures 7D,E**).

To further assess the effects of TSA treatment on modulation of cytokine production during food allergy, we examined the local and systemic production of Th2 type cytokines in allergic mice. As anticipated, examination of jejunal tissue for mRNA transcripts revealed the induction of a number of Th2-type cytokine genes in OVA-sensitized and challenged animals compared to controls (**Figures 8A–F**). In contrast, except for IFN-γ, decreased expression of these cytokines was observed in the intestines of most of the TSA-treated mice, suggesting that TSA is able to modulate cytokine production during food allergy development (**Figures 8A–F**). To further investigate the effects of TSA on NF-κB expression *in vivo*, we also examined jejunal tissue for NF-κB transcripts as a measure of transcriptional regulation during food allergy development. While OVA-sensitized and challenged mice exhibited increased levels of total NF-κB mRNA in jejunal tissue compared to control mice, the expression of NF-κB was significantly reduced in the intestines

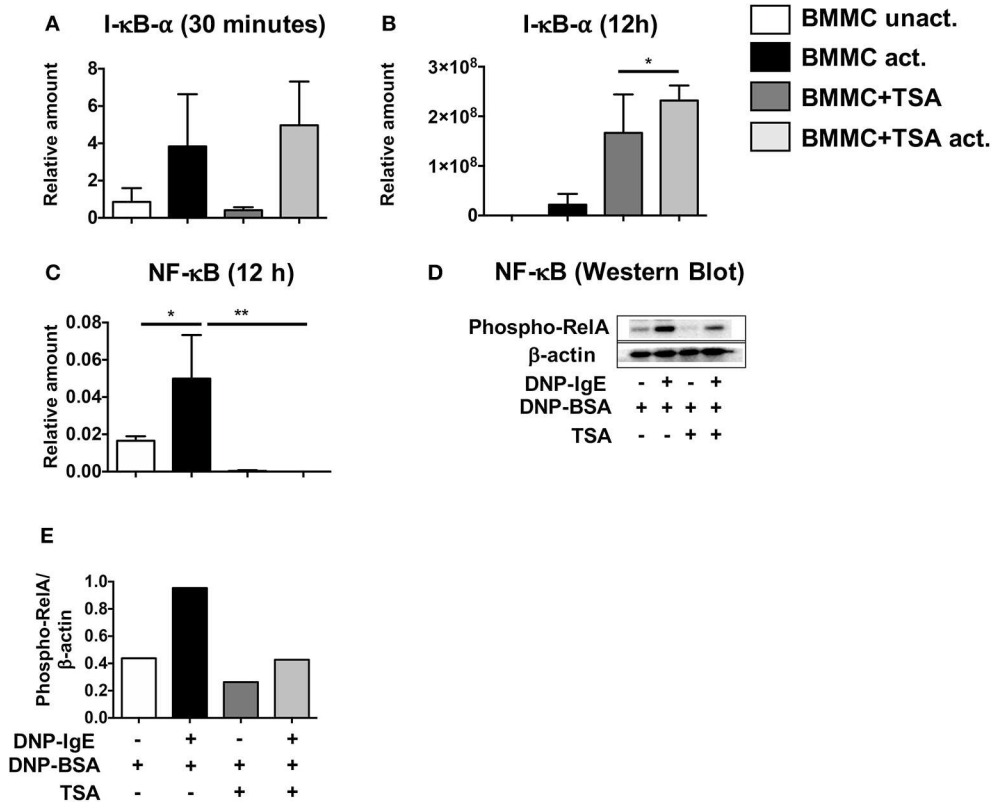

FIGURE 6 | TSA induces I-κBα expression and inhibits NF-κB activation. **(A–C)** BMMCs were pre-treated with vehicle or 500 nM TSA overnight prior to activation with DNP-IgE and DNP-BSA. The expression of I-κBα and NF-κB was assessed by RT-PCR. **(D,E)** Western blot was performed on whole cell lysate protein extracts from BMMCs pre-treated with vehicle or TSA and activated with DNP-IgE and DNP-BSA. The amounts of phospho-relA and β-actin were assessed. Data are representative of 2 independent experiments. *$p < 0.01$; **$p < 0.001$ by Students *t*-test.

of similarly challenged and TSA-treated animals, confirming the results we had observed in cell culture with BMMCs (**Figure 8G**).

To further confirm the effects of TSA on modulation of cytokine production by T cells, we examined the production of cytokines in mesenteric lymph node (MLN) cells from experimental animals in response to stimulation with OVA or T cell agonists. In comparison to controls, exposure to OVA induced the production of Th2 cytokines in MLN cells from WT OVA mice that had been sensitized and challenged with OVA (**Figures 9A–D**). In contrast, MLN cells from several OVA-sensitized and challenged TSA-treated animals exhibited a tendency toward lower levels of cytokine production overall compared with untreated animals (**Figures 9A–D**). Interestingly, however, TSA treatment had no effect on modulating the potential for cytokine production, since polyclonal activation of T cells in both groups resulted in equivalent levels of cytokine production (**Figures 9A–D**). These data therefore suggest that TSA can regulate the expression of cytokines in intestinal tissue and differentially modulate the production of cytokines by mast cells and T cells during food allergy development.

DISCUSSION

Epigenetic regulation of immune cell behavior is becoming increasingly accepted as a likely mechanism by which immune cell subsets mediate responses to widely differing stimuli. Mast cells, like many other immune cell types are extremely versatile and perform numerous functions contributing to the development of both innate and adaptive immune responses (63–66). As cells that can be rapidly activated during immune responses, they respond to diverse stimuli including alarmins such as IL-33, pathogen components such as TLR ligands, and antigen engagement *via* antibodies such as IgE and IgG. It is therefore extremely likely that the activation and function of mast cells during immune responses is regulated *via* epigenetic modifications induced by environmental exposure such as dietary antigens (67, 68).

In this report, we show for the first time that the inhibition of HDAC enzymes has a significant effect on mast cell activation and function during food allergy. Furthermore, we demonstrate that HDAC inhibition profoundly inhibits mast cell activation, degranulation and cytokine production *in vitro* in response to both IgE-dependent and independent stimuli, suggesting that

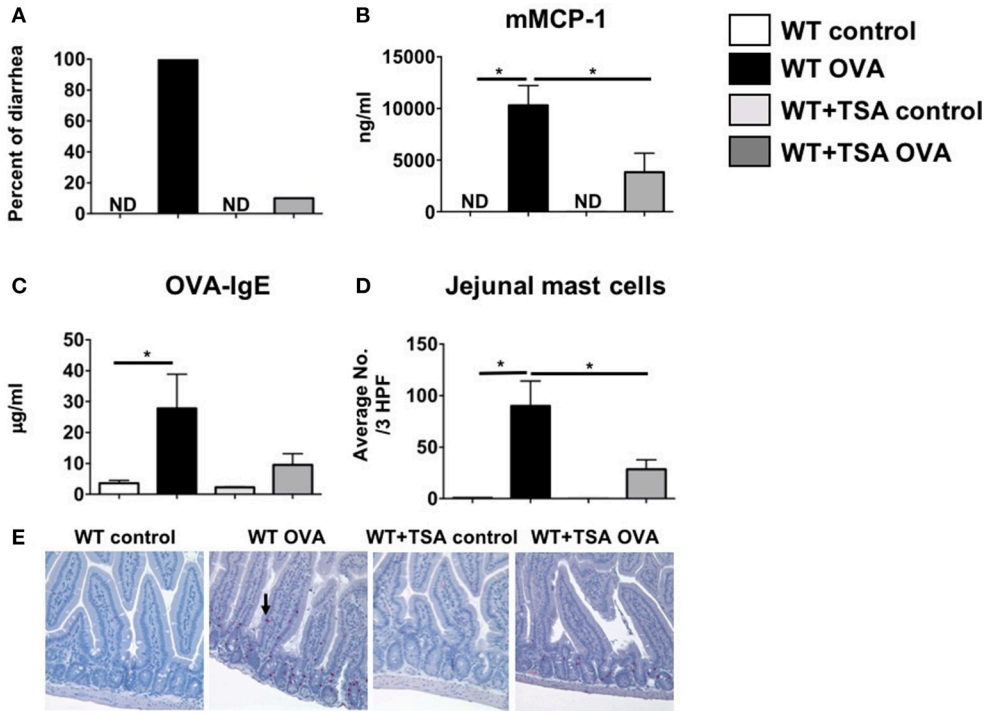

FIGURE 7 | Treatment with TSA inhibits the development of allergic diarrhea and mast cell activation in OVA-sensitized and challenged mice. Mice were sensitized and challenged with OVA as described in Materials and Methods. Beginning 1 day prior to OVA challenges, mice were treated with vehicle or 75 µg TSA *i.p.* daily until sacrifice. **(A)** Percent of mice exhibiting diarrhea **(B)** serum mMCP-1 levels **(C)** serum OVA-IgE levels and **(D)** average numbers of jejunal chloroacetate esterase-positive mast cells/ 3 high powered fields (HPF) are shown. **(E)** Representative histological sections from individual mice are shown. Mast cells are indicated by an arrow. Data are representative of 3 independent experiments. $n = 6$ mice/group. $^*p < 0.01$ by Students *t*-test. ND, not detected.

modification of histone acetylation may be a putative mechanism involved in modulating mast cell function.

TSA is a broad spectrum HDACi that has several pleiotropic effects on cellular gene expression, including both the upregulation and downregulation of genes involved in cellular functions. Examination of TSA in a number of disease models suggests that in general it has beneficial effects on cellular function, promoting the downregulation of chronic inflammation and the induction of apoptosis and anti-oxidative pathways (49, 52, 54, 57, 69–73). This has resulted in the FDA approval of several HDACi (not including TSA) as therapeutic agents, as well as further pursuit of the mechanisms by which HDACi promote or inhibit cellular function (33, 42, 44).

We have previously shown that frequent ingestion of curcumin, the active ingredient of the curry spice turmeric, results in the suppression of mast cell responses and the attenuation of mast cell-mediated experimental food allergy (28). Several studies have suggested that the effects of curcumin *in vivo* are mediated via epigenetic modulation of HDACs as well as HATs, prompting us to investigate whether the observed effects of curcumin on mast cells in our previous study were similarly induced *via* modification of histone acetylation (31). Emerging evidence from a number of studies suggests that both the expression of genes governing immune cell differentiation as well as the induction of allergic sensitization may be epigenetically modulated, further warranting examination of the effects of TSA treatment in our model of food allergy (25, 26, 74). Lastly, while some studies examining the effects of histone deacetylase inhibition on T cell and ILC2-mediated allergic inflammation have been published, its effects on mast cell-mediated allergic responses have not yet been examined (51, 52, 54, 55).

Examination of the effects of TSA in animal models of allergic airway disease (AAD) have yielded mixed results. TSA treatment in models of both acute and chronic AAD resulted in inhibition of airway inflammation, cytokine production, collagen deposition, mucus production and airway hyperresponsiveness (AHR) (52, 55). In contrast, another study reported that TSA treatment inhibits AHR, but not airway inflammation in a model of asthma (54). The effects of TSA in this model were induced by blocking calcium mobilization and inhibiting intracellular calcium release in airway smooth muscle cells. More recently, Toki et al. and Thio et al. demonstrated that TSA also inhibits ILC2-mediated and IL-33-dependent innate allergic inflammation (51, 58).

In this study, we sought to examine the effects of TSA treatment on mast cells in both cell culture as well as *in vivo* during the development of food allergy. Our data demonstrate that TSA can modulate the function of both resting mast cells, as well as mast cells activated *via* IgE and non-IgE pathways. A previous study suggested that TSA may have dichotomous effects on mast cells, inhibiting IL-6 production after IgE-mediated activation, but not when stimulated with LPS (75). In contrast,

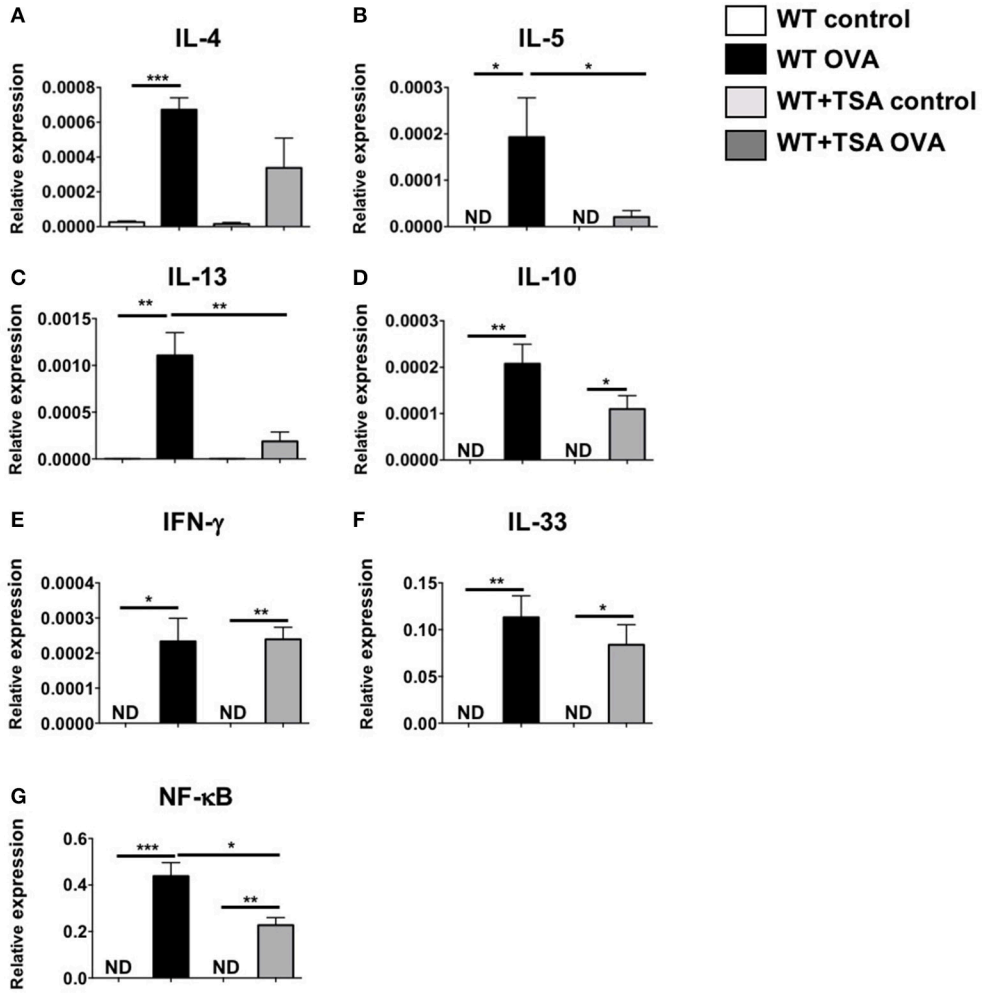

FIGURE 8 | TSA modulates intestinal cytokine and NF-κB gene expression in allergic mice. **(A–G)** OVA-sensitized and challenged mice were sacrificed and RNA and cDNA were prepared from jejunal extracts. The expression of jejunal cytokines was assessed relative to GAPDH using Taqman probes. $n = 6$ mice/group. Data are representative of 2 independent experiments. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$ by Students *t*-test. ND, not detected.

we demonstrate that the inhibitory effects of TSA are conferred irrespective of the type of stimulus and can be induced in both activated as well as resting mast cells. This is further corroborated by our observations demonstrating that while downregulation of FcεRI expression by TSA may partly account for the observed decreases in cytokine production, cytokine production is also inhibited in IL-33-stimulated mast cells, suggesting that the effects of TSA are mediated independently of the IgE pathway. Interestingly, however, TSA treatment immediately after IgE-mediated activation had occurred resulted in the reversal of cytokine inhibition, suggesting that the effects of TSA may be mediated by increasing the expression of an upstream negative regulator of transcription such as I-κBα, which is turned on soon after antigen-induced cross-linking has occurred.

Treatment with TSA also resulted in the apoptosis of mast cells over time and decreased their proliferation and survival, as has been observed with other cell types such as eosinophils and neutrophils (61). The anti-proliferative effects of TSA may partly contribute to the decreased cytokine production in activated mast cells, but it does not explain the complete suppression of cytokine production as observed in **Figures 1–4**, since there are still a significant number of nonapoptotic cells after 24 h of treatment with TSA and we observe significant downregulation of cytokines as early as 1 h after treatment.

Since we had previously shown that the effects of curcumin in our model of food allergy were mediated *via* inhibition of NF-κB activation, we examined the effects of TSA treatment on the induction of I-κBα and NF-κB, hypothesizing that TSA treatment may enhance the expression of I-κBα in activated mast cells. Examination of BMMCs 30 min after IgE-mediated activation demonstrated equivalent expression of I-κBα in both untreated and TSA-treated cells. However, this is not surprising since the expression of I-κBα is regulated through complex interactions with NF-κB in nuclear and cytoplasmic compartments involving an inducible autoregulatory pathway which results in increased I-κBα induction by activated NF-κB (46, 76, 77). As such, further

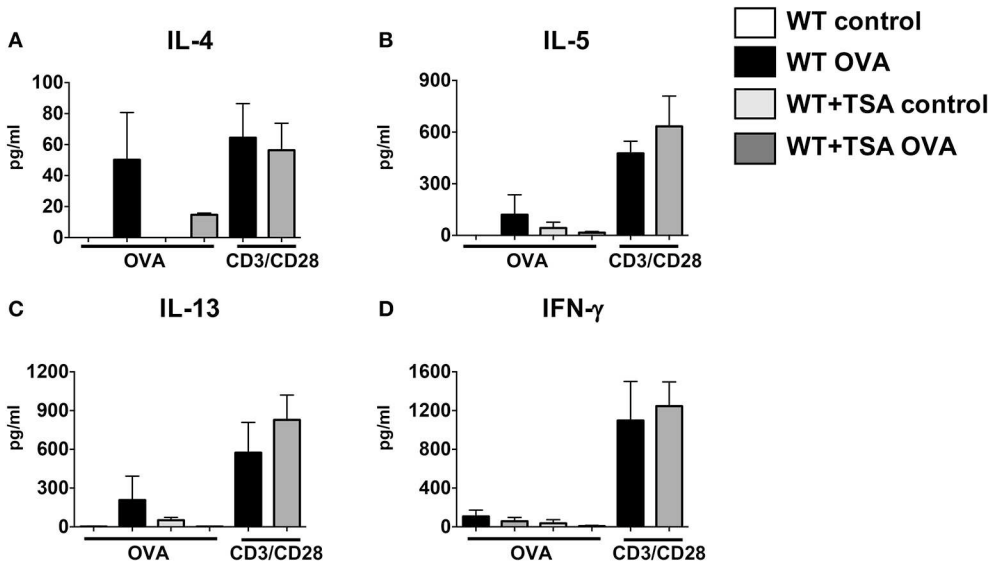

FIGURE 9 | TSA modulates cytokine secretion in OVA-stimulated mesenteric lymph node (MLN) cells. **(A–D)** MLN cells were collected from experimental animals sensitized and challenged with OVA and stimulated for 96 h with either OVA or anti-CD3 and anti-CD28 as described in section Materials and Methods. Culture supernatants were evaluated for secretion of cytokines. $n = 6$ mice/group. Data are representative of 2 independent experiments.

examination several hours later revealed a significant increase in expression of I-κBα in TSA-treated cells compared with untreated controls. Taken together with the decreased NF-κB expression and phospho-relA levels in BMMCs, this suggests that TSA may modulate BMMC function by altering I-κBα transcription and NF-κB transcription and activation.

The suppressive effects of TSA on mast cells were also observed *in vivo* during the development of food allergy. TSA treatment during the challenge phase resulted in the inhibition of allergic diarrhea, the attenuation of mast cell activation and intestinal mast cell numbers, and the suppression of Th2 cytokine genes, suggesting that histone deacetylase inhibition can modulate mast cell function *in vivo* and ameliorate the mast cell-mediated effects of food allergy such as intestinal anaphylaxis. Furthermore, the protective effects of TSA were conferred during the challenge phase in already sensitized animals, suggesting that TSA can modulate the mast cell-dependent phase of the response and attenuate mast cell-mediated effects during acute episodes of allergic inflammation.

Further studies aimed at elucidating the mechanisms by which HDACi modulates mast cell function are warranted. In particular this includes examining the roles of upstream negative regulators of transcription such as I-κBα as well as genes involved in mitotic pathways such as the MAP kinase genes. The effects of TSA on mast cell responses during food allergy also need to be further examined. It will especially be important to assess the differential effects of TSA on mast cell homeostasis and function vis-à-vis its known epigenetic effects on histone acetylation as well as its effects on the recruitment and survival of mature mast cells during allergic responses. Furthermore, TSA is a pan-HDACi and potentially has a wide range of other effects. As such, examination of the effects of specific HDACs in modulating allergic inflammation will provide further insight into the epigenetic regulation of mast cell function. In this context, a recent study demonstrated therapeutic effects of both HDAC6 and HDAC8 inhibitors in a mouse model of asthma (78). Similarly, other studies have demonstrated that HDAC enzymes such as HDAC6 and HDAC8 have a number of effects on non-histone targets including α-tubulin, actin and HSP90 (79–82). Thus, inhibition of these proteins has the potential to modulate the cytoskeleton as well as cellular morphology, migration and cellular interactions, which may contribute to the observed effects.

In summary, our data demonstrate that HDAC inhibition by TSA has a profound inhibitory effect on the activation and function of mast cells both in cell culture and during the development of food allergy, suggesting that the activation of mast cells is epigenetically regulated and that exposure to epigenetic modulators, including dietary components can alter the outcome of allergic disease in sensitized patients.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Animal Use and Care Committee of Western New England University. The protocol was approved by the Institutional Animal Use and Care Committee.

AUTHOR CONTRIBUTIONS

CM and SK conceived the study design and directed the project. DK, EK, JR, SP, CT, CD, JS-D, SS, SK and CM contributed to experiments and analyzed data. DK, SK and CM prepared figures and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02414/full#supplementary-material

Supplementary Figure 1 | TSA suppresses cytokine production in peritoneal mast cells. Peritoneal lavage was isolated from naïve mice and pooled. Cells were incubated with DNP-IgE in the presence or absence of 500 nM TSA overnight. The next day, cells were activated with DNP-BSA and cultured with Brefeldin A for 6 h. **(A)** Intracellular cytokine staining was performed on mast cells and the percent of IL-13 and TNF-α producing cells were assessed. **(B)** The total numbers of cytokine producing cells relative to absolute numbers are shown. **(C)** β-hex activity in cell culture supernatants was assessed.

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A Distinct Esophageal mRNA Pattern Identifies Eosinophilic Esophagitis Patients With Food Impactions

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Eosinophilic esophagitis (EoE), a Th2-type allergic immune disorder characterized by an eosinophil-rich esophageal immune infiltrate, is often associated with food impaction (FI) in pediatric patients but the molecular mechanisms underlying the development of this complication are not well understood. We aim to identify molecular pathways involved in the development of FI. Due to large variations in disease presentation, our analysis was further geared to find markers capable of distinguishing EoE patients that are prone to develop food impactions and thus expand an established medical algorithm for EoE by developing a secondary analysis that allows for the identification of patients with food impactions as a distinct patient population. To this end, mRNA patterns from esophageal biopsies of pediatric EoE patients presenting with and without food impactions were compared and machine learning techniques were employed to establish a diagnostic probability score to identify patients with food impactions (EoE+FI). Our analysis showed that EoE patients with food impaction were indistinguishable from other EoE patients based on their tissue eosinophil count, serum IgE levels, or the mRNA transcriptome-based p(EoE). Irrespectively, an additional analysis loop of the medical algorithm was able to separate EoE+FI patients and a composite FI-score was established that identified such patients with a sensitivity of 93% and a specificity of 100%. The esophageal mRNA pattern of EoE+FI patients was typified by lower expression levels of mast cell markers and Th2 associated transcripts, such as *FCERIB*, *CPA3*, *CCL2*, *IL4*, and *IL5*. Furthermore, lower expression levels of regulators of esophageal motility (*NOS2* and *HIF1A*) were detected in EoE+FI. The EoE+FI -specific mRNA pattern indicates that impaired motility may be one underlying factor for the development of food impactions in pediatric patients. The availability of improved diagnostic tools such as a medical algorithm for EoE subpopulations will have a direct impact on clinical practice because such strategies can identify molecular inflammatory characteristics of individual EoE patients, which, in turn, will facilitate the development of individualized therapeutic approaches that target the relevant pathways affected in each patient.

Keywords: eosinophilic esophagitis, food impaction, machine learning classification, medical algorithm, esophageal motility, eosinophils

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INTRODUCTION

Eosinophilic esophagitis (EoE) is an allergic disorder that is characterized by an eosinophil-rich immune infiltrate of the esophagus. Recent epidemiological reports estimate the incidence of EoE at 1/10,000 new cases per year with the current prevalence of EoE as 25.9–56.7/100,000 in the United States and suggest a rapid increase in incidence in developed countries (1–4).

EoE remains a challenge to diagnose and manage due to the high clinical variations of the disease (5–7). The diagnosis is made clinicopathologically by demonstrating at least 15 eosinophils per high-power field in at least one esophageal biopsy, along with the presence of typical EoE symptoms (8). Evaluation of 2–4 biopsies from both proximal and distal esophagus is recommended because of the patchy nature of the immune infiltrate in EoE (9). Symptomology is age and gender dependent with younger children commonly presenting with nonspecific symptoms such as failure to thrive, feeding difficulties, and choking on solid foods. Older children on the other hand, more capable of communicating their symptoms, frequently complain of abdominal pain, dysphagia, and vomiting (10–13). In contrast to children, adolescents and adults typically present with symptoms more specific to esophageal dysfunction and ongoing fibrosis, such as dysphagia, food impaction, and esophageal strictures (10, 14). In addition to the wide array of presenting symptoms the biological mechanisms underlying disease onset and progression are varied, which has led to the definition of many EoE subpopulations (15). This increasing prevalence and the heterogeneous nature of the disease underlie the pressing need to improve disease management, treatment, and diagnosis.

Currently, the use of machine learning diagnostic algorithms using mRNA pattern stamps is at the forefront of emerging diagnostic strategies for EoE. These approaches rely on an automatized evaluation of the transcriptional profile of the inflamed esophagus to calculate probability scores (16, 17). The use of a machine learning approach allows for an unbiased assessment of patient biopsies independent of human error and is designed to be self-improving with the addition of data from new patients as they become available.

The automatization of data analysis in EoE also opens the possibility of an individualized approach to EoE diagnostics and therapy. This strategy will allow clinicians to evaluate the phenotype of EoE presentation of each patient and potentially assign patients into subpopulations of EoE. Currently, multiple subpopulations of EoE patients are defined by traits such as clinical symptoms, responses to therapies (PPI-REE), and/or underlying gene expression patterns (LTC4S-EoE, IL23-EoE, iNKT-EoE, IGHEhi-EoE) (15–20). In a recently published medical algorithm, a diagnostic score for patients with increased local IgE production has been developed as a secondary diagnosis loop to identify patients that potentially suffer from local esophageal allergies (17). Further expansion of this algorithm to define additional subpopulations of EoE has the potential to improve the understanding of the disease, and to assist in the prediction of symptom onset, response to therapy, and stratification of the underlying EoE etiology.

A significant portion of patients with EoE suffer from food impactions, making EoE the leading cause of food impaction and dysphagia in the pediatric population (21). Nevertheless, the pathologic mechanisms that lead to food impactions in EoE are not well understood (22, 23). Among patients with EoE, food impaction can result from both obstructive anatomical features of the esophagus, such as esophageal stenosis, narrow-caliber esophagus, and strictures, as well as motility dysregulations such as achalasia, and diffuse esophageal spasms (23–27). The majority of food impactions in EoE patients are thought to be the result of a natural progression from an inflammatory phenotype of EoE to a fibrotic one (28–30). However, particularly in the pediatric population, there are EoE patients who present with food impactions without showing any endoscopic features of fibrosis (27).

Hence, the goal of the current study was to examine a large dataset of mRNA pattern stamps of EoE patients to define a transcriptional signature that could identify EoE patients with food impactions and yield insights about possible pathological causes for this condition in the context of EoE. We further aimed to modify our medical algorithm that calculates a composite probability score for EoE (pEoE) to establish an additional diagnostic score for the identification of EoE patients with food impaction.

MATERIALS AND METHODS

Study Population

The patients included in this study were enrolled in an observational longitudinal cohort study, performed at Boston Children's Hospital that concentrates on the understanding of the pathophysiology and diagnosis of EoE (17–19, 31, 32). Children between 1 and 18 years of age, who were scheduled for an elective upper gastrointestinal endoscopy at the Division of Gastroenterology at Boston Children's Hospital, due to a clinical suspicion for EoE (such as presenting with dysphagia, regurgitation, feeding intolerance or failure to thrive), were invited to participate. Following written informed consent by the patients and/or their legal guardians, caregivers filled out a questionnaire regarding the subject's medical history, current and past symptomatology, allergic comorbidity, and dietary habits. In addition to clinical biopsies, two study biopsies, one from the proximal and one from the distal esophagus, were obtained during endoscopy from each patient. Additional information on each subject's medical history was obtained by retrospective chart review. All patients were treated independently of this study. Patients were approached to provide follow-up information and additional esophageal biopsies at follow-up hospital visits and endoscopies. Approval for this study was obtained by the

Abbreviations: FI, Food impaction; AUC, area under the curve; EoE, eosinophilic esophagitis; EoE+FI, eosinophilic esophagitis presenting with food impaction; GERD, gastroesophageal reflux disease; PPI-REE, proton pump responsive eosinophilic esophagitis; NO, nitric oxide; *HIF1A,* hypoxia induced factor 1a; *CCL26,* C-C motif chemokine ligand 26; *NOS2,* Nitric oxide synthase 2, inducible nitric oxide synthase *IL4,* Interleukin 4; *IL5,* Interleukin 5; *FCER1B*, Fc fragment of IgE, high affinity I, receptor for; beta polypeptide, High affinity IgE receptor beta polypeptide; *CPA3*, carboxypeptidase a3.

institutional Investigational Review Board of Boston Children's Hospital (Harvard Medical School, Boston MA, approval number: 07-11-0460).

Clinicopathological Diagnosis by Reference Standards

Patients were classified as having EoE according to existent clinicopathological diagnostic guidelines at the time of sample collection, using the gold standard criteria: esophagitis that is histologically characterized by ≥15 eosinophil per high power field in at least one biopsy obtained after ≥4 weeks of treatment with a PPI and exclusion of other causes of esophageal eosinophilia. Patients that have normal esophageal histology with no clinical evidence of an underlying esophageal disease were classified as controls. Gastroesophageal reflux disease was diagnosed when there was evidence of esophagitis (<15 eosinophils per hpf after >8 weeks PPI treatment) and symptoms associated with reflux. These patients were excluded from analysis along with those with an unknown or ambiguous diagnosis, received steroid therapy or other immunomodulatory medications at the time of inclusion and/or showed evidence of a narrow esophagus or esophageal strictures.

Food impaction in EoE patients (EoE+FI) was defined as following: (1) Experiencing an episode of food impaction that requires removal of the food bolus via endoscopy or surgery, (2) experiencing an episode in which the patient presents to an emergency department and has radiological evidence of impaction and/or relief of symptoms following administration of esophageal relaxants, (3) experiencing an episode of self-reported food impaction that was resolved by bolus regurgitation. EoE patients that do not meet any of these criteria were designated as EoE without food impaction (EoE no FI). Patients who have been diagnosed with esophageal motility disorders such as achalasia, or who have anatomical abnormalities in the esophagus such as esophageal atresias, or tracheoesophageal fistulas were excluded from the study.

Biopsy Processing and Digital mRNA Profiling

Study biopsies from the esophagus were collected in RNA*later* (Qiagen, Valencia, CA) and stored at −80°C. For mRNA profiling, the biopsies were thawed and homogenized in RTL buffer (Qiagen). Further processing of the samples was done with the nCounter® Prep Station and Digital Analyzer, following the manufacturer's instructions (nCounter® system; NanoString Technologies, Seattle, WA; www.nanostring.com) using a previously published panel as established based on the published EoE transcriptome (33).

Statistical Analysis

Transcript data was normalized by performing background subtraction and normalizing to the geometric mean of the internal positive controls and to the geometric mean of 5 housekeeping genes. The normalized data was analyzed using Kruskal-Wallis test with Dunn's multiple comparisons test. Disease probability scores were calculated using an established algorithm (17). Statistical analysis of clinical characteristics of patients was performed with Fisher's exact test. All statistical tests were performed using python 3.5.2 with the following modules: scipy 0.18.1, numpy 1.11.1, pandas 0.18.1, scikit-learn 0.18, and matplotlib 1.5.3, or IBM SPSS Statistics for Windows, Version 23.0 (Armonk, NY), or GraphPad Prism 7.00 for Windows (La Jolla, CA).

RESULTS

Basic Characteristics of EoE Patients With Food Impactions

The incidence of FI in the EoE population was 12.1% (26/215) referred to as EoE+FI for the rest of the manuscript). While the median age of onset in the pediatric EoE cohort was 10.38 (1.23–18.90), the median age of EoE+FI patients was 14.79 (10.70–16.99), implying that FI is found in a subpopulation of patients with onset and/or diagnosis later in childhood. When restricting the analysis to EoE patients that were diagnosed after the age of nine, the incidence of FI increased to 29.2% (26/89). To control for age as a confounding factor for the mRNA pattern comparison, we randomly selected 13 age-matched EoE patients who did not present with a food impaction event prior or at least 2 years after diagnosis and 18 age-matched control patients with no esophageal eosinophilia or esophageal inflammation **(Figure 1)**. This patient population was used for comparative mRNA pattern analysis and machine learning strategies presented in the rest of the manuscript.

EoE is predominantly found in males (34). In our entire cohort, 67.0% (144/215) of patients were male. In a sub-analysis of the age–matched study population an even higher male predominance of cases presenting with FI was observed with 93% (13/14) of EoE+FI being male compared to 62% (8/13) in the age-matched EoE group without FI.

The incidences of common gastrointestinal symptoms such as epigastric pain, reflux symptoms, and vomiting were comparable between EoE patients who did or did not present with FI. In contrast, dysphagia was significantly more common in the EoE+FI group. The gross endoscopic findings, such as esophageal pallor, edemas, furrowing, loss of vascularity, and the presence of exudate, were comparable between EoE patients with and without FI **(Table 1)**.

EoE Patients With Food Impactions Cannot be Differentiated From Other EoE Patients Based on Standard Diagnostic Measures

Histological and transcriptional measures of eosinophil infiltration were analyzed to determine whether common markers of disease severity could be used to distinguish EoE from EoE+FI patients. No differences in the degree of eosinophilia in either proximal or distal biopsies **(Figures 2A–C)**, or in the maximum count throughout the esophagus was noted **(Figure 2D)** between the two patient groups. Additionally, the esophageal expression of the eosinotrophic chemokine *CCL26* was not significantly different between EoE no FI and EoE+FI patients **(Figure 2E)**. A recently published diagnostic algorithm uses machine learning approaches to calculate a probability

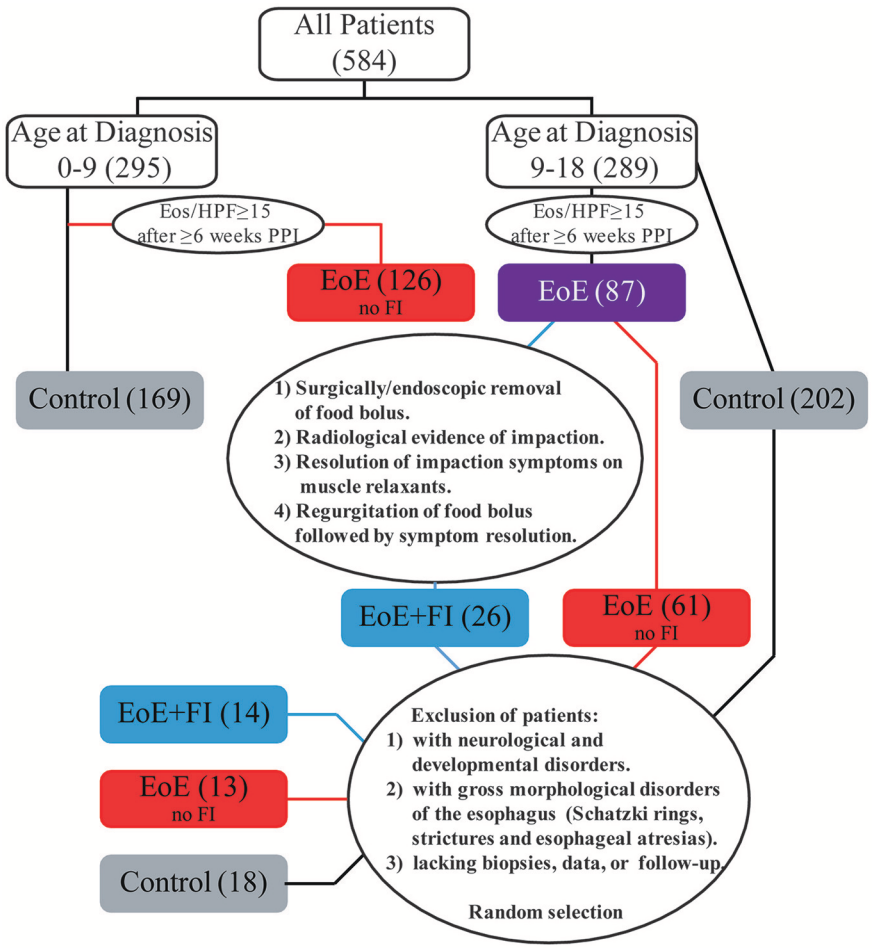

FIGURE 1 | Patient cohort overview.

score for EoE diagnosis (p(EoE)) based on the transcriptional profile of the esophageal tissue in EoE patients (17). Based on this algorithm, an EoE diagnosis could be made when p(EoE) values are >25. When calculating the three probability values for EoE, GERD and control patients, EoE no FI, and EoE+FI patients cluster together and separate from control patients **(Figure 2F)**. The p(EoE) of patients with and without FI was not significantly different **(Figure 2G)**. This data suggests that the presentation of food impactions in EoE does not result from a quantitative difference in tissue eosinophilia, which indicates that an additional analysis loop is needed to expand the medical algorithm to better define the EoE+FI subpopulation.

EoE Patients With Food Impaction Present With Comparable Measures of Systemic Allergy but Decreased Levels of Esophageal Allergy Based on the IGHE Score

Since EoE is classified as an allergic disorder, allergic comorbidities were analyzed as a potential factor in distinguishing the clinically-defined EoE+FI patient subpopulation. No significant difference in the occurrence of food allergies, asthma, eczema, or seasonal allergies was observed in EoE+FI patients compared to EoE patients without FI **(Figures 3A,B)**. The frequency of sensitized patients as determined by RAST or skin prick tests was also comparable between both patient groups **(Figure 3C)**. Furthermore, there was no significant difference in total serum IgE titers **(Figure 3D)**. These data suggest that there is no relationship between IgE mediated allergic comorbidities and food impactions in EoE patients. It is important to note here, however, that the correlation between serum IgE titers and EoE is low (35, 36). We recently established esophageal IgE production as an additional readout for tissue allergy in EoE patients (17). To test if esophageal tissue allergy can be used to identify EoE+FI patients, we analyzed the composite IGHE score. This score was defined as a secondary analysis loop of the published EoE diagnostic algorithm as a correlative measure of increased esophageal allergic Th2-type inflammation (17). Using the published cutoff score of 37.5, we found that 6/13 patients without FI presented with an elevated IGHE-score while none

TABLE 1 | Patient characteristics and cohort composition.

Parameter	EoE+FI	EoE no FI	Controls	*P*-values		
				EoE-FI vs. EoE no FI	EoE-FI vs. Controls	EoE no FI vs. Controls
n	14	13	18			
Age at diagnosis (in years; median, range)	14.26 (10.70–17.58)	13.41 (9.08–15.72)	13.39 (10.63–17.98)	0.528	>0.999	0.466
Male gender	13/14 (93%)	8/13 (62%)	6/18 (33%)	0.077	<0.001	0.157
SYMPTOMS IN THE PAST YEAR						
Dysphagia	14/14 (100%)	9/13 (69%)	5/18 (28%)	0.041	<0.001	0.033
Food impaction	14/14 (100%)	0/13 (0%)	0/18 (0%)	NA	NA	>0.999
Chest pain	3/14 (21%)	0/13 (0%)	2/18 (11%)	0.222	0.631	0.497
Epigastric pain	4/14 (29%)	6/13(46%)	9/18 (50%)	0.440	0.289	>0.999
Reflux symptoms	4/14 (29%)	6/13(46%)	11/18 (61%)	0.440	0.087	0.481
Feeding difficulties	0/14 (0%)	0/13 (0%)	0/18 (0%)	1.000	>0.999	>0.999
Vomiting	2/14 (14%)	4/13 (31%)	2/18 (11%)	0.385	>0.999	0.208
ENDOSCOPY						
Pallor	3/14 (21%)	1/13 (8%)	1/18 (6%)	0.596	0.295	>0.999
Edema	1/14 (7%)	0/13 (0%)	0/18 (0%)	>0.999	0.438	>0.999
Loss of vascularity	7/14 (50%)	2/13 (15%)	0/18 (0%)	0.103	0.001	0.168
Furrowing	11/14 (79%)	9/13 (69%)	3/18 (17%)	0.678	<0.001	0.008
Exudate	6/14 (43%)	5/13 (38%)	0/18 (0%)	>0.999	0.003	0.008
ALLERGIC/ATOPIC CONDITIONS						
Serum IgE levels (median, range)	214 (63–503)	100.5 (4–1920)	98 (35–189)	0.733	0.519	>0.999
Eczema	5/14 (36%)	3/13 (23%)	1/18 (6%)	0.678	0.064	0.284
Asthma	8/14 (57%)	5/13 (38%)	1/18 (6%)	0.449	0.004	0.059
Allergic rhinoconjunctivitis	7/14 (50%)	9/13 (69%)	4/18 (22%)	0.440	0.142	0.013
Food allergy	4/14 (29%)	3/13 (23%)	0/18 (0%)	>0.999	0.028	0.064
Positive RAST or skin prick test against food antigens	11/14 (78%)	8/13 (61%)	0/5 (0%)	0.420	0.005	0.036
TISSUE EOSINOPHILIA (PEAK VALUE)						
Proximal (median, range)	25 (0–110)	25 (0–100)	0	>0.999	<0.001	<0.001
Distal (median, range)	50 (3–80)	70 (25–150)	0	>0.999	<0.001	<0.001
Maximum eosinophil count (median, range)	89 (0–115)	70 (25–150)	0	>0.999	<0.001	<0.001

p-values calculated by Fisher's exact test.

of the patients with FI did **(Figure 3E).** Based on this analysis, the systemic allergic phenotype is unlikely an eliciting and/or contributing factor for the development of food impaction in EoE patients, however the extent of the local esophageal allergic phenotype may negatively correlate with FI symptoms.

Esophageal Inflammation in EoE Patients With Food Impaction Differs From Those Without Food Impaction

EoE is a complex multifactorial disease, so in order to understand the immunological pathways contributing to the differences in disease presentation the transcriptional profile of 74 genes was analyzed **(Figure 4A)**. EoE+FI patients present with significantly lower transcript counts of *CPA3*, a mast cell specific transcript that encodes for carboxypeptidase a3, as well as lower *FCER1B*, the mast cell and basophil specific beta chain of the high-affinity IgE receptor **(Figures 4B,C)**. In agreement with the IGHE score, this data set suggests that EoE+FI patients present with a lesser contribution of the esophageal mast cell compartment to the local inflammation. *CCL2* is the primary chemokine responsible for the recruitment of mast cell precursors. In line with the probable lower frequency of mast cells, patients presenting with food impactions express lower transcript levels of *CCL2* in their esophageal biopsies indicating that the recruitment of this cell type might be altered **(Figure 4D)**. Finally, it is important to note that the Th2-type cytokines *IL4* and *IL5* are significantly less expressed in patients with food impaction, suggesting a less pronounced Th2-type inflammatory tissue environment in patients with FI **(Figures 4A,E)**. Combined these data demonstrate that patients with food impaction present with similar levels of tissue eosinophilia but the mast cell infiltration and Th2 inflammation are reduced.

Establishing an Analysis Loop That Allows for the Differentiation of EoE Patients With Food Impactions

Given the unique characteristics in immunological mRNA transcript pattern of EoE patients with FI, we hypothesized that

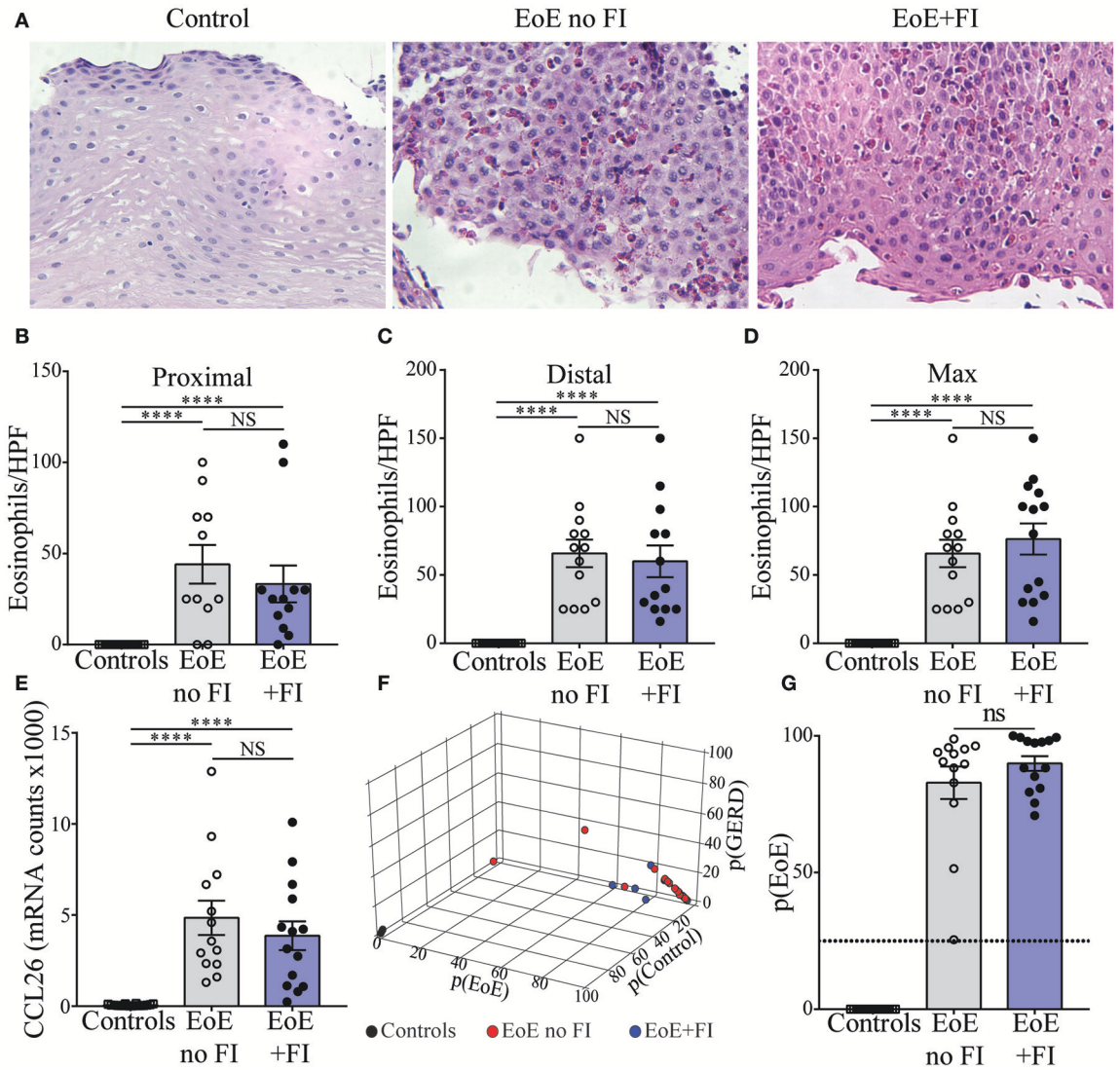

FIGURE 2 | Measures of EoE severity.**(A)** Representative hematoxylin and eosin staining of distal esophageal biopsies of Control, EoE no FI, and EoE+FI patients. Comparison of eosinophil counts in **(B)** proximal and **(C)** distal esophageal biopsies. **(D)** Maximum eosinophil infiltration. **(E)** CCL2 mRNA transcript levels in the esophagus. **(F,G)** disease probability scores (p(EoE), p(Control), p(GERD)) in Control, EoE no FI, and EoE+FI patients. ****$p < 0.0001$ as calculated by Dunn's multiple comparison test after Kruskal-Wallis test.

an algorithm-based score could be developed to differentiate EoE patients with and without FI. By weighing transcripts based on the fold difference in their expression and their adjusted significance, transcript weights were calculated (**Figures 5A,B**). These weights were used to calculate a raw composite FI-score for each EoE patient using weighted factor analysis. This raw score was then standardized and a cutoff determined by ROC analysis. With the calculated cutoff of 0.03 the FI-score distinguished patients with and without FI with a sensitivity of 0.93 and a specificity of 1.00 (**Figures 5C,D**). The AUC for the ROC was 0.99 with a standard error of 0.02 and a positive predictive value of 100.00% and a negative predictive value of 92.86%. This secondary analysis can be used to expand the previously published diagnostic algorithm and may serve to predict the risk of food impactions prospectively.

The Inflammatory mRNA Pattern of EoE+FI Patients Indicates Underlying Esophageal Dysmotility

When analyzing the mRNA pattern for transcripts with potential influence on motility, we found that the inducible nitric oxide (NO) synthase *NOS2* was significantly less expressed in EoE patients with FI (**Figure 6A**). Therefore these patients may not produce sufficient esophageal NO, a key signal for relaxation

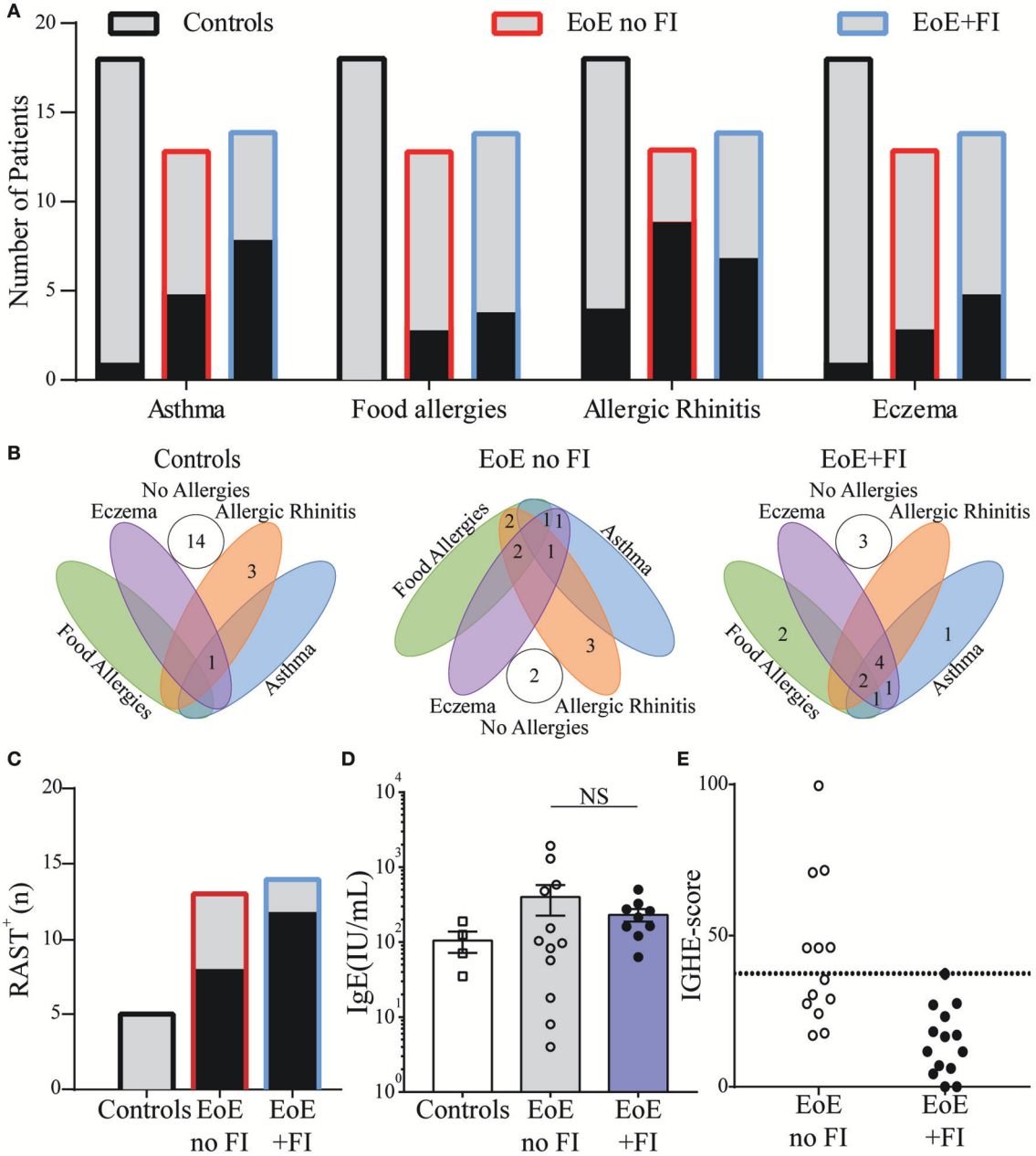

FIGURE 3 | Comparison of clinical allergies and measurements of allergic sensitization in EoE no FI and EoE+FI patients. **(A)** Frequency of individual allergic comorbidities, and **(B)** distribution throughout the patient population. **(C)** Patients with a positive RAST to a food allergen. **(D)** Serum concentrations of IgE. **(E)** Esophageal allergy scores (IGHE score).

of smooth muscles (37, 38). Additionally, the expression levels of the transcription factor hypoxia induced factor 1a (*HIF1A*, **Figure 6B**) were significantly lower in EoE+FI patients. Loss of *HIF1A* expression has previously been described to result in an increase in smooth muscle contractility (39, 40). Combined, these results show altered expression levels of key regulators of motility, which implies that esophageal dysmotility may underlie the development of FI in EoE.

DISCUSSION

Our work confirmed that EoE patients who suffer from food impactions cannot be identified as a distinct subgroup of EoE using gold standard diagnostic markers, such as eosinophil infiltration, histological features, or comorbidities. Yet, we were able to identify a unique esophageal mRNA profile in EoE patients with food impactions who did not show any evidence

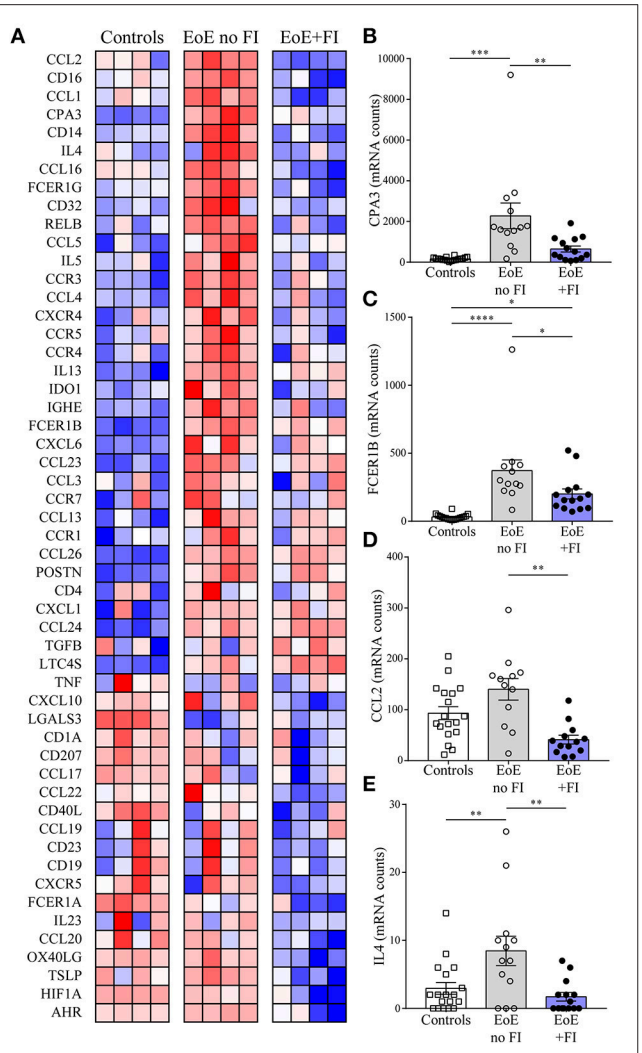

FIGURE 4 | EoE+FI specific mRNA pattern. **(A)** Heat map comparison of esophageal mRNA patterns in control, EoE no FI, and EoE+FI patients. Relative expression of **(B)** CPA3, **(C)** FCER1B, **(D)** CCL2, **(E)** IL4 in control, EoE no FI, and EoE+FI patients. $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$,$^{****}p < 0.0001$ as calculated by Dunn's multiple comparison test after Kruskal-Wallis test.

of anatomical narrowing of the esophagus by endoscopy. A modification of a published medical algorithm used the FI-specific mRNA pattern stamp to create a diagnostic score for this patient subpopulation (EoE+FI), which is characterized by lower expression levels of mast cell specific transcripts, Th2 cytokines, and decreased expression of key regulators of smooth muscle contractility and relaxation.

Food impaction is a common complication in EoE. In adult onset EoE, FI occurs with high frequency (55%) and is one of the main indicators of the disease (41). However, in our pediatric cohort, it occurred in only 12% of our cohort, which probably reflects the fact that in children FI start to occur in older children. As in adults we also observed a male predominance (35). Currently, no diagnostic tool for the identification of patients prone to develop this comorbidity exists. Generally, EoE is a chronic inflammatory disorder that leads to fibrosis and strictures in the esophagus and it has been thought that food impactions occur due to the fibrostenotic complications. Indeed strictures are found and identified as the cause of food impaction in some of the patients. However, in many patients, food impactions occur in the absence of such anatomical problems or any other identifiable cause. These observations imply that impaired esophageal motility contributes to food impactions among pediatric EoE patients (27). The hypothesis that food impactions are not necessarily connected to fibrosis is strongly supported by the findings from our study, which indicate that markers of mast cell expansion and Th2 cytokines, both of which are key regulators of fibrosis, are downregulated in EoE patients with food impactions. The observed inflammatory mRNA pattern implies that FI in pediatric EoE patients may develop independent of fibrosis and rather, manifests as a result of esophageal dysmotility.

Transcripts for inducible nitric oxide synthase (*NOS2*) were significantly downregulated in EoE+FI patients. Lower expression of *NOS2* suggests diminished production and lower bioavailability of NO. This may lead to an increase in smooth muscle cell contractility because lack of NO prevents smooth muscle relaxation, which in turn results in poor peristalsis. Additionally, *HIF1A* was downregulated in EoE+FI patients in our cohort. HIF1A expression levels are acknowledged for contributing to the regulation of vascular smooth muscle tone and low levels of *HIF1A* expression have been shown to be associated with hypertension due to hypercontractile vascular smooth musculature (39, 40). It is currently unknown whether the role of *HIF1A* in smooth muscle contractility applies to esophageal smooth muscle cells. However, it is tempting to speculate that the reduction in esophageal *HIF1A* expression levels contributes to motility dysfunction by exacerbating the difficulty of esophageal smooth muscles to relax during swallowing. The combination of low *NOS2* expression to regulate relaxation and low *HIF1A* expression to regulate smooth muscle tone and contractility can potentially result in smooth muscle cramping and spasm, and subsequently to food getting stuck in the esophagus in a fibrosis-independent manner.

Using both the immunological and non-immunological transcriptional differences in the expression patterns of EoE patients with food impactions, we modified our existing algorithm for diagnosing EoE patients to include a secondary analysis for assessing the risk for food impaction. The predictive power of the current study is limited by the small number of pediatric EoE patients with food impactions. It will, therefore, be important to confirm the accuracy of our algorithm by recruiting additional patients who can be integrated into the current analysis in a forward-feeding way to expand the training set and to generate an additional test set. Ideally, such an analysis would be performed as a multicenter study. Another limitation of the current study is its retrospective nature which allows for the identification of a gene signature that identifies patients with food impactions as a subpopulation but cannot predict the development of this complication. For the latter purpose, a longitudinal cohort study needs to be designed to

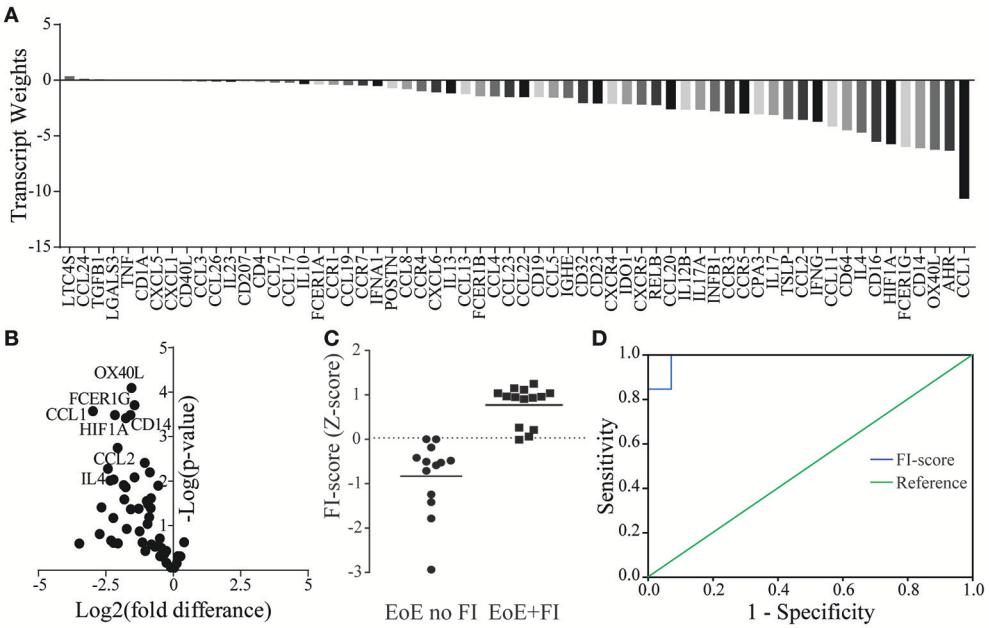

FIGURE 5 | FI-score in EoE no FI and EoE+FI patients**. (A)** Transcript weights of the factors differentiating EoE and EoE+FI. **(B)** Volcano plots of normalized mRNA transcripts displayed as fold difference (x-axis) and significance (y-axis) used for the calculation of the factor weights. **(C)** Calculated standardized FI-score. **(D)** ROC analysis for differentiating EoE no FI and EoE+FI patients based on FI-score (AUC = 0.99, Sensitivity = 0.93 Specificity = 1).

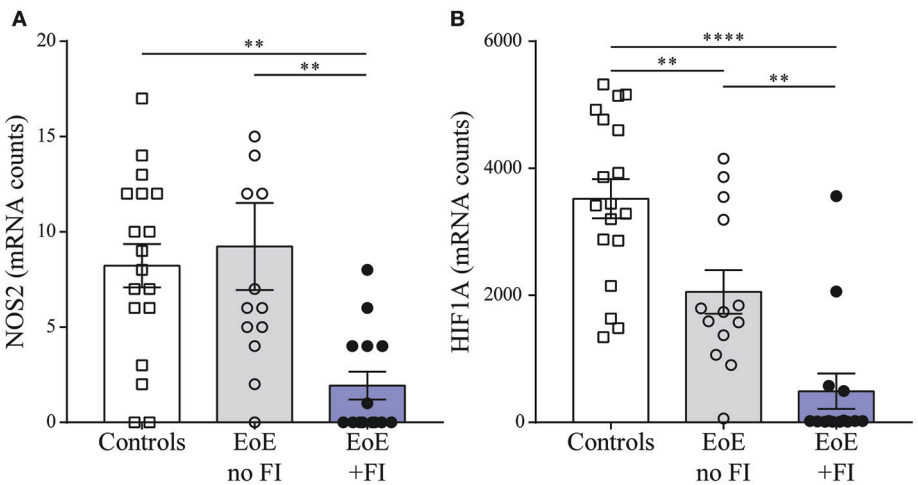

FIGURE 6 | Motility related transcript levels**.** Comparison of **(A)** NOS2 and **(B)** HIF1A expression between patient groups. $^{**}p < 0.01$, $^{***}p < 0.001$ as calculated by Dunn's multiple comparison test after Kruskal-Wallis test.

predictively analyze the esophageal mRNA signature changes in EoE patients as they age and as they develop food impaction, or phenotypes specific to other subpopulations of EoE. Such analysis will determine if the differences in gene expression, which we have identified as specific for EoE+FI can be observed before the first food impaction event. Furthermore, at the inception of our cohort, the definition of EoE still included a lack of response to 8 weeks of PPI, so all patients included in the present cohort are EoE patients that did not respond to PPI. We do not have any patient with PPI responsive eosinophilia, in the study cohort so it is not clear if the mRNA pattern of those patients will be the same.

Currently, the unpredictability of EoE therapy response and disease progression necessitates physicians to find the best treatment strategy for each patient using a trial and error approach monitored by gold standard diagnosis which significantly affects the quality of life of the EoE patients (42). For this purpose, EoE algorithms and their future expansions with

secondary diagnostic loops appear as attractive strategies because they will permit the prediction of the development of symptoms and treatment responsiveness and can, thus, help inform the diagnosis and treatment of EoE reducing the time between initial presentation and effective treatment. Improved diagnostic strategies will assist physicians in educating EoE patients and their families if their score implies the risk of developing food impactions, thus helping the child and family anticipate how the disease will impact them particularly. Additionally, the FI-score will assist physicians in assessing the risk for food impactions in each patient objectively, making it a highly useful diagnostic tool when dealing with patients that are unable to accurately express their symptoms.

AUTHOR CONTRIBUTIONS

BS, UA, CK, RR, SB, and EF designed experiments, performed research, and analyzed data. KH, RR, JV, JG, and SN were involved in patient recruitment, evaluation of clinical patient status, evaluation of biopsies, and analysis of human serum data. BS, SN, and EF wrote the first draft of the manuscript. All co-authors contributed to writing the final manuscript and approved its last version.

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Baseline Gastrointestinal Eosinophilia Is Common in Oral Immunotherapy Subjects With IgE-Mediated Peanut Allergy

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Rationale: Oral immunotherapy (OIT) is an emerging treatment for food allergy. While desensitization is achieved in most subjects, many experience gastrointestinal symptoms and few develop eosinophilic gastrointestinal disease. It is unclear whether these subjects have subclinical gastrointestinal eosinophilia (GE) at baseline. We aimed to evaluate the presence of GE in subjects with food allergy before peanut OIT.

Methods: We performed baseline esophagogastroduodenoscopies on 21 adults before undergoing peanut OIT. Subjects completed a detailed gastrointestinal symptom questionnaire. Endoscopic findings were assessed using the Eosinophilic Esophagitis (EoE) Endoscopic Reference Score (EREFS) and biopsies were obtained from the esophagus, gastric antrum, and duodenum. Esophageal biopsies were evaluated using the EoE Histologic Scoring System. Immunohistochemical staining for eosinophil peroxidase (EPX) was also performed. Hematoxylin and eosin and EPX stains of each biopsy were assessed for eosinophil density and EPX/mm^2 was quantified using automated image analysis.

Results: All subjects were asymptomatic. Pre-existing esophageal eosinophilia (>5 eosinophils per high-power field [eos/hpf]) was present in five participants (24%), three (14%) of whom had >15 eos/hpf associated with mild endoscopic findings (edema, linear furrowing, or rings; median EREFS = 0, IQR 0–0.25). Some subjects also demonstrated basal cell hyperplasia, dilated intercellular spaces, and lamina propria fibrosis. Increased eosinophils were noted in the gastric antrum (>12 eos/hpf) or duodenum (>26 eos/hpf) in 9 subjects (43%). EPX/mm^2 correlated strongly with eosinophil counts ($r = 0.71$, $p < 0.0001$).

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Conclusions: Pre-existing GE is common in adults with IgE-mediated peanut allergy. Eosinophilic inflammation (EI) in these subjects may be accompanied by mild endoscopic and histologic findings. Longitudinal data collection during OIT is ongoing.

Keywords: peanut food allergy, Eosinophilic Esophagitis, eosinophil, adverse event, biopsy, endoscopy, gastrointestinal, oral immunotherapy

INTRODUCTION

Peanut allergy is a potentially fatal disease affecting 0.5–1% of the general population (1–3) with rates as high as 3% in young children (4). Possibly fueled by an era of widespread early peanut avoidance (5, 6), the disease has doubled over the past decade (7) and tends to persist through adulthood for a majority of individuals (8). The current standard of care for patients entails food avoidance and acute management of allergic reactions (9). Emerging evidence suggests that desensitization can be achieved by a method of graduated peanut administration termed oral immunotherapy (OIT) (10–12). Following an initial escalation phase, OIT subjects are desensitized to the offending food antigen during a series of gradual dose adjustments until a pre-specified maintenance dose is reached. Rates of clinical desensitization are excellent ranging from 80 to 90% for food allergy (10, 13, 14); however, the durability of this immunologic response remains in question. In most patients, continuation of "regular" therapy appears to be necessary in order to maintain desensitization (15, 16). However, a few trials have now shown that desensitization can be maintained for some time after a period of discontinuation of therapy (allergen avoidance) (12, 17–19). This outcome is termed "sustained unresponsiveness (SU)," and refers to patients who successfully pass an oral food challenge after a defined period of allergen avoidance following OIT (20).

Questions regarding safety are the principal obstacles to broader acceptance and availability of OIT. Specifically, 5–36% of subjects withdraw from clinical trials because they cannot tolerate the treatment (11, 21–23). Moreover, adverse events (AEs) are not limited to a small subset of individuals. A recent retrospective study of AEs during peanut OIT suggested 80% of subjects experience OIT-related AEs (22). Although serious AEs, such as anaphylaxis are rare, the frequency of OIT-related side effects has led some investigators and allergists to question whether this intervention is superior to avoidance (24). The most common AEs related to OIT discontinuation that have been reported are gastrointestinal symptoms, specifically abdominal pain (25). While OIT-induced gastrointestinal symptoms are more common during the buildup phase, they may occur at any point during therapy (26, 27).

Most concerning is the occurrence of eosinophilic gastrointestinal disease (EGID) in some subjects undergoing OIT (28). EGIDs are a group of diseases characterized by eosinophil-rich inflammation affecting different locations of the digestive tract: the esophagus, stomach, and intestine. Eosinophils can normally reside in the mucosa of the stomach and intestine, but are not normally found in the esophagus (29). Eosinophilic esophagitis (EoE) is the most common EGID and is typically a chronic immune disorder mediated by antigen exposure. EoE is defined by clinical and histopathological criteria, in the absence of other causes. Clinical symptoms may include the following: reflux-like symptoms, abdominal pain and/or vomiting that is refractory to reflux treatment, dysphagia and/or food impaction in conjunction with histological evidence of dense eosinophilic infiltration of the mucosa ($\geq$15 eosinophils per high-power field [eos/hpf]) (30).

A meta-analysis (31) and a recent retrospective review (25) estimated the incidence of EoE during OIT at rates of 2.7 and 5.1%, respectively. Both are likely underestimates as most subjects with gastrointestinal symptoms do not routinely undergo upper endoscopy for diagnostic confirmation. Indeed, some centers estimate rates of OIT-induced EoE as high as 8–14% when rates of discontinuation due to gastrointestinal symptoms (i.e., abdominal pain or vomiting) are used as a surrogate measure (32). Notwithstanding, diagnosis of EoE based on symptoms alone is inherently limited. This phenomenon has sparked debate within the food allergy community given the potential risk of generating a chronic disease *de novo*. A confirmed diagnosis of EoE requires long-term treatment with a proton pump inhibitor (PPI), swallowed corticosteroids, or food avoidance despite time already spent in the desensitization process. Fortunately, resolution of EoE is observed in most patients with OIT-induced EoE on discontinuation of OIT (28).

In a prospective study evaluating OIT and the incidence of EGID, 8 of 128 participants undergoing OIT for milk and/or egg were diagnosed with EGID after 15–48 months of starting OIT (six with EoE and two with eosinophilic gastroenteritis). Interestingly, OIT was maintained in five of the six EoE patients while PPI therapy with or without swallowed steroids was initiated. One patient refused treatment with medications and thus, OIT was discontinued. In all six patients, symptoms resolved and in the three of five patients with repeat esophagogastroduodenoscopies (EGDs), there was histological remission (27).

Some have suggested that subjects with OIT-induced EoE may have pre-existing, subclinical disease. This notion is supported by estimates suggesting that the prevalence of EoE in patients with IgE-mediated food allergy (1:20) is 125 times more common than in the general population (1:2,500) (32). Currently, there is a critical need to determine whether food-allergic patients who are avoiding culprit allergens have underlying eosinophilic inflammation (EI) in the gastrointestinal tract and whether the presence of EI will affect their outcomes with OIT. Unfortunately, no biomarkers or minimally invasive measurement techniques have been validated to diagnose EoE or EGIDs without endoscopy (33). The objective of this study was to evaluate the

presence of gastrointestinal eosinophilia (GE) in a small cohort of adult subjects with IgE-mediated peanut allergy prior to initiation of OIT.

METHODS

Study Population

Participants were recruited as part of a randomized, double-blind, placebo-controlled, phase II clinical trial studying peanut OIT at the Sean N. Parker Center for Allergy and Asthma Research at Stanford University from April 2014 to March 2016 (clinicaltrials.gov; NCT02103270) (34). All aspects of the studies from which data was obtained were authorized by the Stanford University School of Medicine Institutional Review Board (Stanford, CA). Peanut allergy was confirmed with clinical history, skin prick tests, and a positive challenge to peanut during a double-blind, placebo-controlled food challenge (DBPCFC). A clinical history of EGID was a key exclusion criterion. A subset of participants, aged >18 years old, was consented to participate in an IRB approved sub-study, with a separate data safety monitoring board, involving EGDs prior to starting OIT. A comprehensive gastrointestinal symptom questionnaire was given to participants to assess clinical symptoms (see **Supplementary Material**). Symptoms were assessed within 1 month of upper endoscopy. The gastrointestinal symptom questionnaire included a 1 month recall period and assessed symptoms of reflux, abdominal pain, constipation, poor appetite, dysphagia, time required for eating, food refusal, and vomiting. None of the subjects enrolled in this study had previously undergone an upper endoscopy.

Esophagogastroduodenoscopies

All participants were consented for the research study and for the procedure. EGDs were performed under conscious sedation by trained gastroenterologists (N.F.B). Endoscopic biopsies were obtained from five sites: the proximal esophagus (PE), middle esophagus (ME), and distal esophagus (DE), as well as the gastric antrum and proximal duodenum. Four passes were performed at each location and one biopsy was obtained with each pass. Given the need to conserve samples for future mechanistic studies, only one biopsy from each site was analyzed by histology. Standardized reporting of endoscopic findings was undertaken, using the Eosinophilic Endoscopic Reference Score (EREFS) (35). The inflammatory and fibrostenotic scores were calculated as per Dellon et al. (36).

Evaluation of Gastrointestinal Pathology

Sections from the PE, ME, DE, gastric antrum, and proximal duodenum were stained with hematoxylin and eosin (H + E). A gastrointestinal pathologist (N.K.), who was blinded to the clinical characteristics and demographic data of the individual participants, quantified peak eosinophils counts in a single hpf in an area of highest density and scored esophageal biopsy slides using the Eosinophilic Esophagitis Histologic Scoring System (EoEHSS), as previously described (37). Briefly, the EoEHSS not only quantifies EI but also informs as to the severity and extent of inflammation and histological abnormality, and consists of 8 features: eosinophilic inflammation (EI), basal zone hyperplasia (BZH), dilated intercellular spaces (DIS), lamina propria fibrosis (LPF), eosinophilic abscess (EA), eosinophil surface layering (SL), surface epithelial alteration (SEA), and dyskeratotic epithelial cells (DEC) (37). Each feature is scored separately for grade (severity) or stage (extent) of abnormality using a 4-point scale (0 = normal; 3 = most severe or extensive). These features were analyzed for each participant for the PE, ME, and DE biopsies. Greater than 5 eos/hpf in the esophagus was considered abnormal and any eosinophils above the published upper limits of normal in the stomach (>12 eos/hpf) or duodenum (>26 eos/hpf) were considered abnormal (29).

Immunohistochemical (IHC) Staining for Eosinophil Peroxidase (EPX)

Tissue sectioning and IHC staining was performed at the Pathology Research Core (Mayo Clinic, Rochester, MN) using the Leica Bond RX stainer (Leica). Formalin-fixed-paraffin-embedded (FFPE) tissues were sectioned at five microns and IHC staining was performed on-line. Slides for EPX stain were retrieved for 20 min using Epitope Retrieval 1 (Citrate; Leica) and incubated in Protein Block (Dako) for 5 min. The EPX primary monoclonal antibody [clone MM25-82.2 (38)] was diluted to 1:750 in Background Reducing Diluent (Dako) and incubated for 15 min.

The detection system used was the Polymer Refine Detection System (Leica). This system includes a hydrogen peroxidase block, post-primary and polymer reagent, DAB, and hematoxylin. Immunostaining visualization was achieved by incubating slides 10 min in DAB and DAB buffer (1:19 mixture) from the Bond Polymer Refine Detection System. To this point, slides were rinsed between steps with 1X Bond Wash Buffer (Leica). Slides were counterstained for 5 min using Schmidt hematoxylin (instead of the hematoxylin provided with the Refine kit) and molecular biology grade water (1:1 mixture), followed by several rinses in 1X Bond wash buffer and distilled water. Once the immunochemistry process was completed, slides were removed from the stainer and rinsed in tap water for 5 min. Slides were dehydrated in increasing concentrations of ethyl alcohol and cleared in 3 changes of xylene prior to permanent cover-slipping in xylene-based medium.

Analysis of EPX Stains

Tissue sections were digitized (Aperio AT Turbo, Leica Biosystems, Buffalo Grove, IL) and peak eosinophil counts (PEC) were evaluated using an area equivalent to 1 hpf (0.24 mm^2). EPX deposition was quantified by an automated pixel algorithm with Aperio ImageScope software (version 11.2.0.780, Aperio Technologies, Vista, CA). Only pixels that stained strongly or moderately positive according to the algorithm were considered positive (see **Supplementary Figure E1**). The peak number of EPX positive pixels within 1 hpf was divided by the epithelial area (mm^2) analyzed. Automated measurements of EPX/mm^2 were made in tissue sections cut from each gastrointestinal biopsy. Manual counts of EPX positive nuclei were also performed in a single hpf in an area of highest density.

Statistical Analysis

Descriptive statistics were reported for baseline characteristics, EREF scores, eosinophil counts, EPX measurements, and EoEHSS scores. The inflammatory score was calculated by summing the exudate, edema, and furrows scores, and the fibrostenotic score was the sum of the rings and stricture scores. The total score was the sum of the inflammatory and fibrostenotic scores. Spearman's test was used to determine correlations between eos/hpf and EPX/mm^2. Comparisons of eos/hpf by H + E and eos/hpf by EPX immunohistochemistry were performed using a Mann-Whitney test. All analyses were conducted using GraphPad Prism version 7.0f for Windows and R v3.4.3.

RESULTS

Clinical Characteristics

Twenty one adults, median age 27 years old, were enrolled in the study and underwent baseline EGDs. The majority of participants were Caucasian males. All participants had histories of peanut allergy with elevated peanut-specific IgE (median 16.81 kU/L) and a median peanut skin prick test of 12.5 mm as well as a confirmatory positive reaction on a DBPCFC. Overall, the cohort included allergic individuals with elevated median total IgE of 617.32 kU/L, median absolute eosinophil counts of 200 cells/uL, and other allergic comorbid conditions, such as asthma (81%), allergic rhinitis (86%), and a history of atopic dermatitis (52%). Forty-eight percent of adults had other food allergies in addition to being peanut allergic (**Table 1**; **Supplementary Table E2**). Details of other food allergies and concurrent medication use in regards to proton pump inhibitors (PPIs) and inhaled corticosteroids are in **Supplementary Table E2**. None of the participants were on concurrent PPI at the time of endoscopy.

Gastrointestinal Symptoms

All patients were generally asymptomatic at the time of endoscopy. Questionnaires (**Supplementary Material**) were administered within 1 month of EGD. None of the participants reported a history of dysphagia. Five participants reported mild abdominal pain < 3 times per month and one subject reported mild reflux (not associated with food), <3 times per month. Two of the five participants with reported abdominal pain and one participant with mild reflux had mild abnormalities on endoscopy. None of the participants with mild abdominal pain or reflux had any appreciable eosinophils in the PE, ME or DE by H + E staining, while others who were found to have any eosinophils in the esophagus ($n = 6$) did not report symptoms. Two participants with abdominal pain <3 times per month had PEC of >26 in the duodenum by H + E staining; the remainder had eosinophils in the gastric antrum or duodenum, but less than noted thresholds (**Supplementary Table E1**).

Endoscopic Findings

During EGD, EREFS was documented in all participants. The overall median EREFS was 0 (min = 0, max = 5). No participants

TABLE 1 | Baseline characteristics.

Characteristic	Subjects ($n = 21$)
Age at baseline (y), median (IQR)	26.5 (22.5–34.5)
Males (*n*, %)	16 (76)
White (*n*, %)	15 (71)
Atopic conditions (*n*, %)	
Asthma	17 (81)
Allergic rhinitis	18 (86)
Atopic dermatitis	11 (52)
Other food allergies	10 (48)
Total IgE level (IU/L), median (IQR)*	617.32 (215.94, 1,125.45)
Peanut-specific IgE level (kU$_A$/L), median (IQR)	16.81 (7.22, 166.90)
Peanut-specific IgG4 level (μg/mL), median (IQR)	0.31 (0.11, 0.43)
Peanut skin prick test (mm), median wheal size (IQR)	12.5 (7.5, 17.5)
Absolute eosinophil counts (cells/μL), median (IQR)	200 (112.5, 322.5)*

**Missing for 1 subject.*

TABLE 2 | Endoscopy findings, EREFS.

Score	Total
Exudates*, *n* (%)	
0	21 (100%)
1	0
2	0
Score, median (min, max)	0 (0, 0)
Rings*, *n* (%)	
0	20 (95%)
1	1 (5%)
2	0
3	0
Score, median (min, max)	0 (0, 1)
Edema*, *n* (%)	
0	18 (86%)
1	3 (14%)
Score, median (min, max)	0 (0, 1)
Furrows*, *n* (%)	
0	18 (86%)
1	3 (14%)
2	0
Score, median (min, max)	0 (0, 1)
Stricture*, *n* (%)	
0	21 (100%)
1	0
Score, median (min, max)	0 (0, 0)
Inflammatory score, median (min, max)[a]	0 (0, 4)
Fibrostenotic score, median (min, max)[b]	0 (0, 3)
Total score, median (IQR)[c]	0 (0, 5)

EREFS: eosinophilic esophagitis endoscopic reference score.
**Maximum score per participant over the three sites.*
[a] The inflammatory score is the sum of the exudate, edema, and furrows scores.
[b] The fibrostenotic score is the sum of the rings and stricture scores.
[c] The total score is calculated from the sum of the exudate, edema, furrows, rings, and stricture scores.

had evidence of exudates or strictures. One participant had mild rings (score = 1), three participants had evidence of mild edema (score = 1), and three participants had evidence of mild furrows (score = 1) (**Table 2**; **Figures 1A,B**). **Figure 1C** displays EREFS by PE, ME, and DE. The inflammatory and fibrostenotic scores are reported in **Table 2** as the max score across the 3 sites reported in the esophagus. The inflammatory score is the sum of the exudate, edema, and furrows scores (median 0, min = 0, max = 4). The fibrostenotic score is the sum of the rings and stricture scores (median 0, min = 0, max = 3). The total score is calculated from the sum of all 5 findings and in our cohort the median total score was 0 (min = 0, max = 5).

Histopathology

Esophageal eosinophilia (>5 eos/hpf) was present in 24% of participants (**Figures 2**, **3**). Eosinophils were detected by H + E staining in one participant in the PE, two participants in the ME, and five participants in the DE, with three participants reaching the histologic threshold for EoE (≥15 eos/hpf). Further assessment of intact eosinophils and their degranulation products by EPX staining suggest the extent of esophageal eosinophilia may be even greater (**Figure 3**; **Supplementary Figure E2**). EPX deposition was greatest in the DE when compared to other esophageal segments and correlated strongly with eos/hpf ($r = 0.71$, $p < 0.0001$). Increased eosinophils were noted in 5 (23.8%) participants in the gastric antrum (>12 eos/hpf) and 6 (28.6%) participants in the duodenum (>26 eos/hpf). **Supplementary Table E1** details eosinophil counts for each subject in the various locations of the gastrointestinal tract. While some participants had GE in the stomach and duodenum (29, 39), none met the histologic criteria for eosinophilic gastritis or eosinophilic duodenitis based on H + E stains (40, 41). In addition, none of the biopsies showed intraepithelial eosinophils in the surface or crypt epithelium. Non-eosinophilic histological abnormalities, such as chronic inflammation and intraepithelial lymphocytosis, were found in esophageal biopsies of 3 participants (**Figure 4**). Histopathologic findings in the PE, ME, and DE using the EoEHSS are summarized in **Figure 5**; **Table 3**. Overall, the grade and stage were normal or mildly abnormal in the majority of participants. The lamina propria fibrosis was oftentimes inadequately assessed due to sampling techniques. The median final grade and stage scores were 0.05 across all sites (**Figure 6**).

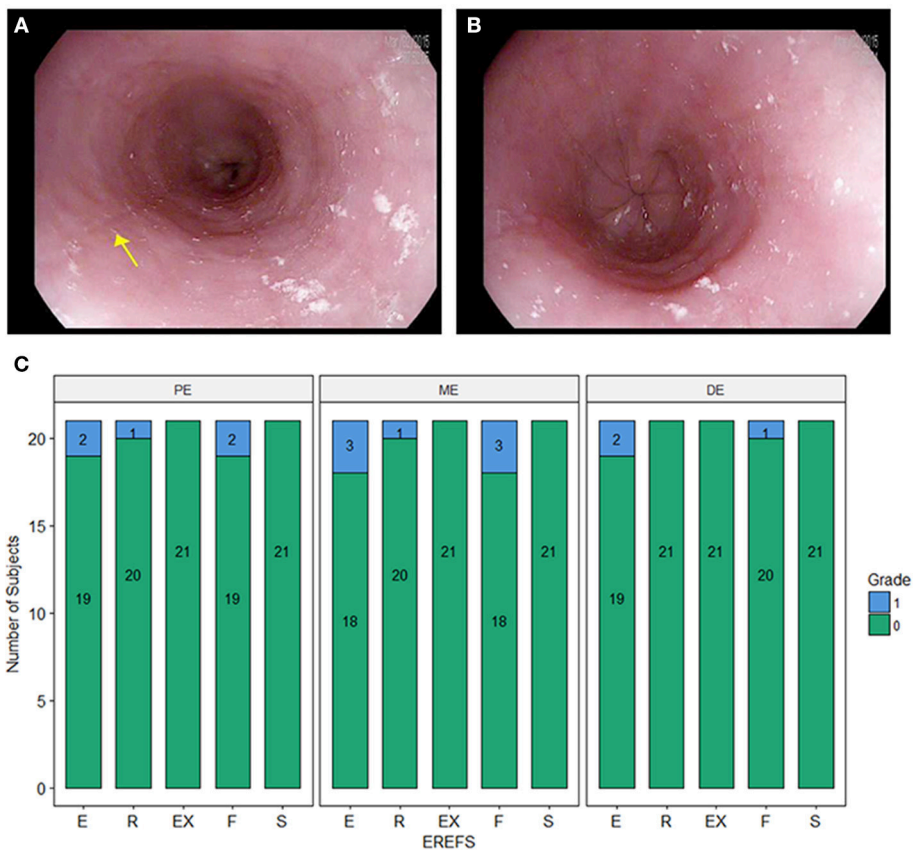

FIGURE 1 | (A) Endoscopic picture of the esophagus showing mild longitudinal furrows (e.g., yellow arrow) and mild edema. **(B)** Endoscopic picture of the distal esophagus with mild edema. **(C)** Esophagitis Endoscopic Reference Score (EREFS) distributed over the proximal esophagus (PE), middle esophagus (ME), and distal esophagus (DE). E, edema; R, rings; Ex, exudate; F, furrows; S, strictures.

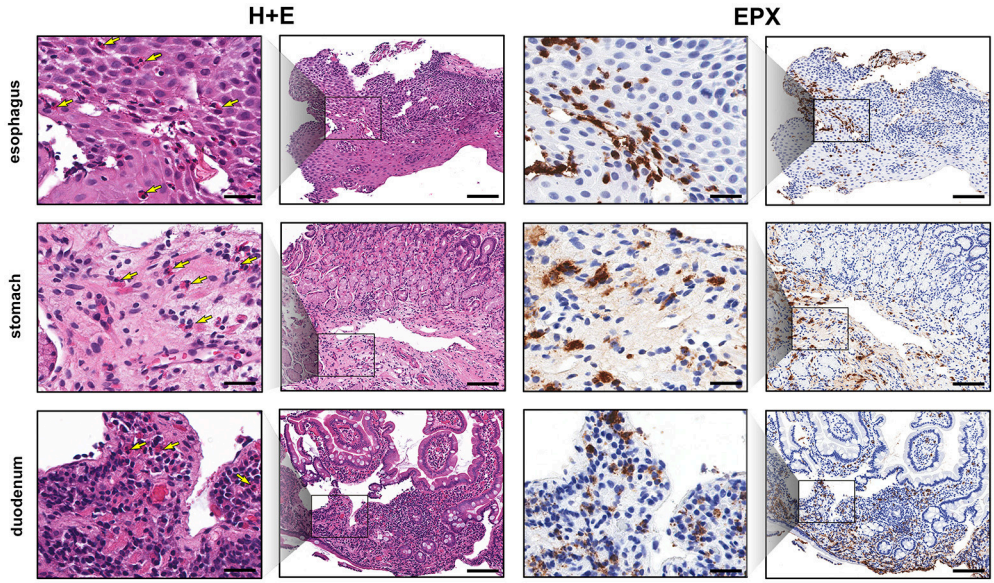

FIGURE 2 | EPX immunohistochemistry highlights gastrointestinal eosinophilic inflammation. Low and high magnification view of serial sections (H&E, columns 1 and 2, and EPX, columns 3 and 4) demonstrating eosinophilia in the esophagus (top row), gastric antrum (middle row) and proximal duodenum (bottom row). Yellow arrows denote eosinophils on H&E stains. Scale bars at high power (columns 1 and 3) and low power (columns 2 and 4) are 25 and 100 microns, respectively.

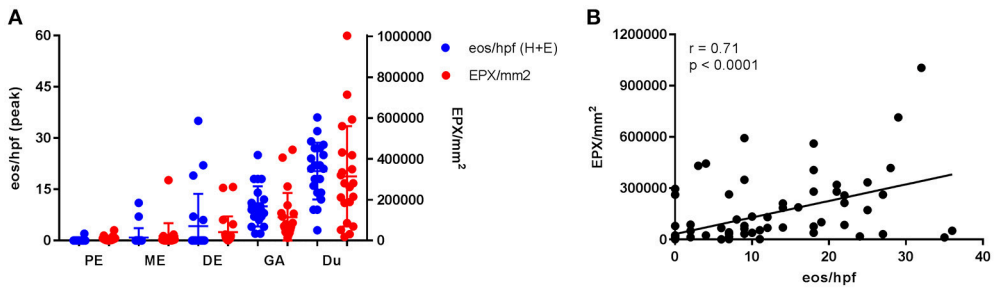

FIGURE 3 | Gastrointestinal eosinophilia is common in adults with IgE-mediated peanut allergy. Eosinophil distribution and EPX deposition **(A)** in the proximal esophagus (PE), middle esophagus (ME), distal esophagus (DE), gastric antrum (GA), and duodenum (Du). Blue circles correspond to the left axis (eos/hpf) and red circles correspond to the right axis (EPX/mm^2). Eosinophil counts were obtained from H + E stains. When plotted against one another, EPX/mm^2 correlates strongly with eos/hpf **(B)**.

DISCUSSION

This is the first study to assess GE by performing endoscopic biopsies in asymptomatic adults with IgE-mediated peanut allergy prior to initiating OIT. We found that 24% of subjects had esophageal eosinophilia at baseline and 14% exceeded the established diagnostic cutoff for EoE, based on PEC. Most of the subjects with esophageal eosinophilia also had additional histologic findings (basal cell hyperplasia, dilated intercellular spaces, and/or lamina propria fibrosis), but these were very mild. The histological abnormalities beyond eosinophilia (as determined by PEC) were assessed by EoEHSS, a newly validated scoring system. EoEHSS objectively and comprehensively evaluates the spectrum of histological changes seen in the esophagus in the setting of EoE. Scoring of eight pathological variables assesses both severity (grade) and extent (stage) of disease. In addition, this system has been shown to be reliable with strong to moderate interobserver agreement among pathologists (37, 42). Based on EoEHSS, despite having baseline eosinophilia in a subset of our cohort, the overall pathology is clearly mild and only a few of the histological abnormalities were noted on endoscopy. Although some of these histopathologic changes resulted in mild endoscopic findings, such as esophageal edema or longitudinal furrows and rings, there were few clinical symptoms and no subjects reported dysphagia. Furthermore, we found that 43% of subjects had increased eosinophils in the gastric or duodenal mucosa, though none met histologic criteria for eosinophilic gastroenteritis based on H + E staining. While there are no current consensus guidelines for cutoff values for eosinophilic gastroenteritis, an average of 30 eos/hpf in 5 hpf is a widely agreed upon histologic threshold for eosinophilic gastritis. Lowichik and Weinberg (39) and Debrosse et al. (29)

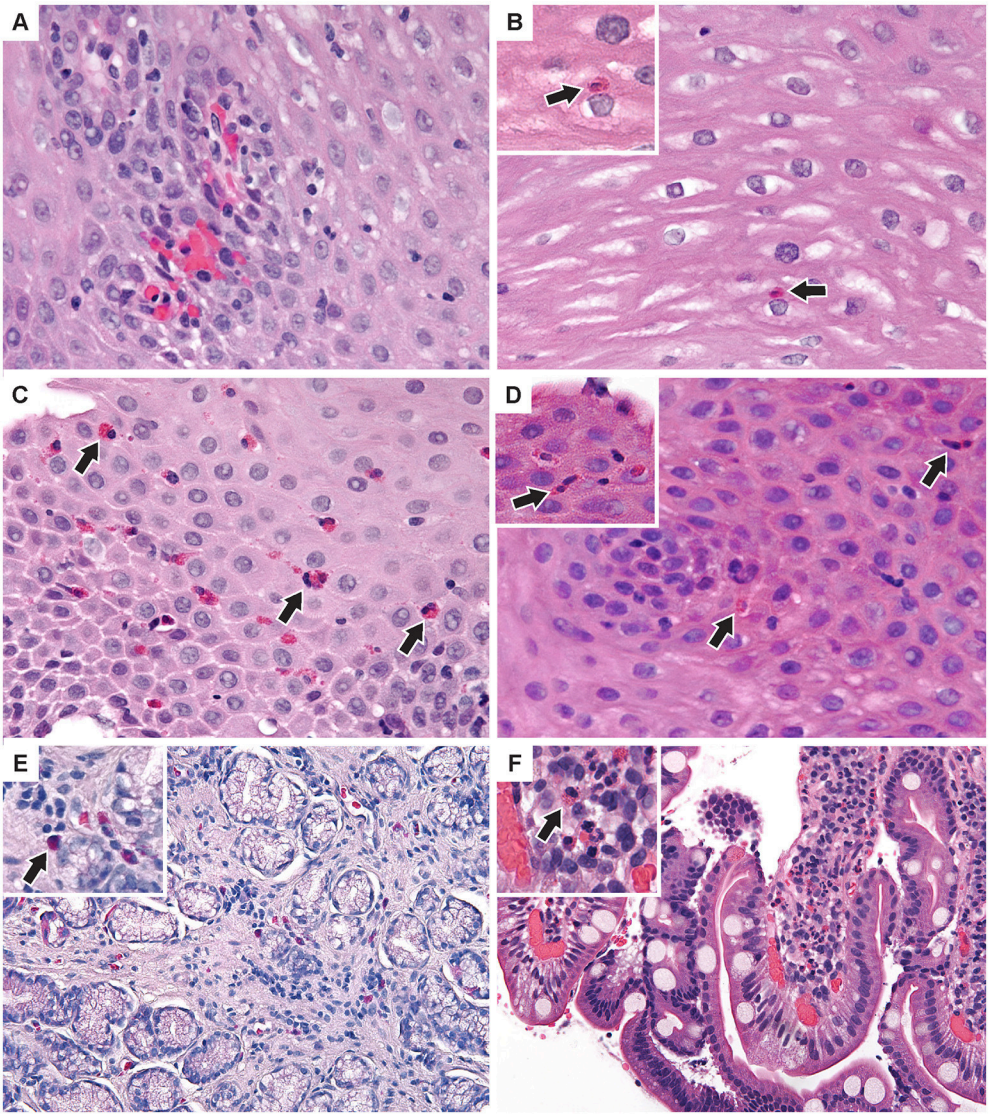

FIGURE 4 | Additional pathologic findings **(A)** Proximal esophagus with increased intraepithelial lymphocytes and congested papillae. Two intraepithelial eos/hpf were seen elsewhere. **(B)** Middle esophagus with normal epithelium and a single intraepithelial eosinophil (inset). **(C)** Distal esophagus with mild basal cell hyperplasia and up to 35 intraepithelial eos/hpf **(D)** Middle esophagus with reactive epithelial nuclei and increased intraepithelial eosinophils (inset) up to 7/hpf. **(E)** Antral stomach mucosa with focal mild non-specific inflammation with lymphocytes and eosinophils (inset). **(F)** Duodenal mucosa with focal lamina propria neutrophils (inset) in one villus. (H&E images ×400). Arrows indicate eosinophils.

have both identified the upper limit of normal as 8 eos/hpf in the gastric antrum and 26 eos/hpf in the duodenum in pediatric subjects. Lwin et al. (41) reported higher gastric eosinophil counts (upper limit of normal 11.63 eos/hpf averaged over 5 hpf) in a population of adult and pediatric subjects; however, biopsy samples were obtained from both the corpus and gastric antrum. Although our biopsies were consistently obtained from the gastric antrum, we conservatively defined gastric eosinophilia as >12 eos/hpf for this study.

We found that eosinophil counts were significantly higher based on EPX immunohistochemistry (**Supplementary Figure E2**), suggesting this may be a more sensitive method for eosinophil detection. However, current reference values (29, 39) and diagnostic cutoffs (40, 41, 43) have been established using routine H + E stains. Moreover, EPX is not a nuclear stain and it assesses both intact eosinophils and degranulation products potentially related to biopsy trauma (38). As a result, there may be an overestimation of eosinophilia in areas of marked degranulation with EPX immunostain. In this cohort, neither eosinophil counts nor EPX deposition corresponded with clinical symptoms as all patients were asymptomatic at the time of endoscopy. Two of the subjects reporting <3 episodes of abdominal pain per month on the GI symptom questionnaire did have increased EPX deposition

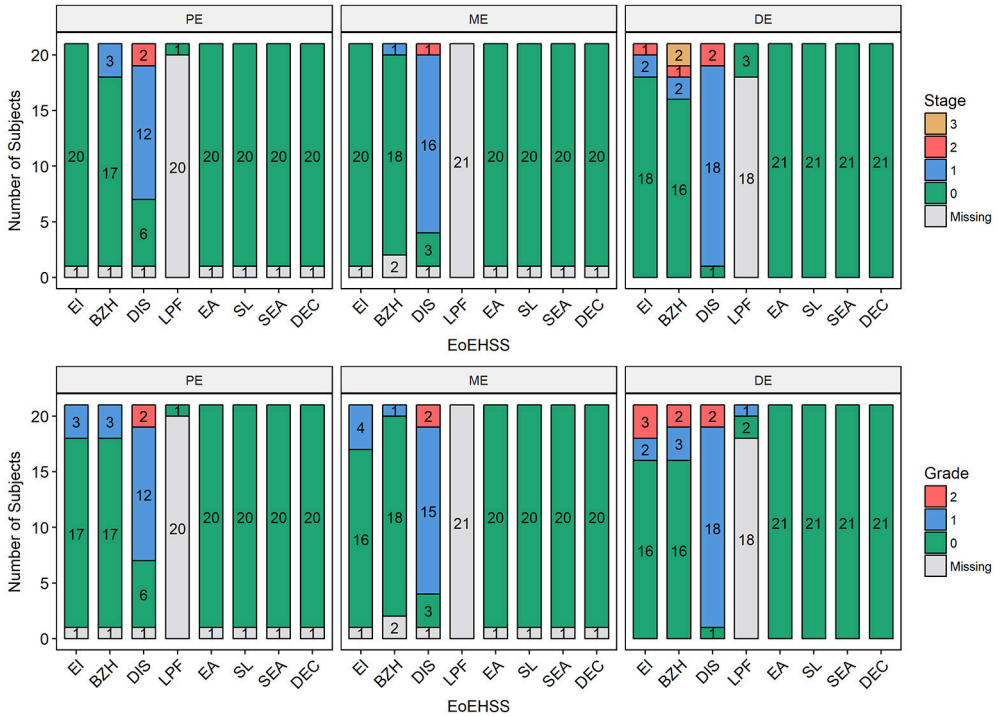

FIGURE 5 | Eosinophilic Esophagitis Histologic Scoring System (EoEHSS). Frequency of histological findings in the proximal esophagus (PE), middle esophagus (ME), or distal esophagus (DE) based on Collins' grading to include eosinophilic inflammation (EI), basal cell hyperplasia (BZH), dilated intercellular spaces (DIS), lamina propria fibrosis (LPF), eosinophilic abscess (EA), eosinophil surface layering (SL), surface epithelial alteration (SEA), and dyskeratotic epithelial cells (DEC). Top panel represents Stage (extent of disease); bottom panel represents Grade (severity of disease).

TABLE 3 | Histology findings: EoEHSS, peak eosinophil counts (PEC) and EPX deposition.

	Esophageal site		
	Proximal	**Middle**	**Distal**
Max EoEHSS, Grade (*n*, % of cohort)			
EI	1 (3, 14%)	1 (4, 19%)	2 (3, 14%)
BZH	1 (3, 14%)	1 (1, 5%)	2 (2, 10%)
DIS	2 (2, 10%)	2 (2, 10%)	2 (2, 10%)
LPF	0 (1, 5%)	NA	1 (1, 5%)
EA	0 (20, 95%)	0 (20, 95%)	0 (21, 100%)
SL	0 (20, 95%)	0 (20, 95%)	0 (21, 100%)
SEA	0 (20, 95%)	0 (20, 95%)	0 (21, 100%)
DEC	0 (20, 95%)	0 (20, 95%)	0 (21, 100%)
Non-eosinophilic features, *n* (%)	1 (5%)	0 (0%)	3 (14%)
Peak eosinophil count, median (min, max)	0 (0, 4)	0 (0, 11)	0 (0, 35)
EPX/mm^2, median (IQR)	8587 (2525-14347)	3776 (540.8-16590)	10356 (4910-26559)

EI, eosinophilic inflammation; BZH, basal cell hyperplasia; DIS, dilated intercellular spaces; LPF, lamina propria fibrosis; EA, eosinophilic abscess; SL, eosinophil surface layering; SEA, surface epithelial alteration; DE, dyskeratotic epithelial cells; EoEHSS, eosinophilic esophagitis histologic scoring system; EPX, eosinophil peroxidase.

in the gastric antrum and one had increased EPX deposition in the duodenum. Larger studies examining the correlation between H + E eosinophil counts and EPX/mm^2 are needed to established EPX-based diagnostic cutoffs. Using this histologic parameter may allow a more consistent, unbiased and thorough quantification of eosinophilic inflammation.

EoE is a clinicopathologic diagnosis characterized by symptoms of esophageal dysfunction and eosinophilic

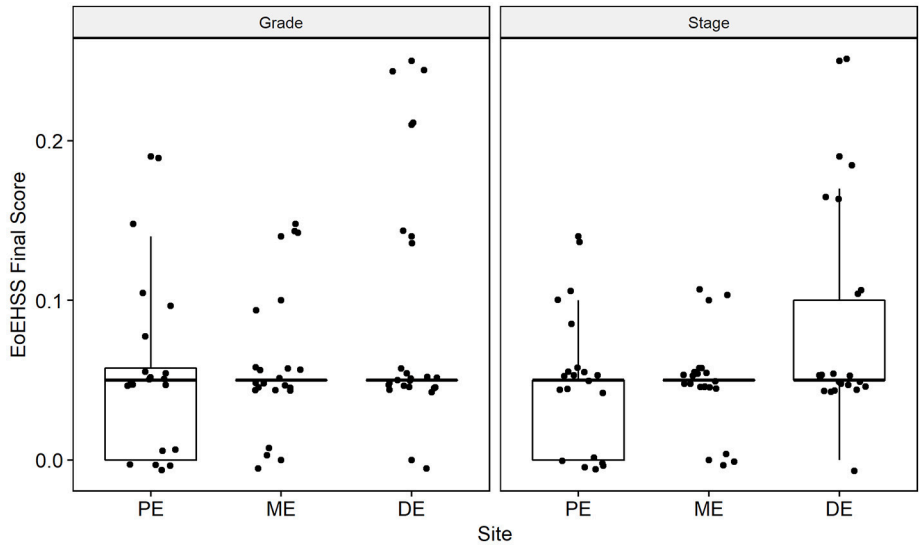

FIGURE 6 | Overall Eosinophil Histologic Scoring System (EoEHSS) at baseline endoscopy. Final score for Grade (left panel) and Stage (right panel) at each site in the esophagus. PE, proximal esophagus; ME, middle esophagus; DE, distal esophagus.

infiltration of esophageal epithelium (43). While symptoms are required for diagnosis, they may not accurately reflect endoscopic and/or histologic remission following treatment (44). Importantly, the initial presentation in some individuals, particularly adults, is food impaction resulting from esophageal narrowing and fibrostenosis (45) likely caused by progression of undetected chronic inflammation (46). It remains unclear whether the patients with esophageal eosinophilia in this study would eventually progress to develop EoE without intervention, or if the chronic antigen exposure associated with OIT would exacerbate pre-existing pathology.

Despite their status as a defining feature of EGIDs, eosinophils are thought to play important homeostatic roles and are normally present in the gastric and duodenal epithelium; albeit, in lower numbers (47). Increased gastrointestinal eosinophils are also seen in other disease states, such as inflammatory bowel disease (48), gastroesophageal reflux disease (49), celiac disease (50), and connective tissue disorders (51). Interestingly, a common element between EoE and food allergy is epithelial barrier disruption (52). While eosinophils are recognized primarily for their pro-inflammatory potential in disease, they also play important regulatory roles in barrier maintenance through mucus and IgA production, tissue repair, and remodeling (48, 53, 54). For example, the recent description of multiple eosinophil subtypes (55) including $Foxp3^{+}$ eosinophils in EoE (56) provides a more nuanced view of the role eosinophils may play in health and disease. Several studies have examined other cell types which may be upstream of eosinophils in the inflammatory cascade. Indeed, animal models suggest mast cell infiltration of the epithelium precedes eosinophilia (57). Furthermore, basophils have been shown to play an important role in eosinophil recruitment (58). Increases in both cell types have been found in esophageal biopsies obtained from patients with active EoE (59, 60). Interestingly, biologics targeting IL-5 have been largely unsuccessful in alleviating clinical symptoms despite their success in achieving substantial reductions in tissue eosinophilia (61, 62). Taken together, these observations suggest that other cellular targets may also be important for treatment of EGIDs.

The prevalence of GE in asymptomatic individuals with IgE-mediated food allergy is largely unknown and difficult to determine due to absence of non-invasive biomarkers for EGID; notwithstanding, our findings are consistent with those recently reported by Barbosa et al. who performed EGDs in 89 subjects with IgE-mediated milk allergy (63). Thirty-eight percent of subjects in their study had evidence of esophageal eosinophilia. While a majority of those with eosinophilic inflammation had at least some gastrointestinal/non-specific symptoms, almost 30% were asymptomatic. GE does not appear to be as common in patients with other atopic conditions, such as asthma (64), though these studies may be confounded by use of inhaled corticosteroids. Importantly, as many as 67% of patients with EoE report comorbid IgE-mediated food allergy (65). As a result, a patient may have resolution of symptoms but have persistent esophageal eosinophilia despite avoidance of EoE food triggers. Consequently, we may question whether asymptomatic patients with IgE-mediated food allergy and esophageal eosinophilia should receive treatment (i.e., diet modification, steroids, PPI) for "silent" EoE to prevent progression to fibrostenosis. This is a critical issue as delayed diagnosis is associated with stricture formation in symptomatic individuals (66, 67). While Echeverria-Zudaire et al. successfully treated a small cohort of patients undergoing OIT who developed EoE with PPI and possible swallowed steroids, it is unclear whether these participants had underlying gastrointestinal eosinophilia at baseline (27). The present study is an analysis of baseline data from a longitudinal study

evaluating the effects of OIT on GE. It has been approved by an IRB and has been closely monitored by a data safety monitoring board of experts in EGID. Due to the absence of clinical symptoms, none of these subjects meet clinicopathologic criteria for diagnosis of EGID, and thus have not received treatment with either PPI or swallowed corticosteroids.

This study has several limitations. First, it is a small, single center study of adult subjects; therefore, it is unclear if similar results would be present in children. In addition, though our population is at much higher risk for EoE, we have applied scoring systems for endoscopic (i.e., EREFS) and histologic (i.e., EoEHSS) findings validated in subjects with EoE (not in IgE-mediated food allergy). Classification of increased gastrointestinal eosinophils distal to the esophagus is also problematic as they are normally present in the stomach and duodenum and reference values are largely derived from pediatric subjects (29, 39). These limitations are balanced by a number of strengths, including a rigorous assessment of symptoms to exclude clinical manifestations of pre-existing EGID, comprehensive endoscopic and histopathologic evaluation, and qualitative and quantitative assessment of GE by EPX immunohistochemistry.

In summary, this study confirms previous speculation that some individuals with IgE-mediated food allergy may have subclinical GE. Endoscopic and histologic findings in the esophagus are mild and are similar to what is seen in EoE that becomes clinically significant. Questions remain regarding the role of eosinophils in IgE-mediated food allergy and the appropriate treatment approach for patients with asymptomatic esophageal eosinophilia. We plan to follow these patients longitudinally to determine the effects of OIT on GE. We will also further examine the role of eosinophils and other cells types during OIT for IgE-mediated food allergy. Surveillance of mucosal responses to OIT may provide a unique window into the pathogenesis of EGIDs.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ICH/GCP/CFR guidelines by the Stanford IRB with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Stanford IRB.

AUTHOR CONTRIBUTIONS

RSC, KN, and SG designed the research study. BW, KS, AD, NF-B, NK, DT, WZ, BB, MM, and RSC performed the research. BW, MR, AD, EJ, NF-B, NK, NP, SB, SG, and RSC analyzed data. BW and RSC wrote the initial draft of the manuscript. BW, MR, AD, EJ, SB, HK, MT, HM, KN, SG, and RSC provided critical assessments during the revision process leading to the final submitted manuscript. All authors have reviewed and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

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Analysis of a Large Standardized Food Challenge Data Set to Determine Predictors of Positive Outcome Across Multiple Allergens

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Background: Double-blind placebo-controlled food challenges (DBPCFCs) remain the gold standard for the diagnosis of food allergy; however, challenges require significant time and resources and place the patient at an increased risk for severe allergic adverse events. There have been continued efforts to identify alternative diagnostic methods to replace or minimize the need for oral food challenges (OFCs) in the diagnosis of food allergy.

Methods: Data was extracted for all IRB-approved, Stanford-initiated clinical protocols involving standardized screening OFCs to a cumulative dose of 500 mg protein to any of 11 food allergens in participants with elevated skin prick test (SPT) and/or specific IgE (sIgE) values to the challenged food across 7 sites. Baseline population characteristics, biomarkers, and challenge outcomes were analyzed to develop diagnostic criteria predictive of positive OFCs across multiple allergens in our multi-allergic cohorts.

Results: A total of 1247 OFCs completed by 427 participants were analyzed in this cohort. Eighty-five percent of all OFCs had positive challenges. A history of atopic dermatitis and multiple food allergies were significantly associated with a higher risk of positive OFCs. The majority of food-specific SPT, sIgE, and sIgE/total IgE (tIgE) thresholds calculated from cumulative tolerated dose (CTD)-dependent receiver operator curves (ROC) had high discrimination of OFC outcome (area under the curves $>$ 0.75). Participants with values above the thresholds were more likely to have positive challenges.

Conclusions: This is the first study, to our knowledge, to not only adjust for tolerated allergen dose in predicting OFC outcome, but to also use this method to establish biomarker thresholds. The presented findings suggest that readily obtainable biomarker values and patient demographics may be of use in the prediction of OFC outcome and food allergy. In the subset of patients with SPT or sIgE values above the thresholds, values appear highly predictive of a positive OFC and true food allergy. While these values are relatively high, they may serve as an appropriate substitute for food challenges in clinical and research settings.

Keywords: food challenge, cumulative tolerated dose, AUC, biomarker evaluation, time-dependent ROC

BACKGROUND

During recent years, the prevalence of IgE-mediated food allergies has steadily increased and has emerged as a significant health crisis (1) affecting 8% of the pediatric population with more than 30% of these children with multiple food (multifood) allergies (2). Not only are childhood food allergies associated with comorbid atopic conditions such as atopic dermatitis, asthma, and allergic rhinitis, but are also associated with impaired quality of life (3–8).

The diagnosis of food allergy is highly complex (9, 10). Skin prick testing (SPT) and food allergen-specific immunoglobulin E (sIgE) are commonly used to determine allergenicity, however outcomes are often variable. High thresholds of both SPT and sIgE have been established for specific foods and tend to correlate with reactivity, such as sIgE > 15 KU/L and SPT > 8 mm associated with 95% positive predictive value (PPV) for tree nuts (11). However, thresholds are less useful for intermediate values where there is already a doubt whether the patient is truly allergic (12–21), and may be associated with false positives (10, 22). Children in particular have a higher rate of sensitization without true allergy (23). Other biomarkers that have been explored include basophil activation tests (BATs) as well as the measurements of allergen-specific IgG, total IgE (tIgE), and component resolved diagnostics, but definitive thresholds remain to be established (24). Due to these limitations, the current gold standard for confirming food allergy is the double-blind, placebo-controlled food challenge (DBPCFC) (9, 10), which is typically performed in the research setting as part of inclusion into clinical trials; however, DBPCFCs are not without a number of limitations. While food challenge guidelines have been recommended in the literature, dosing strategies are not allergen-specific (25). DBPCFCs require multiple days of challenges for multi-food allergic individuals, which can significantly increase the cost. The most significant limitation is that food challenges carry the risk of potentially inducing severe anaphylaxis which may require hospitalization or care in the intensive care unit (26).

In this paper, we attempt to identify potential prognostic indicators for multi-food allergic individuals associated with outcomes during oral food challenges (OFCs) which could aid in risk stratification for designing challenge protocols for clinical trials. We tested data obtained from eligible participants from several food allergy trials that required either baseline DBPCFCs or unblinded food challenges as an inclusion criteria. In our analysis, we attempt to identify factors that may better predict food allergy outcomes in the research and clinical setting and provide guidance toward dosing strategies.

METHODS

Data Source

All clinical trial participant data from food allergy studies conducted under IRB approved protocols were entered into a standardized database. The database was created by a board certified Allergy/Immunology physician and all food challenges were conducted, evaluated, and documented by trained research clinicians. Data entry was performed by trained research staff. Quality checks of data were performed by our data entry and statistics team.

Skin Prick Tests, IgE Blood Tests, and Oral Food Challenges

Between September 2010 to March 2016, participants were recruited to undergo OFCs as part of screening for clinical trial enrollment at 7 sites under an Investigational New Drug (IND) at Stanford University. During the initial screening visit, SPT and IgE values were obtained for each participant in the clinic at the time of the visit or from previous testing at a physician's office, depending on clinical trial inclusion criteria. SPT consisted of a positive histamine control, a negative saline control (both from Hollister-Stier) and allergen extracts from Greer. SPTs were performed on the volar surface of the forearm or back after application of the respective allergen solution. Mean wheal diameter was measured after 20 min. Allergen-specific IgE levels were measured by ImmunoCAP fluorescence enzyme immunoassay. Challenges to each food allergen were performed only in participants with suspected food allergy, defined broadly as an sIgE > 0.35 kU/L and/or a positive SPT (>3 mm above the negative control) to the challenged allergen. OFCs were standardized in methodology and escalated to at least 500 mg cumulative food protein to each of the participants' suspected allergens. Participants with previous reactions to food requiring the use of epinephrine for adverse reactions were eligible for screening and challenges under each study; however, those with a past history of intubation or hypotension related to a food allergy were excluded.

While most of the included challenges were conducted as DBPCFCs, some challenges were unblinded OFCs. All food challenges included for the purpose of analyses will be referred to as OFCs, herein, regardless of blinded status. Excluding such differences in blinding, all OFCs were performed using standardized methodology with respect to monitoring, according to validated guidelines (10, 27, 28). Challenges to eleven different food allergens were included in the analyses, consisting of almond, cashew, egg, hazelnut, milk, peanut, pecan, pistachio, sesame, walnut, and wheat. Typically challenges started with as small as 1 mg (for pistachio), then 2, 5, 20, 50, 100, 100, 100, 123 mg (for pistachio) or 124 mg. Pistachio started at 1 mg due to safety concerns since only those positive to a cashew challenge, were also challenged to pistachio. Challenges to allergens other than those mentioned above were defined as "other" and excluded from further analyses given the limited number of challenges performed to such allergens. Each OFC consisted of sequentially escalating doses of food protein ingested by the participant every 15 min as tolerated. Food protein was administered in flour form mixed in an appropriate vehicle, such as applesauce or pudding. During the course of the challenge, vital signs and pertinent physical examinations were repeated at least every 15 min at the discretion of the clinician. Type and severity of each dose-related allergic adverse event were determined and classified according to Bock criteria (27), and participants tolerating 500 mg cumulative protein dose during the challenge were considered to have a negative challenge, for the purpose of analysis. Cumulative tolerated dose (CTD) was defined as the last ingested cumulative protein dose at which no dose-related allergic adverse event occurred. All aspects of the studies from which data was obtained were authorized by the IRBs at each site.

Statistical Analysis

Challenges were censored at 500 mg CTD if the challenge was negative. A cumulative incidence plot and median survival were reported by food, and the equality of the incidence curves was tested using the log-rank test. The survfit function of R's survminer package was used to fit the model (29).

To determine possible predictors of a positive challenge, Cox proportional hazards models containing Gaussian random effects (i.e., frailty models) were fit to the primary outcome as a function of each clinical and demographic feature, adjusting for challenge food with a random effect for participant. The coxme function was used to fit each model (30). Hazard ratios and 95% CIs were reported.

To determine thresholds of SPT, sIgE, and the sIgE to tIgE ratio (IgEr) that best discriminated challenge outcome, the OptimalCutpoints package was implemented using receiver operator characteristic (ROC) curves based on Youden's index (31, 32). Next, a logistic regression model was fit to both SPT and sIgE then to SPT and sIgEr for each food. The ModelGood package was used to calculate the AUC from each multivariable model (33). The set of 5 ROC analyses were compared for each food graphically and by AUC.

To incorporate the dose-varying nature of the food challenge outcome, a dose-dependent ROC was used, predicting the probability of a positive challenge to a maximum cumulative dose of 500 mg. The survivalROC package was used to determine the optimal threshold, while time ROC was used to calculate the AUC, PPV, and negative predictive value (NPV) at the determined threshold by dose (34, 35). Kaplan-Meier curves were plotted based on the determined threshold, and *P*-values from the log-rank test were reported. Within positive OFCs, concordance of SPT and sIgE thresholds and SPT and sIgEr thresholds for each food was assessed and accuracy was reported.

In order to compare the two ROC methods, AUCs were derived from 1,000 bootstrap samples per ROC method, allergen, and marker. We then took the difference in the two AUCs and calculated a 95% confidence interval around the difference.

All analyses were conducted at the 0.05 alpha level. *P*-values were not adjusted where multiple comparisons were made. Analyses were conducted using R v.3.4.3 (36).

Data Management

Any value of sIgE $>$ 100 kU/L was truncated to 101 due to clinical lab processing. If SPT or sIgE were not performed during screening then previously collected SPT and/or sIgE available within 12 months of the OFC were included in the analysis (14). Negative control (saline) SPT was subtracted from the raw food SPTs prior to analysis. Any SPT that was collected after the food challenge or collected more than 12 months before the challenge was excluded. If a subject had more than one value for SPT or sIgE, then the value obtained most recently was used.

To account for differences in maximum challenge doses, positive challenges with CTDs of 500 mg protein or higher were re-coded as having negative challenges. Subjects who had unknown or non-reported ethnicity were coded as missing ethnicity. Subjects with race of Native Hawaiian, other, or not reported were coded as other. Challenges to oat (placebo) were excluded from analyses. Further, challenges reported as negative with CTDs of $<$500 mg cumulative protein were also excluded. Placebo challenges were not included in the analysis. A consort of these steps is illustrated in **Figure 1.**

RESULTS

Baseline Demographics

Four hundred and twenty-seven participants were challenged to at least one food (**Figure 1**). Ages ranged from 1 to 54, with a median age of 9 years. The cohort was comprised of mostly non-Hispanic (97%), Caucasian (61%), and males (61%). The majority of participants also had atopic history, including asthma (62%), allergic rhinitis (77%), and atopic dermatitis (AD) (73%). The median number of doctor diagnosed food allergens was 5, with only 2% of the cohort being mono-food allergic. The median tIgE was 491 kU/L (**Table 1**).

Challenge Overview

Eighty-five percent of OFCs resulted in a positive outcome. Between 41 and 100% of all OFCs conducted across foods were positive (**Table 2**). For instance, all pistachio challenges had positive outcomes, however only cashew allergic participants were challenged to pistachio. Cashew and pecan challenges had

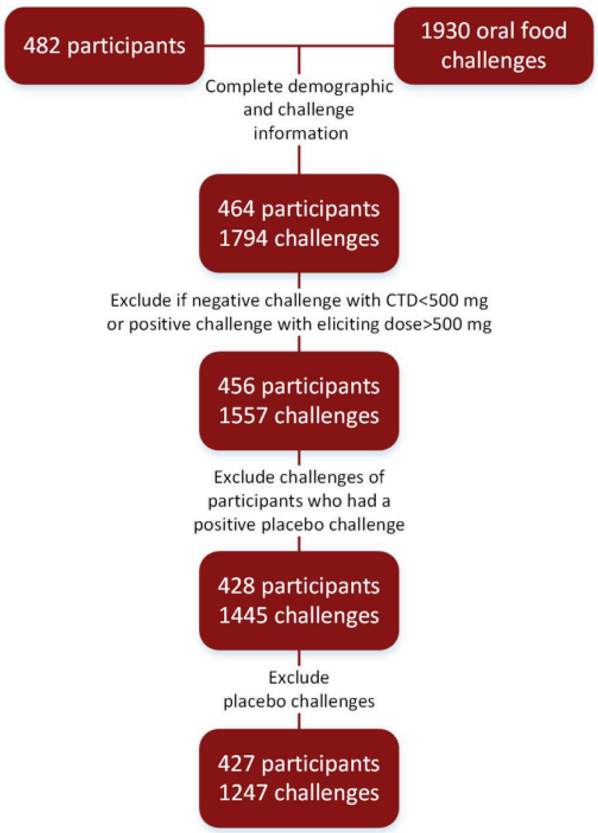

FIGURE 1 | Consort diagram: food challenges conducted in research settings to arrive at final cohort.

TABLE 1 | Baseline demographics.

Characteristic	Total* (n = 427)
Age in years, median (range)	9 (1–54)
Male	259 (61%)
Non-Hispanic	406 (97%)
RACE	
Caucasian	259 (61%)
Black	6 (1%)
Asian	109 (26%)
Multiracial	42 (10%)
Other	5 (1%)
ATOPIC HISTORY	
Asthma	240 (62%)
Allergic rhinitis	294 (77%)
Atopic Dermatitis	279 (73%)
Number of food allergens, median (range)	5 (1–16)
Mono-food allergic	10 (2%)
Total IgE (IU/L), median (range)	491 (17.8–3,366.0)

**Count and percent of total subjects unless otherwise noted.*

TABLE 2 | Challenge summary by allergen.

Allergen	Positive OFCs/ total OFCs (%)	Allergic participants (%)
Almond	30/73 (41)	29 (7)
Cashew	151/163 (93)	150 (35)
Egg	63/71 (89)	60 (14)
Hazelnut	68/102 (67)	65 (15)
Milk	67/77 (87)	66 (15)
Peanut	347/377 (92)	330 (77)
Pecan	88/95 (93)	88 (21)
Pistachio	60/60 (100)	59 (14)
Sesame	30/42 (71)	30 (7)
Walnut	121/138 (88)	120 (28)
Wheat	13/16 (81)	13 (3)
Other	16/33 (48)	13 (3)
Total	1054/1247 (85)	410/427 (96)

Oral food challenge (OFC); Other foods consisted of barley, brazil nut, chickpea, crab, garbanzo bean, macadamia, mustard, rye, shellfish, and soy. Seventeen subjects did have a positive challenge to any food tested. Allergic is defined as the number of participants who had a positive challenge to that food. Participant may have had repeat challenges to the same food.

the next highest percent of positive challenges (93%), followed by peanut (92%). Some participants repeated food challenges to the same food allergen over time, therefore the number of positive OFCs may be higher than the number of unique allergic participants. The largest number of food challenges were conducted for peanut (n = 377) with 77% of participants having positive challenges. Only 41% of almond challenges resulted in a positive challenge outcome.

The highest median CTD at which 50% of participants had no allergic reaction was 28.9 mg (for sesame), while the other challenged foods had lower median CTDs; except for challenges to almond where <50% of participants had a positive outcome (**Figure 2**). No participant challenged to pistachio in our Center tolerated a cumulative protein dose >175 mg and 50% reacted at the first dose (CTD median = 0).

Average SPT values in the cohort ranged from 6.2 mm for almond to 13.6 mm for cashew and peanut (**Table 3**). Peanut had the highest median sIgE (67.55 kU/L) followed by wheat (61.5 kU/L) and almond had the lowest (4.39 kU/L).

Participants with a lifetime history of AD had 1.23-fold higher risk of a positive challenge outcome compared to those without a history of AD (hazard ratio [HR]: 1.23, 95% confidence interval [CI]: 1.00, 1.52) (**Table 4**). The risk of a positive challenge outcome increased by 4% for every additional doctor diagnosed food allergy (HR: 1.04, CI: 1.01, 1.07).

Logistic ROC for Clinical Thresholds

The logistic ROC approach resulted in SPT thresholds that ranged from 4.5 mm for wheat to 14.5 mm for egg for predicting a positive OFC, with AUCs ranging from 0.52 to 0.90 (**Table 5**). The ROC approach using sIgE resulted in thresholds that ranged from 1.2 kU/L for cashew to 52.2 kU/L for wheat, with AUCs ranging from 0.59 to 0.92. AUCs for sIgEr thresholds ranged from 0.65 to 0.89.

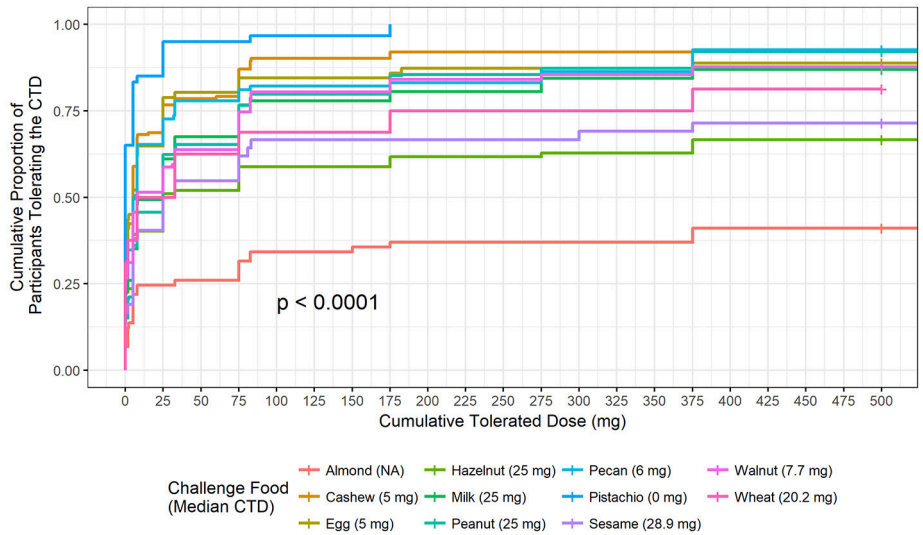

FIGURE 2 | Cumulative tolerated dose by food. Each food is plotted to understand the proportion of participants who tolerated specified cumulative tolerated doses (CTD). Median CTD is the highest cumulative dose at which 50% of participants had no dose-related allergic reaction and is listed in the figure legend in parenthesis.

TABLE 3 | Biomarker summaries per specific food.

Food	*n*	SPT*, mm Mean (range)	*n*	sIgE, kU/L Median (range)	*n*	sIgEr Median (range)
Almond	42	6.2 (0–21.5)	52	4.39 (0–101)	36	0.008 (0–0.085)
Cashew	106	13.6 (0–30.5)	114	10.85 (0–101)	79	0.028 (0.001–0.249)
Egg	43	10.8 (2.5–22.5)	36	10.14 (0.75–100)	28	0.024 (0.002–0.430)
Hazelnut	70	8.9 (0–35.5)	77	11.30 (0–100)	61	0.026 (0.001–0.422)
Milk	53	12.2 (0–26.0)	58	14.55 (0–101)	46	0.029 (0–0.437)
Peanut	302	13.6 (0–33.0)	268	67.55 (0–101)	168	0.080 (0–0.452)
Pecan	64	9.7 (0–21.0)	63	8.30 (0–101)	53	0.018 (0.002–0.200)
Sesame	23	11.2 (0–27.5)	29	9.98 (0–100)	24	0.027 (0–0.209)
Walnut	92	10.0 (0–26.0)	101	13.30 (0–101)	80	0.035 (0–0.347)
Wheat	12	8.3 (0–13.5)	13	61.50 (3.30–101)	12	0.068 (0.012–0.410)

**Subtracting out the negative control.*

In four of the 10 allergens (cashew, egg, peanut, and sesame), the combination of SPT and either sIgE or sIgEr was better at discriminating food challenge outcome than any of the markers individually, and in one instance (for hazelnut), SPT alone was the best (**Table 5**). For cashew, egg, peanut, and sesame where the joint markers were superior, AUCs were 0.80 and above. A comparison of the joint markers and each individual marker by food are displayed in **Figure 3**. The best AUC for each food varied between the clinical markers.

CTD-Dependent ROC for Clinical Thresholds

ROC analyses were also conducted to assess for CTD and challenge outcome to account for the last tolerated dose in the food challenge outcome. Participants with SPTs above the calculated CTD-dependent thresholds were significantly more likely to not only have a positive challenge, but react at lower doses compared to those with values below the threshold for all foods except milk, egg, and wheat (**Figure 4**). AUCs for SPT ranged from 0.65 (almond) to 0.98 (cashew) (**Table 6**). Walnut had the lowest calculated SPT threshold of 4 mm and egg had the highest calculated SPT threshold of 13 mm. While thresholds chosen in the CTD-dependent ROC analysis were similar to those reported for the logistic ROC approach, AUCs were generally higher, though this difference was not significant, in the CTD-dependent ROCs.

Similar to SPT, sIgE values above the threshold were associated with a lower dose to a positive outcome compared to those with values at or below the threshold (**Figure 5**). Cashew had the lowest calculated sIgE threshold of 1.2 kU/L, and wheat was the highest at 43.1 kU/L (**Table 6**). Cashew, pecan, and wheat thresholds had AUCs above 0.80. Hazelnut and sesame had the lowest AUCs. Threshold values were similar to those chosen through the logistic ROC analysis.

TABLE 4 | Univariable associations of positive challenge.

Characteristic	Hazard ratio (95% CI)	Challenges included
Female	1.00 (0.83, 1.20)	1,246
Hispanic	1.44 (0.85, 2.44)	1,228
Race (ref = Caucasian)		1,236
Black	0.82 (0.36, 1.85)	
Asian	1.19 (0.97, 1.45)	
Multiracial	1.24 (0.92, 1.67)	
Other	1.21 (0.58, 2.54)	
Atopic History		
Asthma	1.05 (0.87, 1.27)	1,154
Allergic Rhinitis	0.93 (0.75, 1.15)	1,138
Atopic Dermatitis	1.23* (1.00, 1.52)	1,139
Age	0.99 (0.98, 1.01)	1,247
FEV1	1.00 (0.99, 1.01)	744
FEV1 to FVC Ratio	1.71 (0.35, 8.27)	571
Mono-allergic	0.71 (0.37, 1.37)	1,247
Number of diagnosed foods	1.04** (1.01, 1.07)	1,223
IgE Total (log-scale)	0.93 (0.82, 1.05)	621

Each row corresponds to a single frailty model based on CTD and challenge outcome.
**p<0.05; **p<0.01.*
FEVI, The forced expiratory volume in one second; FVC, Forced vital capacity.

Six of the ten derived sIgEr thresholds had AUCs above 0.80, with a lowest AUC of 0.76. At defined values SPT had the best predictive value compared to sIgE and sIgEr. The PPV for all tested foods was 1 except for sesame, which was 0.95. Within sIgE values, sesame was the lowest at 0.64. The sIgEr had a PPV range of 0.68 to 1 with almond having the lowest PPV (**Table 6**). As with SPT and sIgE, participants with sIgEr values below the threshold were less likely to have a positive challenge at the same CTD as someone with a value above the threshold (**Figure 6**). Significant risk stratification of food-specific challenge outcome by biomarker threshold was found in the majority of foods (**Figures 4–6**).

Among positive challenges, at least 60% of participants had SPT and sIgE values above the reported CTD-dependent thresholds for four of the ten allergens (cashew, peanut, pecan, and sesame), of which cashew displayed the highest level of SPT and sIgE threshold concordance at 90% (**Figure 7**). Among almond, egg, and wheat where accuracy was low, the SPT threshold was more likely to be negative when the sIgE threshold was positive. However, among milk and walnut, the SPT threshold was more likely to be positive when the sIgE threshold was negative. The overall agreement of SPT and sIgE thresholds was 65%. Half of the concordance rates for SPT and sIgEr were higher than those calculated for SPT and sIgE (**Figure 8**). The overall agreement of SPT and sIgEr thresholds was higher than that of SPT and sIgE with 72%.

DISCUSSION

Presently, the gold standard for confirming food allergy remains the DBPCFC, especially in the research setting; however, the procedure can be time consuming, resource intensive, and carries the risk of life-threatening anaphylaxis (9, 10, 26, 27, 37). Recent studies have shown 40–70% of food allergic patients are allergic to more than one food (2), resulting in the need for multiple food challenges to prove or disprove each allergy. Additionally, positive reactions to placebo are not uncommon and can have a varied clinical presentation. In our experience, 12.7% of participants had positive placebo challenges, which is consistent with the published literature (38–44). In light of these significant burdens, there is a great need for a reliable method of diagnosing food allergies without food challenges, in addition to the ability to stratify participants according to potential risk in scenarios where a food challenge cannot be avoided.

Our large dataset of 1247 baseline OFCs allowed us to evaluate CTDs across several allergens and examine the utility of SPT, sIgE, sIgEr, and a combination of these markers in the prediction of food challenge outcome. SPTs and sIgE remain among the most widely used diagnostic markers for the evaluation of a suspected food allergy due to their simplicity and safety, with SPT providing nearly immediate results. Previous literature reports threshold values for each of these markers with high PPVs in the prediction of food challenge outcome and true food allergy (11, 45–50). We implemented similar methods to those described in the literature to derive optimal thresholds of SPT and sIgE for each individual allergen in our dataset. We further derived thresholds for the ratio of sIgE to tIgE to account for relative proportions of each allergen-specific IgE, which has yet to be evaluated in multi-food allergic patients. While a number of our calculated thresholds for SPT and sIgE values appeared to vary in relation to the thresholds at 95% PPV reported in the literature, differences in our cohort may be due to the fact that our participants are multi-food allergic (11, 45, 46, 49).

In addition to their use as individual predictors of food challenge outcome, prior studies have also assessed the utility of a combination of biomarkers (15, 51); however, to our knowledge, this is the first study to evaluate the utility of combining optimized threshold values for SPT with sIgE or sIgEr. While SPT had the highest PPV values compared to sIgE and sIgEr, the combination of SPT cut-off values with those for sIgE or sIgEr resulted in greater AUCs than SPT, sIgE, or sIgEr alone in the prediction of food challenge outcome. While previous data, mostly in the setting of allergen immunotherapy for allergic rhinitis, have demonstrated sIgEr to be promising as a predictive marker for clinical outcome (52–56), the ratio may have underperformed in our population due to limitations in the number of participants with both sIgE and tIgE values.

The methodology described above was also used in evaluating the association between specific allergens, baseline participant characteristics, and food challenge outcome. Our findings indicate that CTDs vary by allergen, suggesting that the use of identical dosing strategies for food challenges across all may not be the optimal, safest approach. Within our dataset, 50% of our participants had reactions before reaching the 10 mg dose for all foods, exluding almonds. When designing clinical trials that include food challenges, smaller incremental dose steps below 10 mg may aid in reducing the severity of reactions.

TABLE 5 | Logistic ROC thresholds for food challenge outcome.

Food	SPT Cutoff, mm (AUC)	sIgE Cutoff, kU/L (AUC)	sIgEr Cutoff (AUC)	SPT + sIgE AUC	SPT + sIgEr AUC
Almond	12.5 (0.52)	12.4 (**0.68**)	0.002 (0.65)	0.63	0.61
Cashew	5.0 (0.90)	1.2 (0.85)	0.002 (0.85)	**0.93**	0.82
Egg	14.5 (0.65)	10.7 (0.71)	0.018 (0.81)	0.84	**0.88**
Hazelnut	7.5 (**0.74**)	15.4 (0.59)	0.025 (0.68)	0.72	0.58
Milk	8.5 (0.73)	22.8 (0.61)	0.017 (**0.83**)	0.56	0.76[x]
Peanut	9.5 (0.71)	11.4 (0.81)	0.017 (0.81)	0.73	**0.82**
Pecan	7.5 (0.76)	2.1 (**0.92**)	0.011 (0.81)	0.97[x]	0.88[x]
Sesame	11.5 (0.81)	8.8 (0.86)	0.069 (0.76)	**0.88**	0.89[x]
Walnut	7.5 (0.80)	13.9 (0.78)	0.021 (**0.82**)	0.93[x]	0.67
Wheat	4.5 (0.82)	52.2 (0.83)	0.057 (**0.89**)	0.82	1.00[x]

Based on ROC analysis of challenge outcome. Bolded values indicate the highest AUC across all markers, excluding those with an x. [x]Denotes quasi-separation/non-convergence of model. AUC from these models may not be informative. Subjects with negative challenge outcomes were not included. SPT, skin prick test; AUC, area under the curve; sIgE, allergen-specific IgE; sIgEr, specific IgE / total IgE ratio.

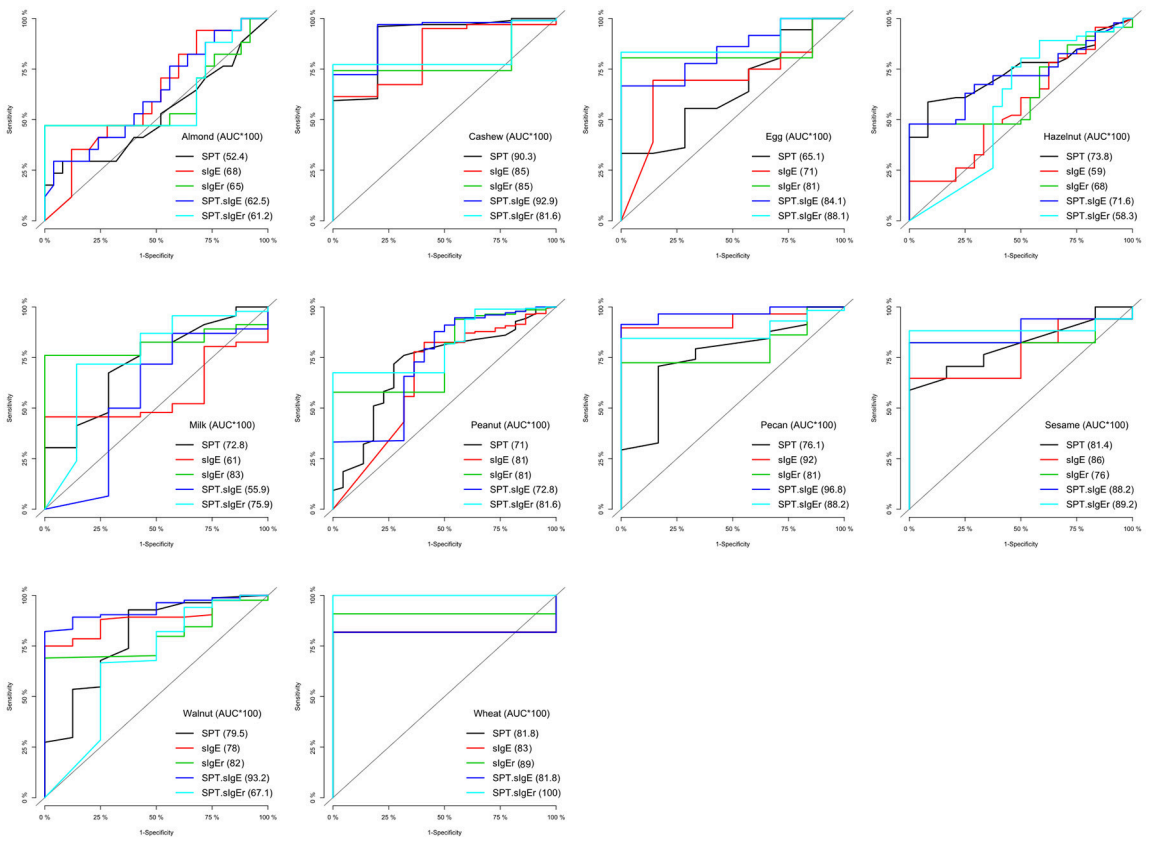

FIGURE 3 | Logistic ROC comparisons of biomarker thresholds by allergen. Comparison of each biomarker to discriminate food challenge outcome. Higher area under the curve (AUC) suggests better discriminative ability. SPT, skin prick test; sIgE, specific Immunoglobin E, sIgEr, ratio of sIgE to total IgE.

Additional findings from our dataset suggest that participants with a history of AD have an increased risk of a positive challenge outcome compared to those without a history of AD. While the presence of AD is often associated with a high rate of false-positives during food allergy testing, especially in children (57–59), our data suggests that among participants who are sensitized to one or more foods, those with a history of AD actually have a higher risk of a positive food challenge than those without a history. This is consistent with the current theory that the impaired skin barrier observed in those with AD may

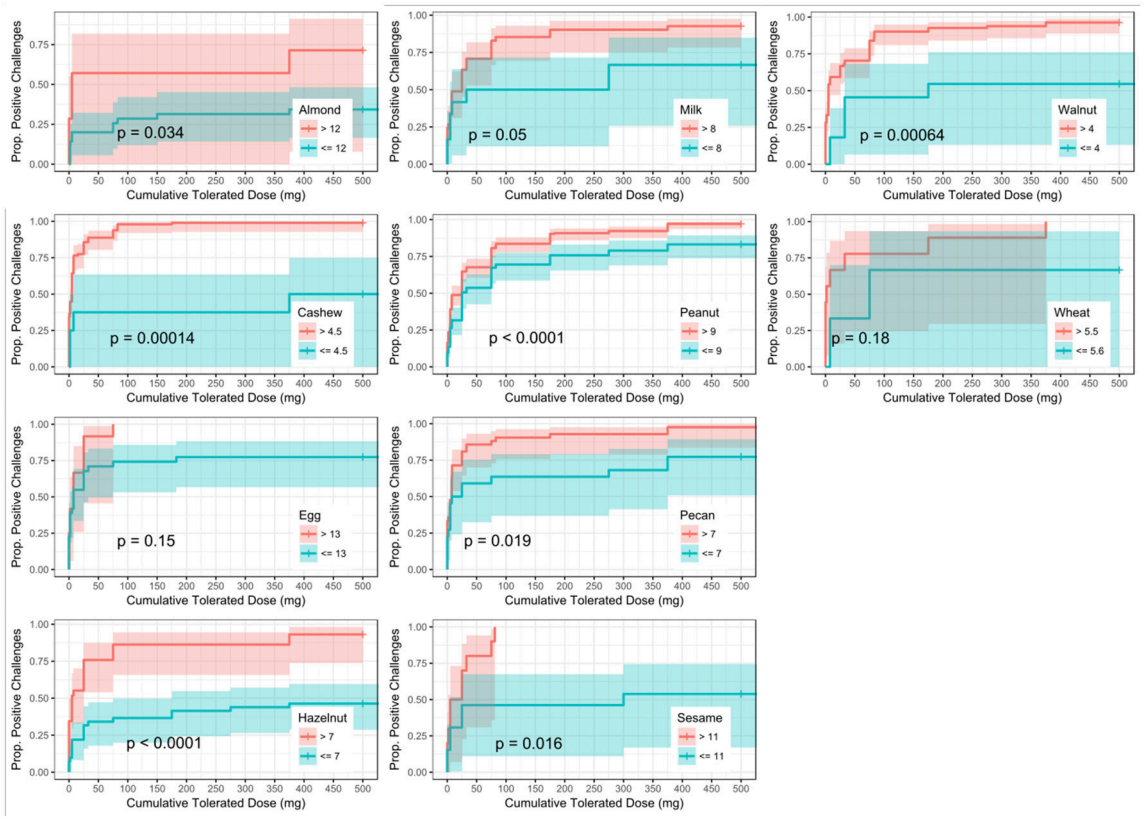

FIGURE 4 | Dose to positive challenge by skin prick test (SPT) threshold and food. Kaplan-Meier curves of dose to positive challenge stratified by the CTD-dependent ROC thresholds for SPT by food. Red lines indicate risk of a positive challenge if SPT is above the threshold, while blue lines indicate risk for participants with SPT at or below the threshold.

facilitate sensitization through environmental exposure to food allergens, and combined with avoidance of regular oral exposure, lead to true food allergy (60, 61). Although, previous literature has found asthma to be a significant predictor of severe reactions (51, 62), our data did not find asthma to be a significant factor associated with positive challenge outcome. Some studies have shown that age can affect IgE and SPT cutoff levels (22, 63, 64), with lower cutoffs typically used in children <2 years of age (65, 66), however our analysis did not reveal strong associations with age, SPT/sIgE/sIgEr cutoff levels and challenge outcomes. This is likely due to the limited number of participants aged <2 years who were challenged in our cohort.

Other studies have similarly explored factors in optimizing predictive outcome. In a retrospective study, DunnGavin et al. used a prognostic model that incorporated gender, age, and prior history of reaction in addition to sIgE, tIgE minus sIgE, and SPT. Their model accurately predicted OFC results 92% percent of the time (67). Cianferoni et al. conducted a retrospective chart review and used a multilogistic regression and discovered that age and history of prior non-cutaneous reactions, when combined with patient's SPT wheal size were predictive of multisystem reactions during food challenges. Simberloff et al. designed and implemented a Standardized Clinical Assessment and Management Plan (SCAMP) to improve sIgE and SPT thresholds to determine which patients would benefit from an OFC. While most studies for food allergy are focused on predictive models to distinguish between a positive or negative food challenge (10, 39, 68), our model also attempts to predict the dose at which a reaction may occur based on biomarkers. We utilized a novel approach to integrate the CTD with food challenge outcome when deriving optimally predictive SPT and sIgE threshold values. Our group has previously found this approach of adjusting for dose to be important in predicting OFC outcomes (62). The primary focus of our analysis was to determine whether the addition of CTD data with food challenge outcome would improve the diagnostic accuracy, as measured by AUC, of derived threshold values for available biomarkers when compared to a logistic ROC approach utilizing food challenge outcome alone. Our analysis did not reveal a statistical difference between these two approaches; however, incorporating CTD into the challenge outcome did allow for risk stratification and the generation of separate Kaplan–Meier curves for individuals with biomarker values above and below the generated thresholds, thus enabling a prediction of the cumulative protein dose that the individual will react to based on biomarker values (**Figures 4–6**). These findings are clinically useful, especially in the research

TABLE 6 | CTD-dependent ROC thresholds at 500 mg CTD.

Food	SPT Cutoff, mm (AUC)	PPV	NPV	sIgE Cutoff, kU/L (AUC)	PPV	NPV	sIgEr Cutoff (AUC)	PPV	NPV
Almond	12.0 (0.65[b])	1	0.68	12.2 (0.71[b])	1	0.72	0.002 (0.83)	0.68	1
Cashew	4.5 (0.98[b])	1	0.56	1.2 (0.83[b])	0.98	0.34	0.002 (0.82[a])	0.99	0.50
Egg	13.0 (0.67)	1	0.23	9.6 (0.80)	1	0.33	0.012 (0.86[a])	1	0.30
Hazelnut	7.0 (0.79)	1	0.56	14.6 (0.56)	0.73	0.38	0.022 (0.83)	1	0.59
Milk	8.0 (0.91)	1	0.47	20.1 (0.68[a])	0.96	0.19	0.016 (0.80[a])	0.97	0.36
Peanut	9.0 (0.86)	1	0.22	10.7 (0.64[b])	0.95	0.17	0.017 (0.77[b])	0.96	0.35
Pecan	7.0 (0.69[b])	0.95	0.19	1.8 (0.94[b])	1	0.46	0.011 (0.82)	1	0.14
Sesame	11.0 (0.79[b])	1	0.46	7.5 (0.40[b])	0.64	0	0.055 (0.76[b])	1	0.47
Walnut	4.0 (0.96)	1	0.57	13.5 (0.77)	1	0.24	0.019 (0.87)	1	0.33
Wheat	5.5 (0.90[a])	1	0.33	43.1 (0.89[a])	1	0.60	0.027 (0.77[a,b])	0.88	0.67

Based on time-dependent ROC analysis for censored survival data. [a]Could only be estimated at 375 mg. [b]Had better predictive ability at a lower dose, but AUC at 500 mg (375 mg for wheat) is reported. SPT, skin prick test; AUC, area under the curve; sIgE, allergen-specific IgE; sIgEr, specific IgE / total IgE ratio; PPV, positive predictive value; NPV, negative predictive value.

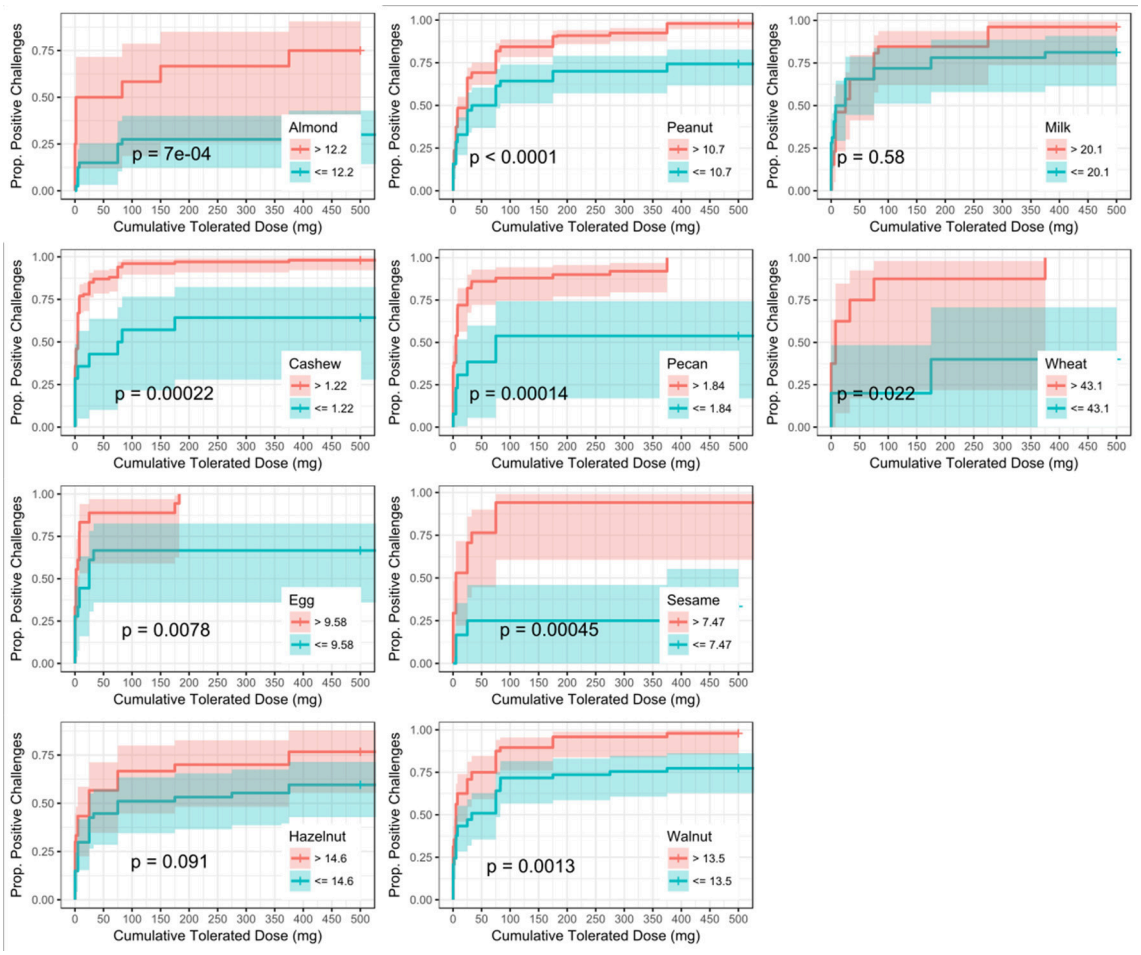

FIGURE 5 | Dose to positive challenge by allergen-specific IgE (sIgE) threshold and food. Kaplan-Meier curves of dose to positive challenge stratified by the CTD-dependent ROC thresholds for sIgE by food. Red lines indicate risk of a positive challenge if sIgE is above the threshold, while blue lines indicate risk for participants with sIgE at or below the threshold.

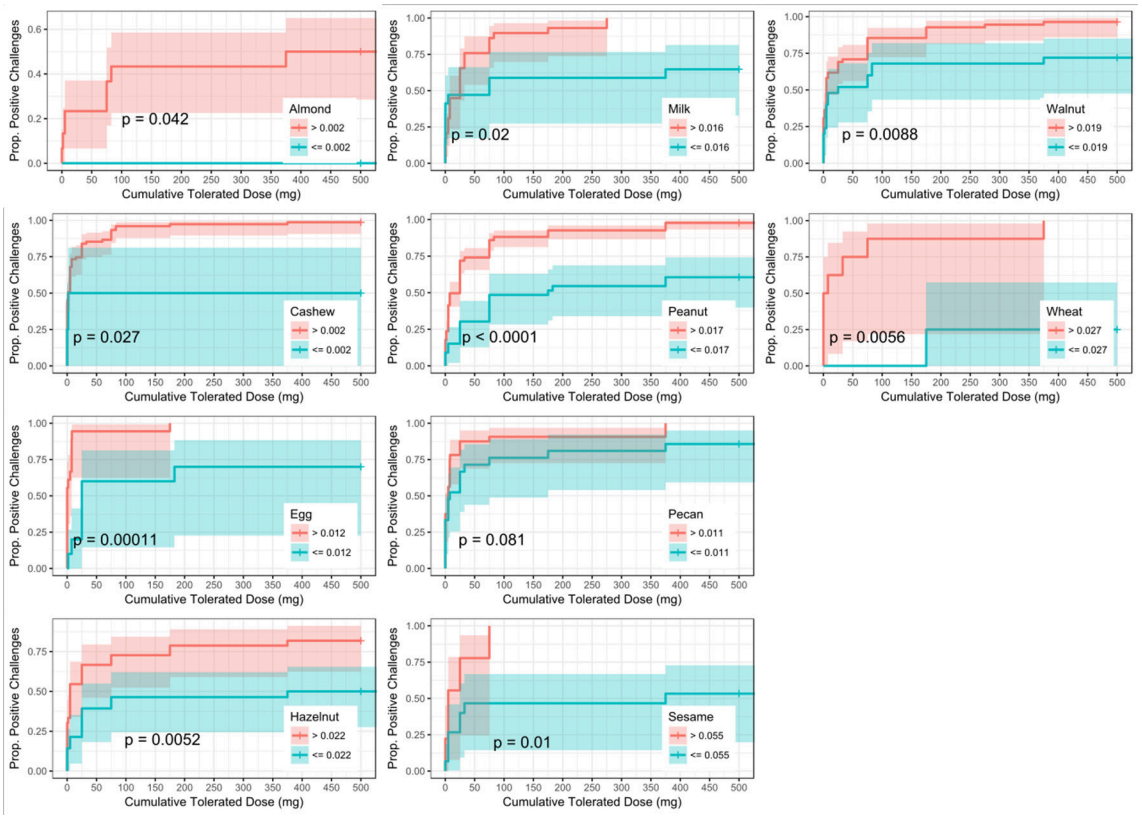

FIGURE 6 | Dose to positive challenge by allergen-specific IgE to Total IgE ratio (sIgEr) threshold and food. Kaplan-Meier curves of dose to positive challenge stratified by the CTD-dependent ROC thresholds for sIgEr by food. Red lines indicate risk of a positive challenge if sIgEr is above the threshold, while blue lines indicate risk for participants with sIgEr at or below the threshold.

setting, in that biomarker values above the threshold were associated with a positive outcome at a lower dose compared to those with biomarker values at or below the threshold. For instance, 50% of participants with an almond sIgE > 12.2 kU/L had a positive challenge by 5 mg CTD, compared to only about 16% of participants with almond sIgE < 12.2 kU/L. Therefore, during an oral challenge, clinicians may incorporate smaller dose increments during the early phase of a challenge for a participant with an sIgE above 12.2 kU/L compared to those below.

The results of our study are strengthened by the large sample size of included food challenges and our novel approach in calculating biomarker thresholds using dose-dependent ROC methodology. To qualify for certain trials the level of SPT and/or sIgE had to meet a certain threshold. Our cohort represents a highly allergic subset with high sIgE and SPT measurements, with values higher than what is typically encountered in the average clinical setting (15) but consistent with the baseline characteristics of patients in the research setting (69–71). sIgE values were capped at 101 kU/L, thus adding additional risk of skewing the sIgE and sIgEr to be falsely low. The thresholds reported in our analysis, though generally consistent with the previously reported thresholds in the literature, are relatively high for SPT, sIgE, and their combination (51); however, given the relatively high AUC levels for the majority of the reported individual and combined threshold values, the thresholds may be a reliable marker to use in clinical trials. In such a setting, the promising AUC levels may provide enough confidence to forego the need for food challenges in confirming allergy and determining study eligibility for a subset of participants. Some limitations of the study include the small sample size for several of the allergens (almond, sesame, and wheat). The results reported here should only be considered as hypothesis-driving and need to be validated in future studies involving larger trials.Our novel approach of utilizing CTD-dependent ROC to develop clinical thresholds was not statistically different than the more commonly used approach of thresholds calculated from logistic ROC; however, CTD-dependent approach allows for risk stratification and for predicting the challenge outcome based on biomarker values. Additionally, having multiple food allergies as well as a history of AD appears to increase the risk of a positive outcome during food challenges. The proposed thresholds may not be applicable for participants with biomarker values falling below the cut-off, and, thus, food challenges may still be unavoidable for such patients. There continues to be a need for newer biomarkers, such as BATs, component result diagnostics, and epigenetic markers, or combinations of these,

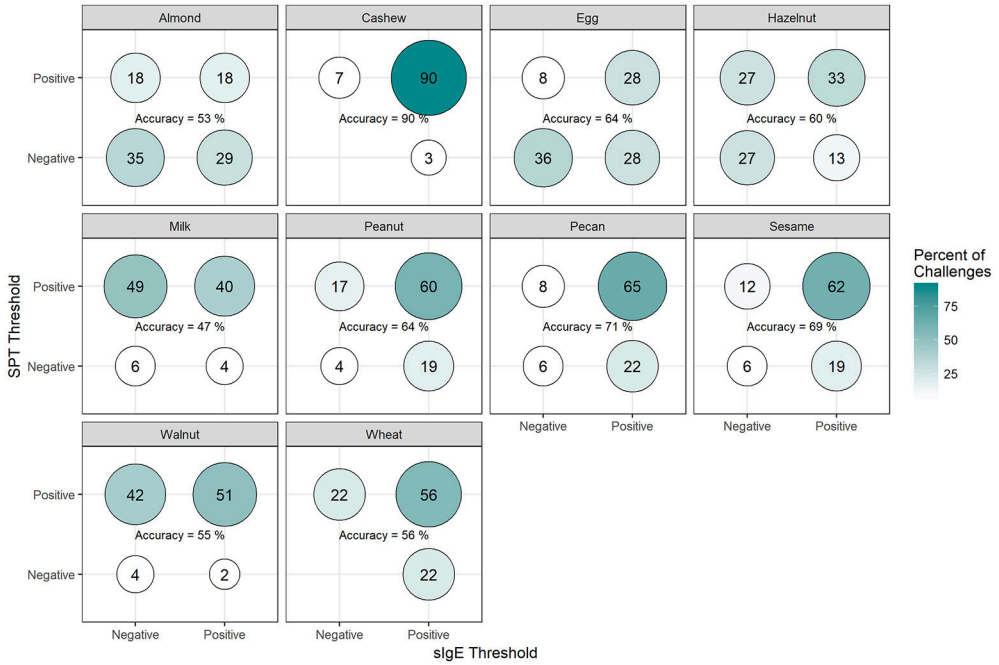

FIGURE 7 | Concordance of skin prick test (SPT) and allergen-specific IgE (sIgE) CTD-dependent thresholds in positive challenges. Among participants with positive challenges, the percentage of participants with each combination of SPT and sIgE values above or below the CTD-dependent ROC thresholds by food. Percentages in each food add up to 100%. Accuracy is the percentage of SPT positive and sIgE positive plus the percentage of SPT negative and sIgE negative.

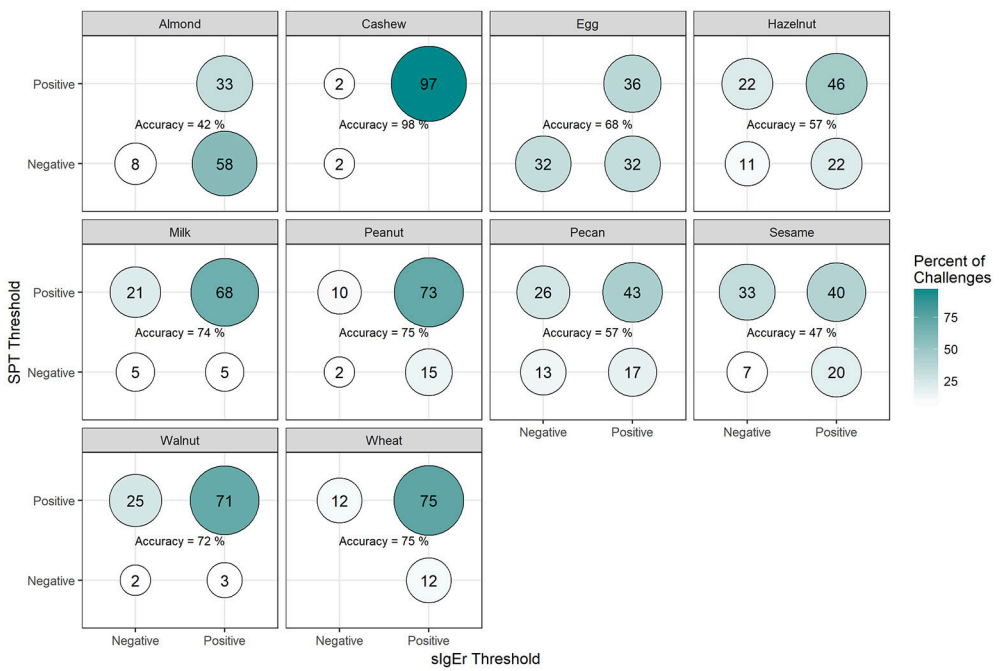

FIGURE 8 | Concordance of skin prick test (SPT) and allergen-specific IgE to Total IgE ratio (sIgEr) CTD-dependent thresholds in positive challenges. Among participants with positive challenges, the percentage of participants with each combination of SPT and sIgEr values above or below the CTD-dependent ROC thresholds by food. Percentages in each food add up to 100%. Accuracy is the percentage of SPT positive and sIgEr positive plus the percentage of SPT negative and sIgEr negative.

which may be predictive tools across all allergens and should be considered in future studies.

CONCLUSION

For the diagnosis of true food allergy, an exact algorithm for determining when an OFC should be performed has yet to be found. Despite remaining the gold standard, food challenges demand significant time and resource requirements and place patients at risk for severe adverse events. As such, dedicated efforts have been made to identify alternative methods of diagnosis. Through our analyses of a large population of standardized food challenges across 11 different foods, we present SPT and sIgE values that are highly predictive of a positive challenge, suggesting food challenges may be unnecessary in the subset of patients with values falling above our reported cut-offs. Additionally our method allows for risk stratification to better predict the dose at which there may be a positive outcome based on biomarker values. While continued efforts will be needed to further refine and identify markers and diagnostic methods outside SPT and sIgE values that are able to fully replace the challenges used today, the ability to potentially forego challenges in the described subset of patients using readily obtainable biomarkers may be an improvement over the current standard of challenges for all patients participating in research.

DATA AVAILABILITY STATEMENT

Data are available on request.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ICH/GCP/CFR guidelines by the Stanford IRB with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Stanford IRB.

AUTHOR CONTRIBUTIONS

Study was designed by SSi, AJL, NP, KN and RSC; study was conducted by SSi, MW, AJL, DK, KN KO, RSC, AA, JP, JS, JT, ST, and JW; data collection and critical review of manuscript was conducted by AA, JP, JS, JT, ST, JW; data collection and analysis was conducted by MC, SS, SSi, NP, SA, DT, KN, and RSC; manuscript was written by SSi, AJL, NP, SJG, KN, and RSC.

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Regulatory Immune Mechanisms in Tolerance to Food Allergy

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Oral tolerance can develop after frequent exposure to food allergens. Upon ingestion, food is digested into small protein fragments in the gastrointestinal tract. Small food particles are later absorbed into the human body. Interestingly, some of these ingested food proteins can cause allergic immune responses, which can lead to food allergy. So far it has not been completely elucidated how these proteins become immunogenic and cause food allergies. In contrast, oral tolerance helps to prevent the pathologic reactions against different types of food antigens from animal or plant origin. Tolerance to food is mainly acquired by dendritic cells, epithelial cells in the gut, and the gut microbiome. A subset of $CD103^+$ DCs is capable of inducing T regulatory cells (Treg cells) that express anti-inflammatory cytokines. Anergic T cells also contribute to oral tolerance, by reducing the number of effector cells. Similar to Treg cells, B regulatory cells (Breg cells) suppress effector T cells and contribute to the immune tolerance to food allergens. Furthermore, the human microbiome is an essential mediator in the induction of oral tolerance or food allergy. In this review, we outline the current understanding of regulatory immune mechanisms in oral tolerance. The biological changes reflecting early consequences of immune stimulation with food allergens should provide useful information for the development of novel therapeutic treatments.

Keywords: dendritic cells, food allergy, food microbiome, oral tolerance, regulatory T and B cells

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INTRODUCTION

Food allergy is defined as an adverse immune response to ingested food proteins. This adverse immune response consists of IgE-mediated immediate hypersensitivity reactions, non-IgE-mediated reactions, and disorders with mixed IgE-mediated and cell-mediated immune reactions (**Figure 1**) (1–4). Food allergy has become an important public health burden in the past few decades, particularly in developed countries (5–7). The prevalence of food allergies is now estimated at 5–10% of the population in developed countries (8). The prevalence of food-challenge-defined allergies for common food allergens was reported to be: 0.6% to cow's milk, 0.2% to egg, 0.1% to wheat, 0.3% to soy, 0.2% to peanut, 0.5% to tree nuts, 0.1% to fish, and 0.1% to shellfish (9). Besides, the World Allergy Organization provided an extensive study using different approaches of food allergy such as oral food challenge, history of the clinical reaction of food-specific IgE, and food allergy questionnaires in 89 member countries. This study revealed that children from Australia, Finland, and Canada at the age of 5 or lower, have the highest prevalence of food allergy (10). The patterns of food allergy were consistent in many regions showing egg and milk were among the most common allergens in preschool children. In other developed countries, the estimation of overall food allergy prevalence has also increased drastically in the past decades for uncertain

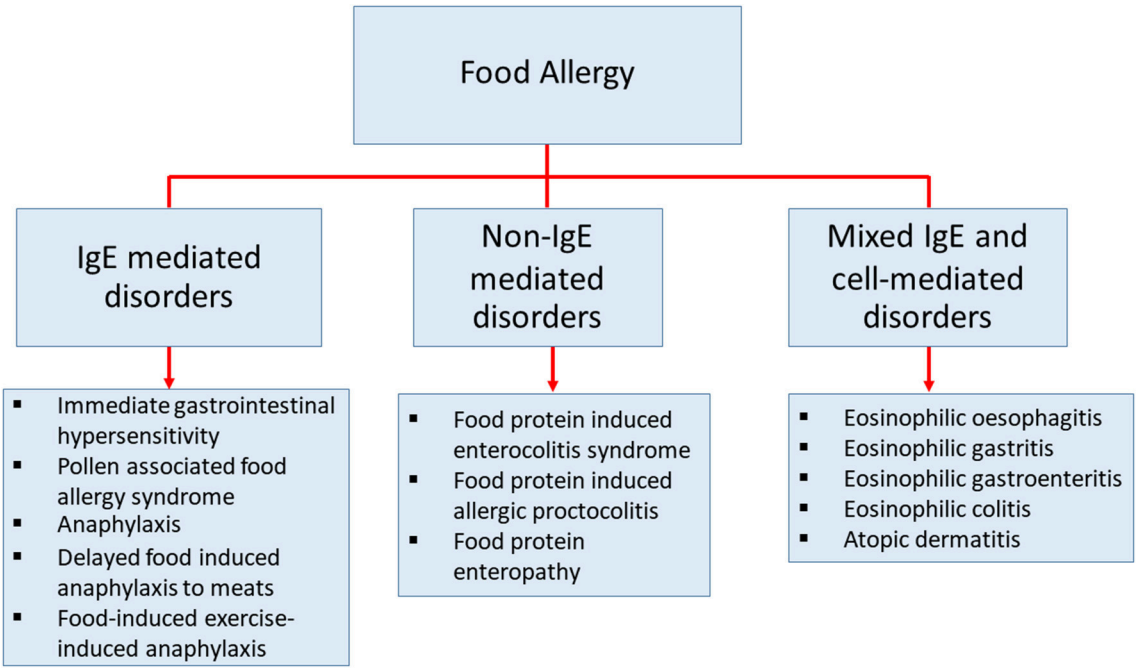

FIGURE 1 | The classification of diseases that cause by food allergy. Classification of food-related diseases by three different immune responses; IgE-mediated immediate disorders, non-IgE-mediated disorders, and disorders with the contributions from mixed IgE-mediated and cell-mediated immune pathways.

reasons (9, 11–14). Therefore, to be able to develop more precise diagnostic approaches, prevention, and medical treatments, a better understanding of the mechanisms in food allergy is necessary (15).

INDUCTION OF ALLERGIC IMMUNE RESPONSES

To know the mechanisms of food allergy, we must understand the role of food allergens in the induction of allergic immune response (16). Food allergens are derived from common naturally-occurring food proteins of plant- and animal-origin (17, 18). The proteins in the food are initially broken down by hydrolytic enzymes in the gastrointestinal track during the digestive process. It is hypothesized that food allergens can be modified into different forms and different structures, which can be processed by antigen presenting cells, exhibited on the major histocompatibility complex class II molecules, and subsequently recognized by antigen-specific T cells (19). The naïve antigen-specific T helper (Th) cells differentiate into effector Th2 cells in the presence of interleukin 4. A set of interleukins such as IL-4, IL-5, IL-10, and IL-13 are massively produced by Th2 cells and induce B cells to differentiate into IgE-producing plasma cells. Antigen-specific IgE antibodies directly bind to high-affinity receptor FcεRI on mast cells and basophils. During the antigen re-exposure, these specific IgE antibodies induce degranulation of mast cells and release of mediators including cytokines, histamine, and proteases which result in allergic symptoms. There are several factors that influence the allergic responses. For example, boiling or frying peanuts can alter the structure of allergens and reduce their allergenicity. The term "allergenicity" is the ability of allergens to initiate a clinical response through an IgE-mediated mechanism (20). The relative amount of major peanut allergen, Ara h 1 is significantly reduced in fried and boiled food preparations, which results in a drastically decreased IgE-binding intensity. Although Ara h 2 and Ara h 3 showed similar relative amounts in fried, boiled, and roasted peanuts, both allergens had lower IgE-binding capacity in boiled and fried peanuts than in roasted peanuts (21). Similarly heating cow's milk and hen's egg, tends to lower allergenicity (22–24).Cow's milk proteins are considered to be the most common food allergens in IgE- and non-IgE-mediated food allergic disorders in children (25). Most individuals with cow's milk allergy are sensitized to caseins and the whey proteins β-lactoglobulin and α-lactalbumin. Caseins are more resistant to high temperatures compared to whey proteins. Cow's milk allergic children who have a high level of casein IgE are less likely to tolerate a baked milk diet compared to those who have a lower level of casein IgE. Specific IgE-binding patterns to casein and β-lactoglobulin peptides could predict the original cause of cow's milk allergy and differentiate subjects between the ones who are more likely to outgrow cow's milk allergy at a younger age vs. and the ones who are more likely to develop allergic symptom at a younger age vs. those with a

Abbreviations: Breg, Regulatory B cell; DC, Dendritic cell; GAP, Goblet-cell-associated antigen passages; LAP, Latency-activated peptide; OIT, Oral immunotherapy; OVA, Ovalbumin; SCFA, Short chain fatty acids; Treg, Regulatory T cell.

more persistent cow's milk allergy (26–28). Besides milk proteins, the two major egg allergens, ovalbumin (OVA), and ovomucoid (Gal d1) are major causes of allergy in children. Bloom et al. showed that Gal d1 has higher heat stability than OVA. OVA showed signs of aggregation after 25 min of heating while Gal d1 stayed stable up to 60 min. However, both egg allergens showed strongly reduced IgE-binding capacity after thermal processing. Additionally the presence of wheat during the heating process also affects the allergenicity and reduces the IgE binding further (28). In contrast, the effect of heating shellfish results in increased IgE reactivity to crustacean allergens. A recent study confirmed that a higher level of IgE reactivity was observed in cooked crustacean extracts compared to raw extracts (29).

To date, there is no clear answer to the question of "what makes a dietary or digestible protein an allergen?" Besides, the mechanisms of food allergy development have not been comprehensively described. The allergic reactions to food are expected to be enhanced by the imbalance of immune suppression (30, 31). As a result of the lack of immune suppression, the induction of immune tolerance to food is not achieved in allergic individuals.

ORAL TOLERANCE

Oral tolerance is the physiological response to ingested antigens. The development of oral tolerance takes place in the gastrointestinal tract. The gut-associated lymphoid tissues play a major role in limiting inflammatory responses to resident bacteria and food proteins (32). To maintain oral tolerance, the gut-associated lymphoid tissues continue to discriminate self from non-self-antigens and recognize the pathogens that can cause tissue inflammation or gut disease. A breakdown in this process occurs when the gut-associated lymphoid tissues fail to perform its functions. Continuously breaking down of oral tolerance leads to the loss of oral tolerance and unwanted allergic responses. (33).There is an enormous unmet need for modern therapeutic treatments to treat patients with food allergy. Therefore, studies dissecting the mechanisms of oral tolerance are very important (34). The possible mechanisms of oral tolerance may involve recognition of food antigens by dendritic cells, robust induction of Treg cells as well as Breg cells. Also, the environment in human gut reinforces and enhances the expansion of the presence of bacteria-derived metabolites and biogenic amines, such as short-chain fatty acids and histamine (35–38).

Dendritic Cells

Sensitization to food allergens starts with the uptake of antigens in the gut. This occurs through special types of epithelial cells: M cells (39, 40) and more importantly specialized Goblet-cells, called goblet-cell-associated antigen passages (GAPs) (41). Antigens, taken up by M cells or GAPs, can then be transferred to dendritic cells (DCs). In the small intestine, there are two major populations of DCs: $CD103^+CX3CR1^-$ DCs and CX3CR1+ DCs. $CX3CR1^+$ DCs are able to directly uptake antigens from the lumen and have more inflammatory potential (42–44). $CD103^+CX3CR1^-$ DCs on the other hand have tolerogenic properties. GAPs exclusively deliver antigens to $CD103^+CX3CR1^-$ DCs and thus are related to the induction of oral tolerance (41).

Besides GAPs, CXCR1+ macrophages can also present antigens from the lumen to CD103+ DCs and induce tolerogenic effects and IL-10 production. (45, 46). After contact with the antigen CD103+CX3CR- DCs can migrate to mesenteric lympnodes in a CCR7 dependent manner (47). In the lympnode $CD103^+CX3CR1^-$ DCs promote the development of T regulatory cells (Treg cells) through the production of TGF-β and RALDH2, an enzyme that converts retinol into retinoic acid (48).

T Regulatory Cells

T cells play an essential role in food allergies. Th2 cells drive the allergic response by producing IL-4, IL-5, and IL-13. However, other types of T cells play a role in developing tolerance: Treg cells. There are different types of Treg cells, some are thymus-derived and are called natural Treg cells (nTreg cells), and some are induced in the periphery and are called iTreg cells. It was shown by Mucida et al. that oral tolerance can be induced in the absence of thymic-derived Treg cells in a mouse model (49). Besides, Lafaille et al. showed that iTreg cells are essential for establishing oral tolerance (50). The best-described tolerogenic T cells are $FOXP3^+$ Treg cells, which are characterized by the expression of CD25 and the transcription factor FOXP3. Treg cells regulate immune responses to allergens through several mechanisms (51, 52). Treg cells can produce different types of inhibitory cytokines, such as IL-10 and TGF-β. Furthermore, they can inhibit antigen-presenting cells by the inhibitory co-stimulators programmed cell dead protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA4). Additionally, Treg cells can prevent the proliferation of effector T cells through CD25, a high-affinity receptor of IL-2, by depriving the effector cells of IL-2 (53). Lastly, Treg cells can produce granzyme A and B, which can cause cytolysis of effector cells (54, 55).

Treg cells play a key role in induction and maintenance of tolerance (53, 56). It was shown that FOXP3 knockout mice developed multi-organ inflammatory responses associated with allergic inflammation (57, 58). Adoptive transfer of Treg cells was able to suppress anaphylaxis in a food allergy model in mice and was able to control the Th2 immune response (59, 60). It was shown that children with IgE mediated food allergy have significantly lower FOXP3 expression compared to healthy controls (61, 62) and children with peanut or egg allergy showed a decrease in Treg cell percentage after allergen exposure (63–65). It was also revealed that children with egg allergy have reduced neonatal regulatory T cell function (66). At last, oral immunotherapy, the only known therapy for food allergies, increases Treg cell function, hypomethylation of FOXP3 gene (67) and the number of FOXP3 positive cells (68). In addition to immunotherapy, low dose IL-2 treatment is also able to induce Treg cells and prevent immune responses (69). A combination of the two treatments has been performed by Smaldini et al. and was effective in reversing IgE-mediated food allergy (70).

Besides conventional $FOXP3^+$ Treg cells, another type of T-cell that plays a role in oral tolerance is a Th3 cell. Th3 cells

do not express FOXP3 or CD25 but express latency-activated peptide (LAP) on their surface, and they are able to produce the inhibitory cytokine TGF-β. It was shown in mice that epicutaneous immunotherapy with OVA-induced Th3 cells that protected against anaphylaxis by suppressing mast cell activation through TGF-β production (71). In an OIT model of cow's milk allergy, fructo-oligosaccharides induced Th3 cells that coincided with protection against anaphylaxis (72). Moreover, Th3 cells were found capable of promoting the development of iTreg cells (73).

TGF-β is not only produced by Th3 cells, but also by conventional FOXP3+ Treg that express LAP and the surface molecule GARP (74). Treg cell expression of GARP is essential for optimal induction of oral tolerance (75). IL-6, IL-21, and IL-35 can inhibit the expression of GARP on FOXP3+ Treg- cells and thereby inhibit LAP and TGF-β. Blocking the IL-6 pathway can enhance oral tolerance in mice (76). Excessive levels of IL-4 also inhibited the induction of allergen-specific Treg cells and caused intestinal inflammation in a mouse model (77).

As mentioned earlier CD103+ DCs produce retinoic acid that is able to amplify TGF-β production and induce FOXP3+ Treg cells (48). Additionally, retinoic acid produced by DCs induces the expression of receptors α4β7 and CCR9 on T cells. These receptors are responsible for T cell homing to the gut (78). It was demonstrated by Hadis et al. that gut-homing and expansion of Treg cells in the lamina propria is necessary to achieve oral tolerance (46). Furthermore, it was shown that homing of IL-10 producing Tregs is important for oral tolerance (79, 80).

Besides the functioning of Treg cells, T cell depletion and anergy can also contribute to oral tolerance. During high dose oral allergen desensitization in cow's milk allergy, a reduction in antigen-specific T cells was observed (81). Additionally, after it was found that during peanut immunotherapy allergen-specific CD4+ T cells can shift toward an anergic Th2 phenotype (82).

B Regulatory Cells

B cells that can differentiate into antibody-secreting plasma cells are commonly considered to administer immune responses by producing antigen-specific antibodies and help to induce optimal $CD4^+$ T cell activation (83). Immunosuppressive Breg cells play a role in the regulation of immune responses by suppressing effector T cells through the production of suppressor cytokines (IL-10, TGF-β, and IL-35) (84). However, the role of Breg cells in oral tolerance remains unclear.

Breg cells moderate immune responses in infection, allergic inflammation, and tolerance, predominantly via IL-10 (85). Van de Veen et al. found that inducible IL-10 secreting B regulatory (Br1) cells contribute to peripheral allergen tolerance in beekeepers. The increment of bee venom allergen-specific, IL-10-producing B cells, has been observed in bee venom allergic patients receiving AIT. Also, Br1 cells produce IgG4 when they switch to plasma cells, which is a non-inflammatory immunoglobulin isotype that prevents IgE-mediated mast cell and basophil degranulation (86). Allergen-specific IgG4 may be involved in promoting immune tolerance in OIT. Santos et al. demonstrated that the ratio of peanut-specific IgG4 to peanut-specific IgE was higher in tolerant patients compared to peanut-allergic patients (87). The essential mechanism of tolerance induction via IL-10-producing B cells was examined with IL-10-overexpressing B cells. IL-10-overexpressing B cells were found to suppress the maturation of dendritic cells, T effector cell proliferation, and IgE production. In addition, IL-10-overexpressing B cells produced the anti-inflammatory IL-1 receptor antagonist and vascular endothelial growth factor and had lower production of pro-inflammatory cytokines (88). Human type 3 innate lymphoid cells (ILC3s) support the maintenance of mucosal tolerance. A recent study showed that human $CD40L^+$ ILC3s provide innate B-cell help and are affected in an innate immunoregulatory mechanism through induction of immature translational Breg cell differentiation, which takes place in palatine tonsils *in vivo* (89).

IL-10-producing $CD5^+$ Breg cells in mesenteric lymph nodes play a role in the regulation of IgE-mediated anaphylaxis following challenge with cow's milk allergens in a murine model (90). Peanut-specific B cells were increased in the blood after oral immunotherapy in peanut allergic patients (91, 92). A recent study showed IL-10 producing B cells are able to induce and maintain Treg cells in rheumatoid arthritis disease (93). The down-regulation of IL-4, and upregulation of IL-10 production result in an increase of IgG4 and a decrease of IgE levels. IL-10 is known to promote heavy chain immunoglobulin isotype switching to IgG4 while IL-4 induces switching to IgE (94). Furthermore, mucosal IgA inhibits uptake of an antigen by the epithelium and may protect against food allergy (95). The proposed mechanisms of food tolerance are shown in **Figure 2**.

Gut Microbiome

The gut microbiome is being increasingly recognized as a major factor in mediating health and disease (96, 97). There are several studies describing the interrelation between microbiota of the gastrointestinal tract, respiratory tract, and skin allergic disorders (98–102). Additionally, it has been shown that the microbiome is associated with oral tolerance (103–105). The human microbiome is capable of inducing Treg cells that suppress Th2-derived responses.

Certain bacterial strains such as *Bifidobacterium longum 35624*, *Clostridia,* and *Bacteroides fragilis* can induce intestinal Treg cells that are able to suppress food allergy and colitis (106, 107). Pattern-recognition receptor activation on DCs is a potential mechanism by which intestinal microbes (Lactobacillus rhamnosus JB-1) may promote Treg cell differentiation (108). A study from the National Institutes of Health, Human Microbiome Project revealed 14 important bacterial strains by sequencing and analytical processing 380 whole-genome shotgun samples (109). In addition, a 16S rDNA gene was sequenced to characterize the oral bacterial composition in saliva samples from healthy and allergic children up to 7 years of age. The result affirmed that early changes in oral microbial composition seem to influence immune maturation and allergy development (110, 111).

The potential role of the gut microbiome in food allergy has been studied in mouse models. Rodriguez et al. demonstrated that intestinal colonization of *Staphylococcus* protects against oral sensitization and allergic response in a mouse model.

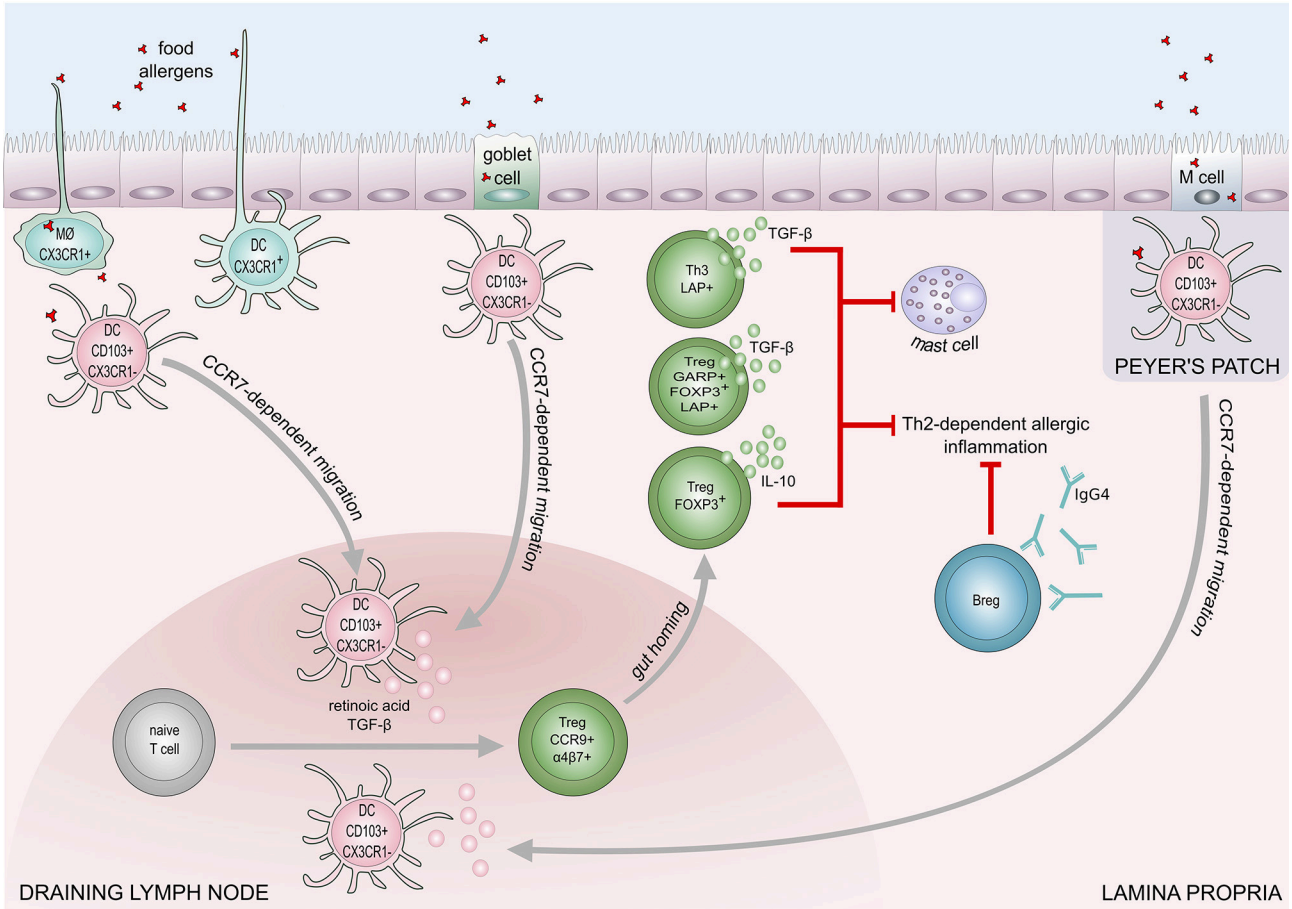

FIGURE 2 | Mechanisms of immune tolerance to food allergens. Induction of food tolerance takes place in the gut when the immune cells encounter food antigens. Several cell types are involved in the antigen uptake: goblet cells, microfold (M) cells, intestinal epithelial cells, CX3CR1$^+$ macrophages (MØ), and CX3CR1+dendritic cells. CX3CR1$^+$ DCs and CX3CR1$^+$ MØ, are capable of extending dendrites to capture antigens on the apical layer of epithelium in the gut lumen. Antigens taken up by CX3CR1$^+$ MØ and goblet cells are transferred to CD103$^+$CX3CR1$^-$ DCs, which subsequently migrate to draining lymph node in a CCR7 dependent manner. Production of retinoic acid and TGF-β foster differentiation of naïve T cells into regulatory T cells (Tregs). Retinoid acid-dependent induction of integrin α4β7 expression on Tregs is responsible for T cell homing to lamina propria. Tregs (Foxp3$^+$), and Th3 cells inhibit Th2-dependent allergic inflammation and mast cell degranulation, through the production of IL-10 and TGF-β. Suppression of Th2-responses also engages regulatory B cells (Bregs) that contribute to food tolerance by producing IgG4.

This was the first study to describe a relationship between alterations within the subdominant microbiota and severity of food allergy (112). Another study showed that allergen-sensitized (Il4ra^{F709}) mice had a different microbial composition compared to wildtype mice with an increased abundance of different bacterial families including Lachnospiraceae, Lactobacillaceae, Rikenellaceae, and Porphyromonadaceae. This different microbial composition increased OVA-specific responses and anaphylaxis when reconstituted in wild-type germ-free mice, which indicates that the microbial composition play a role in food allergy (113).

Rivas et al. demonstrated that disease-susceptible (Il4ra^{F709}) mice with an enhanced interleukin-4 receptor (IL-4R) signaling exhibited STAT6-dependent impaired generation and function of mucosal allergen-specific Treg cells. Their study showed that the Treg cells failed to suppress mast cell activation and expansion (114). Those Treg cells were reprogrammed into Th2-like cells and participated in the development of food allergy (115). Another study determined that microbiota regulates Th2 responses through the induction of RORγt Treg cells and Th17 cells (116).

Moreover, the bacterial metabolites, such as short-chain fatty acids (SCFA's) and biogenic amines produced in the human gut play a role in host immune regulatory activity (117, 118). SCFA'sare able to enhance dendritic cell regulatory activity, leading to the induction of Treg cells and IL-10-secreting T cells (119). SCFA's can be produced by bacteria after digestion of dietary fibers. It was shown that infants with a diet consisting of high levels of fruits and vegetables is associated with less food allergy by the age of 2 years, which could be due to an increased amount of dietary fiber intake (120). It was shown in mice that deficiency of dietary fiber intake increases the susceptibility to

OVA induced allergic airway inflammation (121). Additionally, it was shown that uptake of polyunsaturated fatty acids can increase the production of SCFA's by bacteria (122) and the dietary intake of poly unsaturated fatty acids was inversely associated with atopy in childhood (123).

The secretion of biogenic amines such as histamine has extensive effects on many immune cell types (124, 125). Histamine levels are increased in patients with irritable bowel syndrome, inflammatory bowel disease and in adult asthma patients (126, 127).

Food allergy could be related to changes in microbial exposure in early life, which affect host microbiota composition, modifies the development of host immunity, and causes pathogenic immune responses to food allergens (96). How the microbiome exactly affects food allergy should be further investigated.

CONCLUSION

Loss of oral tolerance can lead to the development of food allergy in children and adults. However, the development of food allergy in terms of molecular and cellular mechanisms has not yet been demonstrated. The induction or loss of oral tolerance is likely modulated by the combination of DCs, Treg cells, Breg cells, and microbiome. DCs are capable of inducing Treg cells, which produce anti-inflammatory cytokines and are able to suppress T effector cells. Additionally, Breg cells can produce anti-inflammatory cytokines as well and can produce IgG4, which is the anti-inflammatory Ig isotype. So far food allergies are mainly managed by strict avoidance of the food allergens and can only be treated with immunotherapy. How immunotherapy exactly works is not entirely understood. Therefore, the underlying mechanisms of induction and loss of oral tolerance need to be more clearly identified so novel therapeutic treatments can be developed.

AUTHOR CONTRIBUTIONS

PS, KJ, AG, WvdV, and MA prepared the manuscript.

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Germ-Free Mice Exhibit Mast Cells With Impaired Functionality and Gut Homing and Do Not Develop Food Allergy

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Background: Mucosal mast cells (MC) are key players in IgE-mediated food allergy (FA). The evidence on the interaction between gut microbiota, MC and susceptibility to FA is contradictory.

Objective: We tested the hypothesis that commensal bacteria are essential for MC migration to the gut and their maturation impacting the susceptibility to FA.

Methods: The development and severity of FA symptoms was studied in sensitized germ-free (GF), conventional (CV), and mice mono-colonized with *L. plantarum* WCFS1 or co-housed with CV mice. MC were phenotypically and functionally characterized.

Results: Systemic sensitization and oral challenge of GF mice with ovalbumin led to increased levels of specific IgE in serum compared to CV mice. Remarkably, despite the high levels of sensitization, GF mice did not develop diarrhea or anaphylactic hypothermia, common symptoms of FA. In the gut, GF mice expressed low levels of the MC tissue-homing markers CXCL1 and CXCL2, and harbored fewer MC which exhibited lower levels of MC protease-1 after challenge. Additionally, MC in GF mice were less mature as confirmed by flow-cytometry and their functionality was impaired as shown by reduced edema formation after injection of degranulation-provoking compound 48/80. Co-housing of GF mice with CV mice fully restored their susceptibility to develop FA. However, this did not occur when mice were mono-colonized with *L. plantarum*.

Conclusion: Our results demonstrate that microbiota-induced maturation and gut-homing of MC is a critical step for the development of symptoms of experimental FA. This new mechanistic insight into microbiota-MC-FA axis can be exploited in the prevention and treatment of FA in humans.

Keywords: germ-free, mouse models, food allergy, mast cells, commensal bacteria

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INTRODUCTION

Food allergy (FA) is a widespread pathological immune reaction which is initiated by generally harmless food antigens. Its global prevalence has been increasing since the 1960s, especially in industrialized countries, suggesting environmental factors play a key role in the susceptibility and etiology of this disorder (1). IgE-mediated FA, which is the most common form of FA, is based on two phases: (i) allergic sensitization and (ii) the effector phase (1, 2). The humoral and cellular immune responses are clearly biased toward a type 2-related phenotype, characterized by the production of specific IgE antibodies and cytokines, such as IL-4, IL-5, IL-13, or IL-10 (3). In the effector phase, allergen-induced crosslinking of IgE bound to mast cells (MC) leads to release of histamine, serotonin and MC proteases as well as cytokines (e.g., TNF-α), resulting in the rapid appearance of symptoms, such as diarrhea and hypothermia (4). Even in individuals with high levels of food allergen-specific serum IgE, the susceptibility to develope these clinical symptoms differs dramatically (5, 6). The mechanisms underlying this phenomenon remain unknown.

The hygiene hypothesis suggests that changes in the commensal microbiota composition and/or function, due to excessive antibiotic use or increased hygiene, can increase the level of allergic sensitization (7, 8). By using germ-free (GF) animals, several studies have shown that the lack of bacteria leads to increased levels of serum IgE in comparison to colonized conventional (CV) mice (9, 10). Interestingly, colonization of GF mice with a single bacterial strain, with a mixture of several strains, or with the microbiota of CV mice through co-housing prevented the development of allergic sensitization and led to decrease of allergen-specific IgE (10–12).

Lactobacillus plantarum is an extremely versatile lactic acid bacterium that has been isolated from a variety of habitats, such as plants, the gastro-intestinal tracts of human and animals as well as raw or fermented dairy products (13). The human isolate *L. plantarum* WCFS1 possesses strong immunomodulatory properties, and has been shown to induce maturation of immune cells *in vitro* (14, 15) and interact with the host immune system *in vivo* (16). Specifically, oral application of *L. plantarum* WCFS1 enhanced activation of intestinal cells and shifted the Th1/Th2 balance toward a Th2 response (17). In a mouse model of peanut allergy, oral supplementation of this strain aggravated the allergic responses associated with increased MC degranulation (14).

MC are innate immune cells which are involved both in the immunological homeostasis as well as in parasitic infection (18–20) and various immunological disorders (21, 22). MC originate from CD34+ progenitors in the bone marrow and then enter the circulation and peripheral tissues, where they undergo maturation (23, 24). Being at the mucosal sites, MC are in close contact with the microbiota. Indeed, commensal bacteria have been shown to modulate several phenotypic and functional characteristics of MC, including their recruitment to the tissue, maturation and survival (23, 25). Along these lines, Kunii et al. have shown that the microbiota is required for the migration of MC to the intestine through the induction of CXCR2 ligands (23). Similarly, in the skin, the microbiota is crucial for recruitment and maturation of dermal MC (25).

Although only low numbers of MC are found in the intestine of naïve mice (26), their numbers increase in food allergy (27). The crucial role of MC in FA has been well-established (27, 28). After MC depletion with anti-c-kit antibody, CV mice do not develop OVA-induced gastrointestinal manifestation (27) and MC are also essential for the full development of hypothermia in the OVA FA mouse model (29). Additionally, transgenic mice with increased numbers of intestinal MC exhibit augmented severity of FA symptoms (30).

The literature on the interaction between microbiota, MC and susceptibility to FA is contradictory. On one hand, it has been demonstrated that GF mice exhibit altered functionality of MC and their impaired migration into the intestinal and skin tissue (23, 25). On the other hand; different studies have shown that GF mice are more susceptible to develop clinical symptoms of FA (10, 31).

In this study we seek to determine the role of commensal bacteria in the induction of FA using GF mice. We observed that GF mice did not develop the clinical symptoms of FA, such as allergic diarrhea and hypothermia, despite having higher titers of allergen-specific Th2-associated antibodies. Furthermore, the lack of commensals resulted in reduced numbers of tissue MC with low maturation status. Importantly, conventionalization of GF mice with complex microbiota through co-housing with CV mice, but not mono-colonization with *L. plantarum* WCFS1, fully recapitulated the FA phenotype observed in the CV mice. These results implicate that signals from complex microbiota are necessary for the homing of MC into the intestinal tissue as well as their maturation, which are prerequisites for developing the clinical symptoms of FA.

METHODS

Animals

Germ-free (GF) BALB/c mice were derived from the conventional BALB/c mice by Cesarean section and kept under axenic conditions in Trexler-type plastic isolators for at least 5 generations. The sterility was controlled as previously described (32). Briefly, sterility was assessed every 2-weeks by confirming the absence of bacteria, molds, and yeast by aerobic and anaerobic cultivation of mouse feces and swabs from the isolators in VL (Viande-Levure), Sabouraud-dextrose and meat-peptone broth and subsequent plating, and aerobic/anaerobic cultivation on blood, Sabouraud and VL agar plates. Conventional (CV) *Helicobacter*-free mice were housed in individually ventilated cages (Tecniplast S.P.A., IT). Experimental animals were obtained by mating of female and male BALB/c mice and their female offspring were weaned at day 28 postnatally. Female offspring were co-housed together until day 60, when they were assigned either to the CV/OVA or CV/Ctrl group, which were housed separately thereafter.

Abbreviations: CV, Conventional; ExGF, GF mice co-housed with conventional mice; FA, Food allergy; GF, Germ-free; IL, Interleukin; LPS, Lipopolysaccharide; MC, Mast cells; MCPT-1, Mast cell protease-1; OVA, Ovalbumin; PCR, Polymerase chain reaction; SCF, Stem cell factor; SPF, Specific pathogen free.

Ex-germ-free (exGF) mice were obtained by co-housing of 28-day old GF mice with age- and gender-matched CV mice. GF mice were mono-associated with *Lactobacillus plantarum* WCFS1 (Lp) and the level of bacterial colonization was evaluated weekly by plating serial dilution of feces on de Man, Rogosa, and Sharpe (MRS, Oxoid, UK) agar plates as described previously (33). Colonization remained stable throughout the experiment and reached levels of 2–3 $\times$ 10^9 CFU/g feces. Ceca from control CV, GF, exGF, and Lp mice were weighed and a picture was taken. Cecum content was frozen for PCR analysis. Animals were kept in a room with 12 h light-dark cycle at 22°C, fed by OVA-free diet Altromin 1410 sterilized by irradiation and water *ad libitum*. Water was sterilized by autoclaving for GF and Lp-colonized mice. This study was carried out in accordance with the recommendations of the Committee for the Protection and Use of Experimental Animals of the Institute of Microbiology Academy of Sciences of the Czech Republic. All protocols were approved by the same committee.

Experimental Protocol

Female 8-week-old CV, GF, exGF and Lp mice were sensitized *i.p.* within two-week interval with 60 μg of OVA (Worthington, USA) together with 100 μl Alu-Gel-S (Serva, DE) adjuvant and PBS in a final volume of 200 μl on day 1 and 14. Control mice received 100 μl of PBS mixed with 100 μl of Alu-Gel-S. Two weeks after the second *i.p.* sensitization, mice were challenged 8 times at 2–3 day intervals (days 28–44) by *i.g.* gavages of 15 mg OVA in a final volume of 150 μl (OVA groups). Control mice received 150 μl of PBS by *i.g.* gavages (ctrl groups). Diarrhea occurrence was monitored for 30–60 min after each *i.g.* exposure. The diarrhea score was assessed according to the following criteria: 0–normal, well-formed stool, 1–soft, sticky well-formed stool, 2–not formed stool, 3–liquid diarrhea, 4–more than two episodes of liquid diarrhea after the antigen gavage during the treatment period. The temperature was measured by Thermocouple Thermometer with mouse rectal probe (World Precision Instruments Inc., USA) 30 min after the last *i.g.* exposure (**Figure 1A**).

Isolation of Bacterial DNA From Cecal Content and 16S rDNA PCR Amplification

Total DNA from 150 mg cecal content was isolated by ZR Fecal DNA kit according to manufacturer's instructions (Zymo Research, USA). The purity, integrity and concentration of nucleic acids were confirmed by agarose gel electrophoresis and UV spectrophotometry as previously described (34). Bacterial 16S rDNA was amplified using PCR with the universal primers 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) as previously described (35). Ten ng of chromosomal DNA from *Escherichia coli* was used as a positive control. Amplification products were separated by electrophoresis in 1.2% agarose gel, visualized using GelRed™ Nucleic Acid Gel Stain (Biotinum, USA) and images were obtained by Fluorescent Image Analyser FLA-7000 (Fujifilm Corporation, JP).

Quantification of OVA-Specific Antibodies

Blood samples were collected at sacrifice and serum was collected after centrifugation. OVA-specific serum IgE, IgG1, IgG2a, and IgA levels were determined by ELISA (36). Briefly, 96-well-microtiter plates were coated with OVA (5 μg/ml). Serum samples were diluted 1/10 for IgE, 1/10,000 for IgG1, 1/100 for IgG2, and 1/10 for IgA. Rat anti-mouse IgE, IgG1, IgG2a, and IgA antibodies (1 μg/ml, Pharmingen, San Diego, CA, USA) were applied, followed by peroxidase-conjugated anti-rat IgG antibodies (1/1,000, Jackson, Immuno Labs, West Grove, PA, USA) for detection. Antibody levels were reported as optical density (OD). The activity of OVA-specific IgE in serum was measured by rat basophil leukemia (RBL) cells degranulation assay as described previously (36).

Cellular Immune Response

At sacrifice, spleens were aseptically removed and single-cell suspensions were prepared in RPMI-1640 containing 10% fetal bovine serum (BioClot GmbH, Aidenbach, Germany) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich). Cells (6×10^5/well) were cultured in a flat-bottom 96-well plate (TPP, Trasadingen, Switzerland) without any stimuli or in the presence of OVA (100 μg/well) for 72 h (37°C, 5% CO_2). Supernatants were collected and stored at −40°C until analyses. Levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ were determined by the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, USA). Values are reported in pg/ml after subtraction of baseline levels of non-stimulated cell cultures.

ELISA for Mast Cell Protease-1 and Cytokines in Jejunal Homogenates

Jejunum was aseptically removed and homogenate was prepared as followed. Protease inhibitor (Roche, DE) supplemented with 0.5% Triton X (Sigma-Aldrich, USA) was added to jejunum samples in the ratio 9:1 (w/w). After cooling on ice, the jejunum was homogenized for 1 min/40 Hz using Tissue Lyzer and stainless steel beads 7 mm (Qiagen, DE), frozen in liquid nitrogen, thawed, and homogenized again. Supernatants were collected after centrifugation and stored at −80°C. Protein content of the homogenates was determined by the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA) using albumin as a standard. Levels of mouse mast cell protease-1 (MCPT-1) in serum and jejunal homogenates was determined by commercial kit Ready-SET-Go!® (eBioscience, USA) according to manufacturer's instructions. Levels of IL-4, IL-13, and TNF-α in jejunal homogenates were measured by the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, USA). MCPT-1 and cytokine levels in jejunal homogenates are represented per 1 mg of total protein.

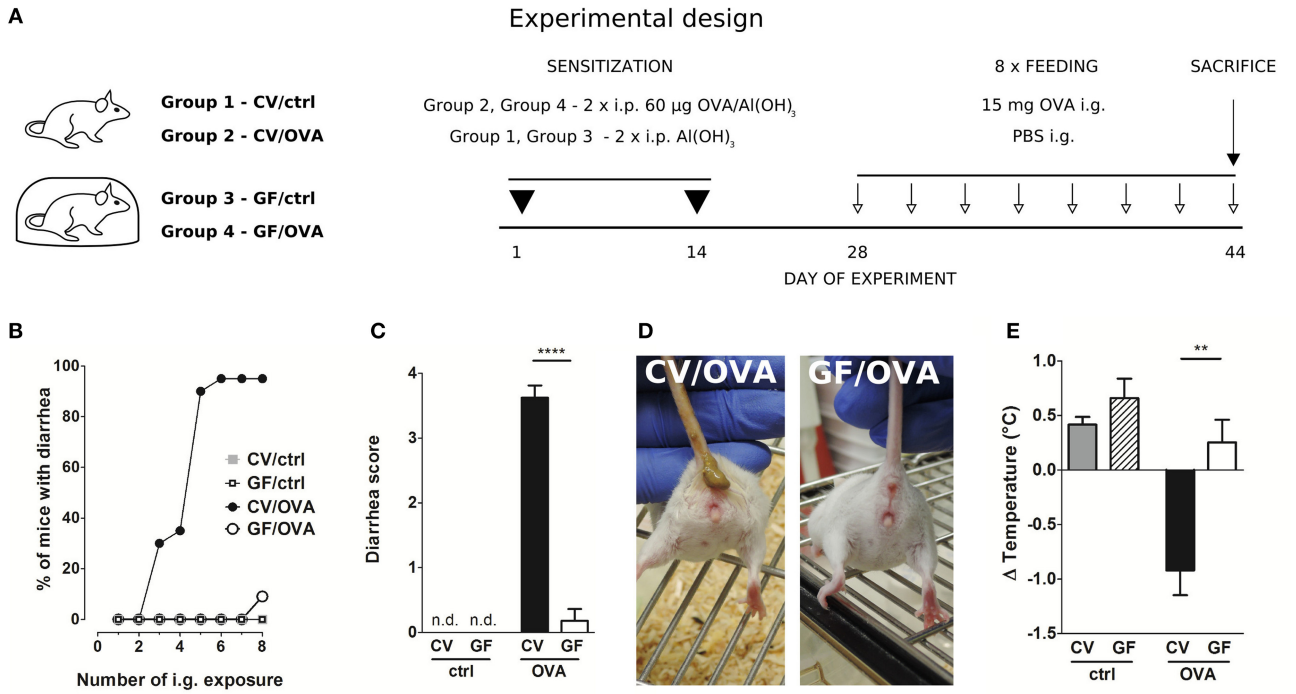

FIGURE 1 | Germ-free mice fail to develop symptoms of OVA-induced food allergy. **(A)** Experimental design: conventional (CV) and germ-free (GF) mice were sensitized twice within a two-week interval by intraperitoneal (i.p.) injection of 60 µg OVA in $Al(OH)_3$ (alum) followed by intragastric gavage (i.g.) with 15 mg OVA in PBS 8 times within 17 days (OVA groups). Age-matched control mice were injected with PBS/alum and gavaged by PBS (ctrl groups). After the last i.g. exposure, rectal temperature was assessed and mice were sacrificed by cervical dislocation. Sera and small intestine samples were collected for further analysis. Graphic art was created in freely available professional vector graphics editor Inkscape 0.92 (https://inkscape.org/). **(B)** The occurrence of diarrhea was monitored for 60 min after each i.g. administration in control CV (gray squares), control GF (open squares), in OVA-treated CV (black circles), and OVA-treated GF (open circles) mice. **(C)** The diarrhea score was assessed according to the scoring method described in Material and Methods section. **(D)** Representative pictures of CV/OVA and GF/OVA mice after the last i.g. OVA exposure. **(E)** The rectal temperature was measured 30 min after the last i.g. exposure at day 44 of the experiment in CV/ctrl (gray bars), and GF/ctrl (dashed bars) control mice, and in CV/OVA (black bars) and GF/OVA (white bars) OVA-treated mice. Difference in the temperature before and after challenge is shown. Data are plotted as mean values ± SEM. Pooled values of at least two independent experiments (CV/ctrl $n = 8$, CV/OVA $n = 14$, GF/ctrl $n = 9$, GF/OVA $n = 11$ mice per group) are shown. $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$.

Isolation of Peritoneal Cavity Cells and Small Intestine Lamina Propria Mononuclear Cells

Naive CV and GF BALB/c mice were euthanized by isofluran and peritoneal cavity lavage was performed twice with 5 ml of cold PBS containing 0.1% sodium azide and 0.2% gelatin from cold water fish skin (PBS-gel) (Sigma-Aldrich, USA). Small intestine mononuclear cells were isolated according to Scott et al. (37) with minor modifications. Briefly, small intestine was excised and washed in cold PBS. Fat tissue and Peyer's patches were removed. The intestine was cut into small pieces and washed by 3% RPMI (Sigma-Aldrich, 3% FCS, pen/strep and HEPES). The tissue was incubated in 30 ml warm 3% RPMI with 5 mM EDTA and 1 mM DTT for 20 min at 37°C on orbital shaker (200 rpm). After washing with RPMI with 5 mM EDTA and PBS, the tissue was minced with scissors, mixed with 10 ml of digest solution [RPMI, 200 mM glutamin, pen/strep, sodium pyruvate, non-essential amino acids, HEPES, 0.1 mg/ml of Liberase (Roche, Switzerland), and 0.5 mg/ml of DNase 1 (Roche, Switzerland)] and incubated 30 min at 37°C on orbital shaker (200 rpm). Ten milliliters of 3% RPMI was added to stop the digestion. The digested tissue was filtered through 70 µm and 40 µm filter and centrifuged at 450 × g for 5 min. Cells were resuspended in 1 ml of complete RPMI.

Flow-Cytometry Analysis

Cells (10^6/well) were blocked for 10 min at 4°C in dark by 20% rat heat-inactivated serum in PBS containing 0.1% sodium azide. Staining was performed with fluorochrome labeled anti-mouse monoclonal Abs: CD45-FITC (eBioscience, USA; clone 30-F11), FcεRIα-phycoerythrin (BioLegend, USA; clone MAR-1), and CD117(c-kit)-APC-eFluor® 780 (eBioscience, USA; clone 2B8) according to the manufacturer's recommendation. After 30 min cells were washed four times by cold PBS-gel and data were acquired by FACSCalibur (BD Immunocytometry Systems, Mountain View, CA) or FACS Aria III (BD Immunocytometry Systems, Mountain View, CA) flow cytometer. Analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

Cutaneous Activation of Mast Cells

Intraplantar injection was performed with compound 48/80 (Sigma-Aldrich, USA; 0.5 µg/10 µl per paw) or PBS alone (10 µl

per paw) into hind footpad of naive GF and CV mice. Changes in paw width were measured with digital caliper (±0.01 mm; Festa, Czech Republic) at time 0, 30, and 60 min. The baseline paw widths (time 0) for each mouse were measured immediately after injection and subtracted from paw widths after application to calculate tissue edema.

Histology

Intestinal tissue specimens were fixed with 4% paraformaldehyde for 24 h followed by storage in 80% ethanol. Right ears were fixed in Carnoy's fluid for 30 min and transferred to 96% ethanol. Collected and fixed tissue specimens were dehydrated by using increasing concentrations of ethanol and transferred into methyl salicylate, benzene, benzene-paraffin and paraffin. Sections (5 μm) were deparaffinized in xylene and rehydrated through an ethanol to water gradient and stained for chloracetate esterase activity which is characteristic for mast cell granula. Reagent solution was prepared by mixing of 4% pararosaniline, 2 mol/l HCl, 4% aqueous sodium nitrite, 0.07 mol/l phosphate buffer (pH 6.5), and substrate solution (Naphthol AS-D chloroacetate dissolved in N-dimethylformamide) (all Sigma-Aldrich, USA). The sections were stained by reagent solution for 30 min in the dark and counterstained with hematoxylin for 2 min. The numbers of mast cells per randomly selected villi in jejunum and per 0.1 mm^2 in the ear tissue were determined.

RNA Isolation and Real-Time PCR

Jejunal tissues were stored in RNA-later reagent (Sigma-Aldrich, USA) overnight at 4°C and kept at −80°C until processed. Tissue samples were homogenized by Precellys 24 tissue homogenizer (Bertin Technologies, FR) at 5,000 rpm for 20 s using tubes with zirconium oxide beads. RNA was isolated via RNeasy Mini kit (Qiagen, Valencia, CA). An iScript cDNA Synthesis Kit (BioRad Laboratories, USA) was used to generate cDNA. Real-Time (RT) PCR was performed on the LightCycler® 480 instrument (Roche, DE) using LightCycler® 480 SYBR Green I Master according to the manufacturer's instructions (Roche, DE). β-actin was used as an internal control to normalize gene expression using the 2–ΔCt method (38). RT PCR primer sequences are listed in **Supplementary Table 1**.

Statistical Analysis

Statistical analysis between multiple groups was performed by ANOVA with Tukey's multiple comparison test. Differences between two groups were evaluated using *t*-test. GraphPad Software was used to evaluate the data (GraphPad Prism 5.04, USA); $P < 0.05$ were considered significant. Data are expressed as means ±SEM.

RESULTS

Germ-Free Mice Exhibit Reduced Susceptibility to Experimental Food Allergy

After intraperitoneal injection of OVA adsorbed to alum, CV and GF mice were challenged by oral gavage of OVA eight times over the period of 2 weeks (**Figure 1A**). Sensitized CV mice were most likely to develop experimental FA after five OVA challenges, with ∼90% of these animals exhibiting allergic diarrhea (**Figure 1B**). In contrast, none of the GF mice developed diarrhea at this stage. Only after the eighth dose was diarrhea detected in 10% of GF animals (**Figure 1B**). This is reflected in the allergic diarrhea score where the majority of sensitized CV animals exhibited more than two episodes of liquid diarrhea after antigen gavage during the treatment period (**Figures 1C,D**). Sensitized CV mice also exhibited reduced core body temperature after the eighth OVA challenge. This was not observed in the GF animals (**Figure 1E**).

High Levels of Th2-Accociated Specific Serum Antibodies and Spleen Cytokines Do Not Correlate to the Low Susceptibility of Germ-Free Mice to Develop Food Allergy

Systemic allergic sensitization and oral challenge with OVA led to the induction of OVA-specific IgE, IgG1, IgG2a, and IgA in sera in both experimental groups (**Figures 2A–E**). In agreement with previous studies (10, 12, 35), sensitized GF mice exhibited increased levels of OVA-specific IgE in comparison to CV animals (**Figures 2A,B**). The functionality of the OVA-specific IgE was tested in a rat basophil leukemia cell degranulation assay. Sera from sensitized and challenged GF animals induced higher levels of β-hexosaminidase release in comparison to sera from CV mice (**Figure 2A**). While levels of IgG1 were comparable between CV and GF mice (**Figure 2C**), higher specific IgG2a (**Figure 2D**) and IgA (**Figure 2E**) levels were detected in mice raised in the presence of microbiota compared to GF animals. Systemic allergen-specific cellular responses were evaluated by re-stimulating splenocytes with OVA *ex vivo* (**Figures 2F–J**). Stimulation of cells derived from sensitized and challenged GF mice led to the induction of Th2 cytokines (**Figures 2G–J**). Levels of IL-5 and IL-13 were significantly higher in GF mice compared to CV mice (**Figures 2H,I**). A lack of bacterial exposure was associated with reduced levels of OVA-specific IFN-γ production (**Figure 2F**).

Absence of Microbial Colonization Leads to a Lower Density of Mast Cells in the Gut and Reduced Levels of Local and Systemic MCPT-1

Given the fact that GF mice were protected from the development of food allergy despite their ability to produce large amounts of OVA-specific IgG1 and IgE as well as pro-allergic systemic cellular responses, we hypothesized that the lack of microbial stimulation resulted in a non-functional effector compartment in the gut. It has been well-established that gastrointestinal symptoms during oral antigen-induced anaphylaxis depend not only on IgE but also on the numbers of MC in the intestine (30). Here we show that sham-treated GF mice displayed significantly lower numbers of mucosal MC compared to CV mice (**Figures 3A,B**), accompanied by the lower levels of MCPT-1 in the intestinal tissue (**Figure 3F**). This phenomenon, although not as pronounced, was also confirmed in the skin where GF mice harbored 20% fewer MC in comparison to CV mice (**Figures S1A,B**). Although the numbers of intestinal MC increased both in CV and GF mice after OVA-treatment, the

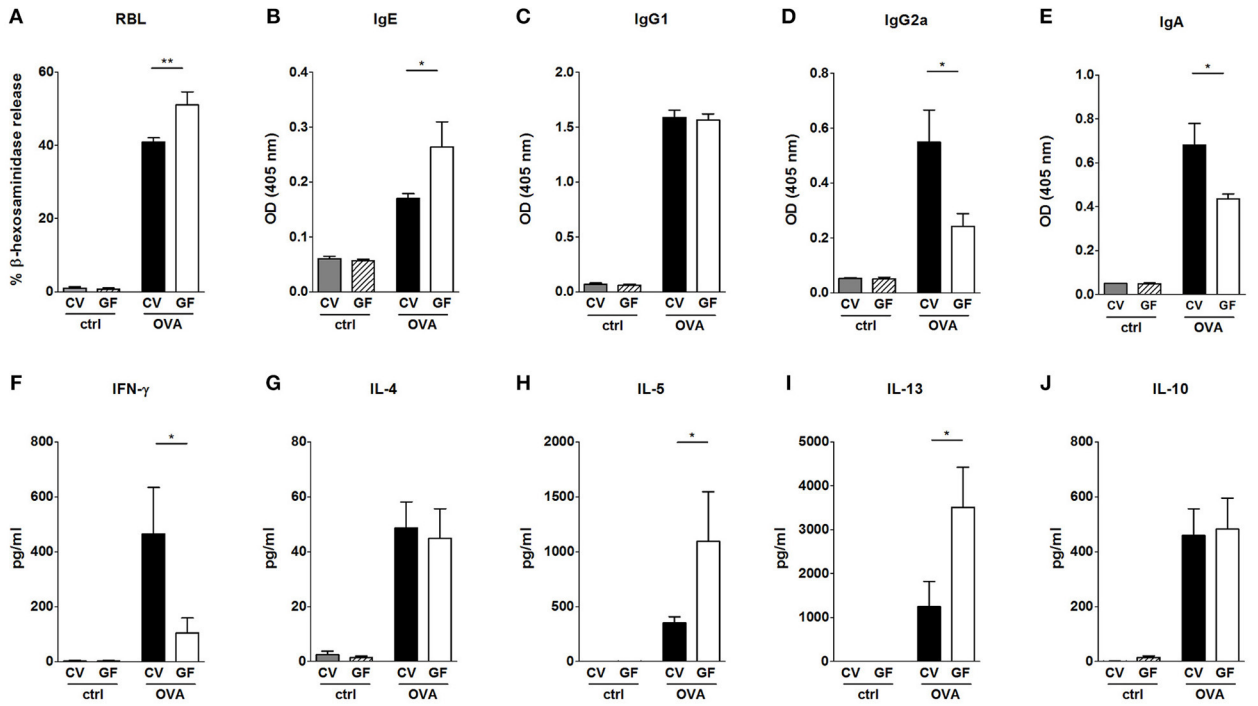

FIGURE 2 | Impact of bacterial colonization on the levels of OVA-specific antibodies in sera and on OVA-induced cytokine production in splenocytes. Levels of OVA-specific antibodies were measured in sera of control CV (gray bars) and GF (dashed bars) mice, and sera of OVA-treated CV (black bars), and GF (white bars) mice. **(A)** Functional IgE in serum was measured by OVA-mediated β-hexosaminidase release from rat basophil leukemia cells (RBL). OVA-specific **(B)** IgE, **(C)**, IgG1, **(D)**, IgG2a, and **(E)**, IgA were measured by ELISA and expressed as optical density (OD). Levels of **(F)**, IFN-γ, **(G)**, IL-4, **(H)**, IL-5, **(I)**, IL-13, and **(J)**, IL-10 in splenocyte culture supernatants were measured by MILLIPLEX Cytokine panel. Cytokine levels are expressed after subtraction of base line levels of unstimulated splenocytes. Data are plotted as mean values ± SEM. Pooled values of at least two independent experiments (CV/ctrl $n = 8$, CV/OVA $n = 14$, GF/ctrl $n = 9$, GF/OVA $n = 11$ mice per group) are shown. $^*P \leq 0.05$, $^{**}P \leq 0.01$.

numbers were significantly lower in GF mice compared to CV mice (**Figures 3A,B**). This was associated with lower expression of intestinal CXCR2 and its ligands CXCL1 and CXCL2, markers associated with the recruitment of MC to the gut (**Figures 3C–E**). Concomitantly, the local production of Th2-associated IL-4 and IL-13, and pro-inflammatory TNF-α was significantly lower in the jejunum of sensitized and challenged GF mice compared to CV animals (**Figures S2A–F**). Levels of MCPT-1 in the gut and in serum were significantly lower in OVA-treated GF mice compared to CV mice (**Figures 3F,G**). Although the numbers of intestinal MC in OVA-treated GF mice reached ∼60% of those detected in CV mice, the production of MCPT-1 in the jejunum reached only 30% of the levels detected in OVA-treated CV mice. These observations suggest that also the maturation status and functionality of MC are influenced by microbiota.

Lack of Microbial Colonization Leads to Reduced Expression of Mast Cells Expansion, Differentiation, and Survival Factor Associated With Reduced Mast Cells Degranulation *in vivo*

Stem cell factor (SCF) is a key factor in MC biology (39). Besides being the major chemotactic factor, SCF is indispensable for MC proliferation and for their survival, differentiation and maturation (24). We therefore determined the SCF expression in the small intestinal tissue. We found that SCF expression was significantly lower in GF mice in comparison CV animals, both in naïve controls and OVA-treated mice (**Figure 4A**). We further assessed the expression of the SCF receptor on the intestinal MC in naïve GF and CV mice by flow-cytometry (gating strategy in **Figure S3**). In the absence of microbiota, the levels of mean fluorescence of surface CD117 were significantly lower on GF MC compared to MC isolated from CV intestines, suggesting their lower maturation status (**Figure 4B**). Furthermore, GF mice exhibit significantly lower numbers of mature and significantly higher numbers of immature MC compared to CV mice as assessed by the determination of the peritoneal FcεRIα$^+$CD117$^+$ granularity by FACS (**Figures S4A–C**). To functionaly confirm the role of the microbiota in MC maturation and function, we injected footpads of GF and CV mice with the compound 48/80, which has been used to induce MC degranulation and is associated with tissue edema *in vivo* (40). We clearly showed that injection of the 48/80 induced edema in footpad of CV mice and this was significantly less pronounced in GF animals (**Figure 4C**).

Reconstitution of Germ-Free Mice With Complex Microbiota but Not With a Single Bacterial Strain Restores the Susceptibility to Food Allergy

To test whether the susceptibility to food allergy can be restored, we colonized GF mice by co-housing them with CV mice or by

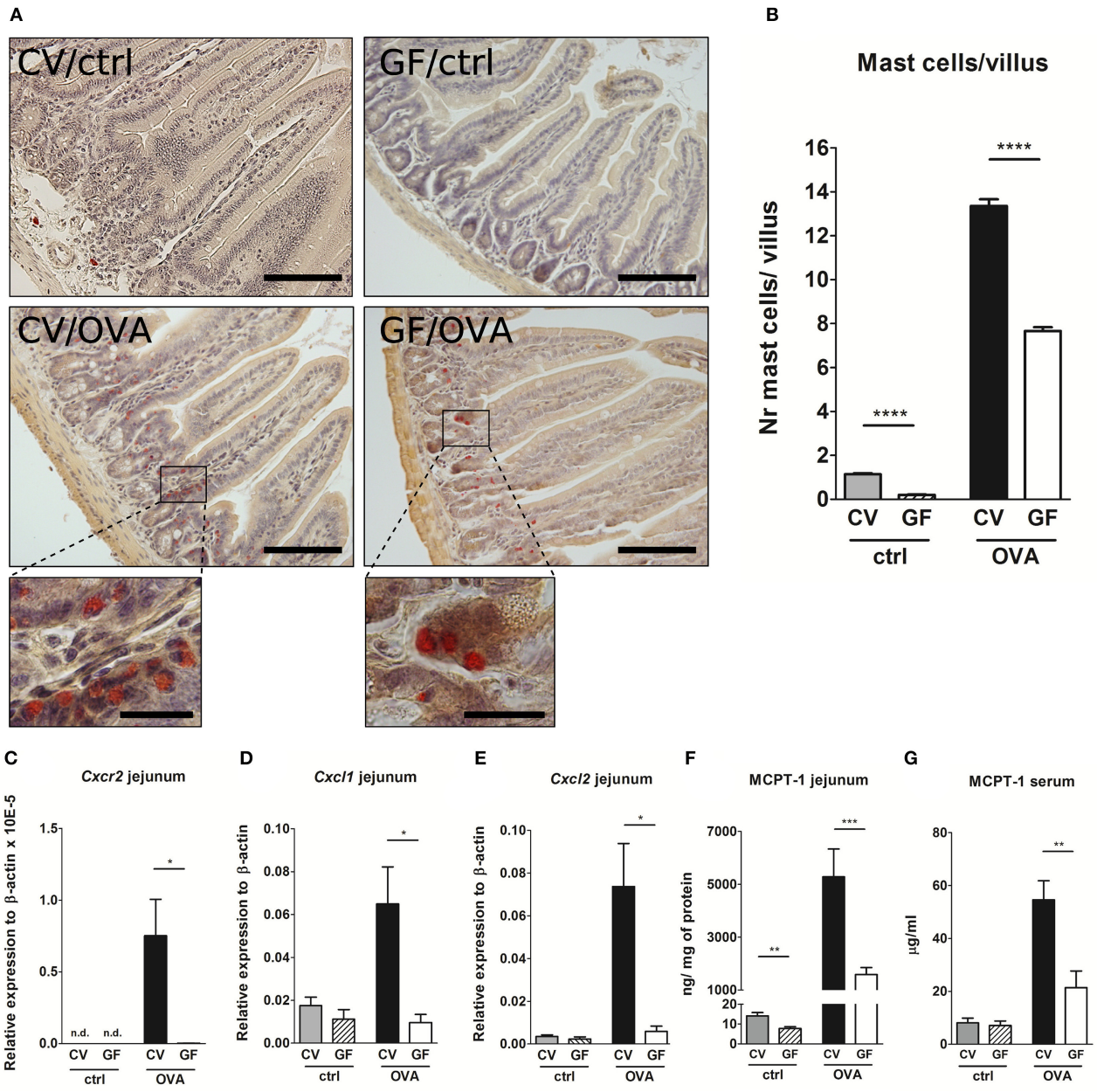

FIGURE 3 | Germ-free mice exhibit low numbers of intestinal mast cells, low expression of *Cxcr2* and its ligands, and low levels of Mast cell protease-1 after OVA-sensitization and challenge. **(A)** Histological staining of jejunal sections for mastocytosis by hematoxylin/pararosaniline was performed on samples from control conventional (CV/ctrl) and germ-free (GF/ctrl), and from OVA-treated CV (CV/OVA) and germ-free (GF/OVA) mice (scale bars, 100 µm; inset scale bars, 25 µm) **(B)**, Quantification of mast cells per villus in jejunal sections (CV/ctrl $n = 3$, CV/OVA $n = 8$, GF/ctrl $n = 5$, GF/OVA $n = 5$ mice per group). Messenger RNA expression of **(C)**, *Cxcr2* **(D)**, *Cxcl1*, and **(E)**, *Cxcl2* (in the jejunal tissues was determined by real-time PCR. Relative expression to β-actin is shown (CV/ctrl $n = 6$, CV/OVA $n = 7$, GF/ctrl $n = 6$, GF/OVA $n = 5$ mice per group). Mast cell protease-1 (MCPT-1) levels were determined in **(F)**, jejunal homogenates and in **(G)**, sera of control conventional (CV; gray bars) and germ-free (GF; dashed bars) mice, and in OVA-treated CV (black bars), and GF (white bars) mice by ELISA. Data are plotted as mean values ± SEM. Pooled values of at least two independent experiments (CV/ctrl $n = 8$, CV/OVA $n = 14$, GF/ctrl $n = 9$, GF/OVA $n = 11$ mice per group) are shown. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

gavaging them with human *Lactobacillus* isolate *L. plantarum* WCFS1. Successful conventionalization was verified by decrease in cecal weight and the presence of bacterial DNA measured by PCR in cecal samples (**Figures S5A,B**). In conventionalized mice (exGF), the incidence of diarrhea, the diarrhea score and the degree of hypothermia reached levels comparable to those observed in CV mice (**Figures 5A–C**). Furthermore, these mice exhibited higher levels of MCPT-1 in the jejunum and serum

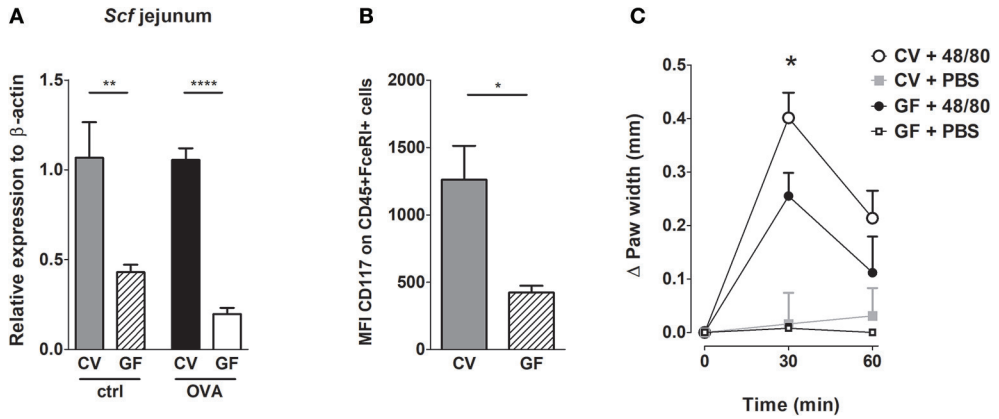

FIGURE 4 | Germ-free mice have less mature mast cells and these cells are functionally impaired. **(A)** Messenger RNA expression of Stem cell factor (*Scf*) in the jejunal tissues was determined by real-time PCR. Relative expression to β-actin is shown. CV/ctrl (gray bars) $n = 5$, GF/ctrl (dashed bars) $n = 6$, CV/OVA (black bars) $n = 5$, GF/OVA (white bars) $n = 5$ mice per group. **(B)** Mean fluorescence intensity of CD117 was determined by flow cytometry on CD45+FcεRIα+ cells isolated from small intestine. CV (gray bars) $n = 3$, GF (dashed bars) $n = 3$ mice per group. **(C)** GF and CV mice were injected with degranulation-inducing compound 48/80 or PBS and edema was recorded. Data are presented as paw width after subtraction of baseline values, GF 48/80 (black circles) $n = 6$, CV 48/80 (open circles) $n = 8$, GF PBS (open squares) $n = 5$, CV PBS (gray squares) $n = 8$ mice per group. Data are plotted as mean values ± SEM. $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{****}P \leq 0.0001$.

(**Figures 5D,E**). In contrast to conventionalized ex-GF mice, GF mice mono-colonized with bacterial strain *L. plantarum* failed to develop clinical symptoms of experimental FA (**Figures 5A–C**). There was no significant difference in the occurrence of diarrhea, diarrhea severity or in the level of hypothermia between Lp mono-colonized mice and GF animals. Moreover, *L. plantarum* did not restore the production of MCPT-1 in the jejunum and serum to those observed in CV mice; levels were similar to those observed in GF mice (**Figures 5D,E**).

DISCUSSION

Here we report that despite showing high levels of allergic sensitization, GF BALB/c mice are protected against the development of OVA-induced FA, exhibiting low incidence of diarrhea and hypothermia. In the intestine, GF mice displayed reduced numbers of MC and lower levels of MCPT-1 after allergic challenge in comparison to CV mice. Further we confirmed the altered MC maturation and function in the absence of microbiota. Finally, colonization of GF mice with conventional microbiota, but not their mono-association with single bacterial strain *L. plantarum* WCFS1, induced the susceptibility of exGF mice to OVA-induced FA.

In accordance with the hygiene hypothesis, GF animals have been shown to develop increased levels of serum IgE after allergic sensitization in comparison to their CV counterparts (9–12). Here we show that sensitization and challenge lead to increased OVA-specific IgE levels in sera and increased production of OVA-specific IL-5 and IL-13 in re-stimulated splenocytes from GF mice compared to CV animals. The concept that signals derived from commensal bacteria are crucial to normalize elevated Th2 responses have been tested previously by us and others. For example, colonization of GF mice with conventional or SPF microbiota, with a well-defined mixture of different strains, or with only a single bacterial strain led to reduced levels of serum IgE and Th2 cytokine production in comparison to GF animals (11, 12, 31, 41). Recently, we have expanded this observation to residual bacterial fragments and showed that the presence of LPS in sterile food was sufficient to reduce high levels of allergen-specific IgE in GF sensitized mice (35).

Surprisingly, despite the elevated systemic OVA-specific humoral and cellular Th2 responses, GF mice were protected from FA symptoms, i.e., they failed to develop allergic diarrhea and hypothermia. This observation is in disagreement with studies by Cahenzli et al., Rodriguez et al. and Stefka et al. who showed that GF mice are more susceptible to FA (10, 31, 42). There are several possible explanations for this discrepancy. First, differences in the sensitization and challenge protocols. We used 2 intraperitoneal doses of 60 μg OVA/Alum followed by eight challenges of 15 mg OVA by gavage, as previously established by Golias et al. (36). In contrast, Cahenzli et al. sensitized mice with one subcutaneous dose of 50 μg OVA/Alum followed by single oral challenge with 50 mg OVA (31). A very different model was used by Stefka et al. and Rodriguez et al., where mice were sensitized by intragastric gavages with antigen admixed with cholera toxin (CT) (10, 42). CT is known as a potent mucosal adjuvant which induces mobilization and maturation of intestinal immune cells (43) as well as the induction of Th1/Th2/Th17 responses (44). Thus, the application of CT to GF mice might provide signals for maturation or recruitment of MC to the gut tissue. Second, there are differences in the readouts and reporting of clinical symptoms. In Cahenzli et al. and in Stefka et al. the allergic sensitization and oral challenge did not induce hypothermia in SPF or CV mice (10, 31). This is surprising since we and others have shown that immunization in the presence of adjuvant followed by oral challenge with or without adjuvant results in clinical symptoms, such as hypothermia or diarrhea in both conventional animals or animals with SPF microbiota

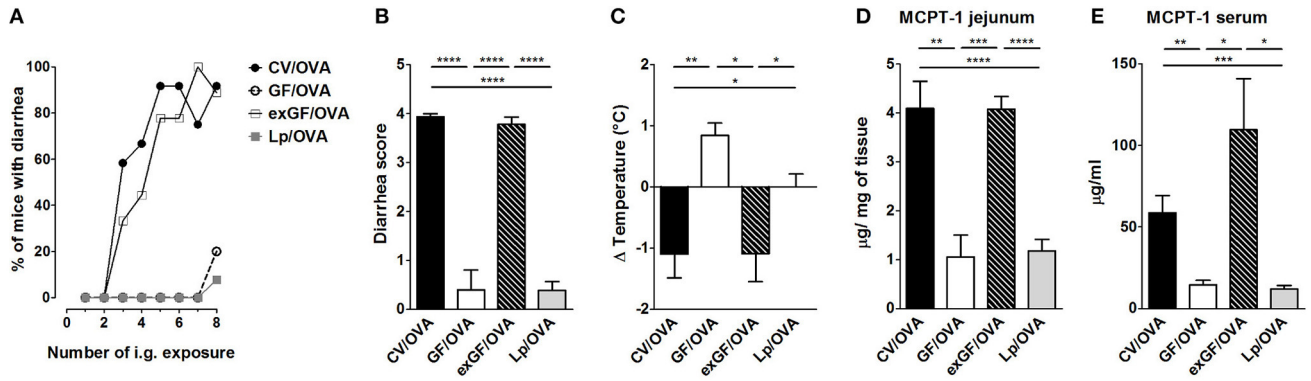

FIGURE 5 | Colonization of germ-free mice by the conventional microbiota but not by single bacterial strain *Lactobacillus plantarum* WCFS1 restores sensitivity to OVA-induced food allergy. Germ-free (GF) mice were colonized by co-housing with age-matched conventional (CV) animals (exGF group) or mono-associated with the strain *L. plantarum* WCFS1 (Lp group). At the age of 8 weeks, all groups were submitted to OVA-sensitization and challenge, as described in **Figure 1**. **(A)** The occurrence of diarrhea during OVA treatment. **(B)** The diarrhea score for each group was assessed according to the scoring described in section Materials and Methods section. **(C)** The rectal temperature was taken 30 min after the last i.g. exposure at day 44. Difference in the temperature before and after challenge is shown. Mast cell protease-1 (MCPT-1) levels were determined in jejunal homogenates **(D)**, and in sera **(E)**, by ELISA. Data are plotted as mean values ± SEM. CV/OVA ($n = 8$), GF/OVA ($n = 5$) and pooled values of two independent experiments for exGF/OVA ($n = 9$) and Lp/OVA ($n = 13$) colonized mice are shown. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.01$, $^{****}P \leq 0.0001$.

(2, 28, 30, 45, 46). Third, there are differencies in sterilization methods of chow for GF animals. In our study, GF animals were fed by γ-irradiated chow, which is in contrast to sterilization method used by Cahenzli et al. where the chow was autoclaved (31). Autoclaving leads to formation of advanced glycation end-products as a result of chemical reaction between reducing sugars and amino acids in proteins or lipides (Meillard reaction) (47). These products has been recently pointed at as one of the possible causes of increasing FA incidence in Western countries (48). This is of special interest, as autoclaved, but not irradiated food has been shown to increase the numbers of intestinal MC in GF rats (49).

To determine the discrepancy between high levels of sensitization and the absence of FA symptoms, we investigated the presence of intestinal MC, which are widely recognized key effectors of allergy in the periphery (27, 28, 46). Here we demonstrate that GF animals exhibit low levels of MC in the gut under homeostatic conditions and that MC numbers are still reduced after allergen sensitization and oral challenge in comparison to CV mice. The reduced numbers of intestinal MC in GF mice were accompanied by decreased expression of *Cxcr2* and its ligands in intestinal tissue. Concomitantly, challenged GF mice had significantly lower production of MCPT-1 both locally in the intestinal tissue and systemically in sera. Further experiments are required to dissect whether the microbiota impacts MC recruitment and/or function directly or indicrectly via the action on intestinal epithelial or innate lymphoid cells.

Together with a previous report about the crucial role of MC for the development of FA symptoms (27), our data suggests that the MC homing to the intestinal effector compartment is impaired in GF animals. Chen et al. have also shown that the severity of FA correlates with intestinal MC numbers as mouse strains without intestinal MC (i.e. C57BL/6 or C3H/HeJ) did not exhibit clinical symptoms of experimental FA after antigen sensitization and challenge (28). However, due to the different mouse strains used in the study by Chen et al. were not littermate controlled, the role of microbiota in the susceptibility to food allergy cannot be ruled out (28).

In our model, intestinal MC numbers in sensitized and challenged GF animals reached 60% of those observed in CV animals. Yet the GF mice were fully protected from the development of allergic diarrhea and hypothermia. This indicates that not only the numbers of local MC in GF mice, but also the functionality and maturation status of MC are impaired in these mice. In a recent paper, Wang et al. clearly demonstrated that the microbiota drives the recruitment and functionality of skin MC through the LTA-TLR2-dependent production of SCF by keratinocytes (25). In agreement with this study, we have shown that the SCF expression was significantly lower in GF mice compared to the CV mice, both in naïve controls and OVA-treated groups. The low expression of intestinal SCF in GF mice was associated with reduced expression of CD117 on FcεRIα+ intestinal MC in these animals. Next, we addressed the impact of the microbiota on maturation status of intraperitoneal MC. Previously Dahlin et al. showed that the low granularity of MC corresponds with their low maturation status (50). We found that there was an increased percentage of MC with low granularity in GF animals compared to CV animals and that these immature MC expressed lower levels of CD117 (data not shown), a known surface maturation marker (25). These data confirmed our hypothesis that GF mice have low MC numbers in the intestine exhibiting lower maturation status.

In order to test the functionality of MC we challenged the GF and CV mice by injection of degranulation compound 48/80. GF mice exhibited significantly lower swelling compared to CV animals confirming the impaired

functionality and lower maturation of MC in the absence of microbiota.

Finally, we could show that conventionalization rendered exGF mice sensitive to FA, as demonstrated by hypothermia, diarrhea, and elevated levels of MCPT-1 in the gut and serum. Interestingly, mice mono-colonized with Gram-positive strain *L. plantarum* remained unresponsive to OVA challenge in contrary to CV mice. This observation is surprising, as *L. plantarum* WCFS1 is a bacterial strain with strong immunomodulatory properties (15, 17, 51) and oral application of this strain aggravated the severity of peanut FA in a mouse model (14). Nevertheless, it has been well-documented that different bacterial strains, and even the strains of the same species, may differ in their immunomodulatory potential (52, 53). Whether mono-colonization by other bacterial strains (e.g., Gram-negative), or supplementing the GF mice with specific bacterial products (e.g., LPS, peptidoglycan, lipoteichoic acid) can impact the maturation and function of intestinal MC and render the mice susceptible to food allergy remains to be determined.

Taken together, we report here that commensal bacteria impact MC migration and maturation in the intestine, thus playing a key role in the susceptibility to food-induced allergy. Our model based on CT-free systemic sensitization and oral challenge leads to full development of hypothermia and diarrhea in CV settings but not GF mice, represents an important tool to investigate the role of microbiota in the development or prevention of infectious or immune-mediated inflammatory diseases. The mechanistic insight into the role of the commensals-MC-FA axis, with a focus on the microbiota-induced recruitment and maturation of MC in the intestinal mucosa, can pave the way to the design of novel strategies for the prevention and treatment of food allergy in humans.

AUTHOR CONTRIBUTIONS

MSch, LT, HK, and IS conceived and designed the experiments. MSch, PH, JG, CZ, TH, DS, and JA performed the experiments. MSch, PH, JG, CZ, TH, MS and DS analyzed the data. MSch, PH, UW, LT, HK, and IS wrote the paper. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Immunology

REVIEW
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Non-classical B Cell Memory of Allergic IgE Responses

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The long-term effectiveness of antibody responses relies on the development of humoral immune memory. Humoral immunity is maintained by long-lived plasma cells that secrete antigen-specific antibodies, and memory B cells that rapidly respond to antigen re-exposure by generating new plasma cells and memory B cells. Developing effective immunological memory is essential for protection against pathogens, and is the basis of successful vaccinations. IgE responses have evolved for protection against helminth parasites infections and against toxins, but IgE is also a potent mediator of allergic diseases. There has been a dramatic increase in the incidence of allergic diseases in recent decades and this has provided the impetus to study the nature of IgE antibody responses. As will be discussed in depth in this review, the IgE memory response has unique features that distinguish it from classical B cell memory.

Keywords: allergy, IgE, memory B cells, plasma cells, anaphylaxis, sequential switching, IgG

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GENERAL MECHANISMS OF HUMORAL MEMORY IN MICE AND HUMANS

IgE antibodies mediate allergic diseases through their ability to bind to high-affinity receptors on mast cells and induce degranulation upon allergen crosslinking (1). Given the increasing prevalence of allergic reactions and allergic diseases, the study of human IgE cells becomes extremely important. Furthermore, the beneficial effect of anti-IgE treatment on allergic asthma and other chronic allergic diseases validated IgE as a therapeutic target (2). While a high titer of serum IgE has long been considered the cardinal marker of atopy, IgE-producing cells are extremely rare in humans and mice, suggesting that IgE production is strongly regulated. IgE antibodies also have protective functions against parasite infections and toxins (3, 4). Possibly as a consequence of this dual beneficial and pathogenic potential, IgE production has been evolutionary conserved but is strongly regulated. Despite the importance of IgE in allergic pathology, very little is known about the origin of human IgE B cells and the mechanisms of humoral IgE memory.

The classical humoral memory of IgM, IgGs and IgA antibodies is mediated by a two-pronged mechanism (5): non-dividing quiescent memory B cells that can be quickly reactivated, and long-lived plasma cells (6, 7) that constantly secrete antibodies during their lifespan (**Figure 1** and **Table 1**).

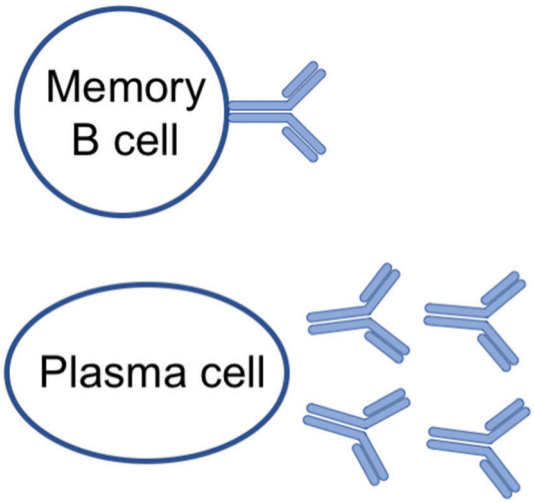

FIGURE 1 | Cellular components of B cell memory.

TABLE 1 | Memory B cells and long-lived plasma cells are the cellular components of B cell memory.

Characteristic	Memory B cells	Long-lived plasma cells
Cell division	Non-dividing	Non-dividing
Activation	Respond with proliferation and differentiation	Do not respond to activation
Differentiation potential	Germinal center cells Plasma cells Memory cells	Terminally differentiated into antibody secretory cells
Tissue location	Circulating between blood and lymphoid organs	Bone marrow and other specialized niches

Comparison of their main characteristics.

Affinity maturation and class switch recombination of antibody genes are the central processes in the establishment of effective B cell memory. Affinity maturation is the mechanism by which B cells improve the recognition of their cognate antigen by refining the affinity of their B cell receptor (BCR). This process occurs in germinal centers and involves the somatic mutation of V(D)J genes followed by selection of higher affinity B cell clones through interaction with both antigen-loaded follicular dendritic cells (FDC) and T follicular helper cells (Tfh) (8–11). Both plasma cells of higher affinity (12), and memory B cells of broad affinity range (13–16) emerge from the germinal center reaction.

Immunoglobulin class switch recombination (9, 17) is the mechanism whereby activated B cells "switch" their constant region while maintaining their antigen-binding domain, resulting in the production of IgG, IgA, and IgE antibodies (**Figure 2**). While V(D)J domains determine antigen specificity, the constant regions endow the antibodies with specific biological activities (18). In mice there are four IgG isotypes: IgG1, IgG2a (or IgG2c), IgG2b, and IgG3. In humans, there are four IgG isotypes: IgG1, IgG2, IgG3, and IgG4, and two IgA isotypes: IgA1 and IgA2. Class switching to different immunoglobulin isotypes is regulated by B cell activation and cytokines (19–22). Class switch recombination to IgE is dependent on IL-4/IL-13 signaling through IL-4Rα and STAT6. In humans, IgE production is usually associated with IgG1 and IgG4 production (23, 24), and in mice, with IgG1 production (25). In terms of function, IgM and IgG antibodies are important for neutralization and clearance of pathogens through complement fixation and binding to receptors of phagocytes (18). IgA antibodies are transported through the epithelium to luminal cavities such as the gut, where they regulate pathogens and maintain homeostasis with the microbiota (18). The main biological activity of IgE derives from its ability to bind in monomeric form to high affinity FcεRI receptors on mast cells and basophils, inducing their degranulation upon crosslinking caused by antigen binding (26). IgE also binds to FcεRII (CD23) on FDC and B cells, regulating antigen presentation and IgE production (1, 27–29).

The combination of class switch recombination, affinity maturation and cell differentiation results in the generation of class-switched memory B cells and long-lived plasma cells that are the essential components of effective B cell memory (30–32).

Memory B Cells

Memory B cells are antigen-experienced quiescent B cells (33) that respond rapidly and vigorously to activation, generating a humoral response that is of higher magnitude and affinity than the primary response (31, 32, 34). Memory B cell survival is independent of the presence of cognate antigen (35).

Memory B cells represent a heterogeneous population. They differ in their origin, immunoglobulin isotype, mutation rates, and differentiation potential. Memory B cells may originate from within or outside of germinal centers. Extrafollicular memory cells are typically generated early in an immune response and carry low number or no somatic mutations. Germinal center-derived memory cells are continuously generated during a germinal center response, comprising clones with diverse affinities. Memory B cells may express IgM or switched immunoglobulin isotypes, and they may be programmed to differentiate into either plasma cells or germinal center cells upon activation (31, 32, 34). IgM memory cells typically have fewer somatic mutations than IgG memory cells, and preferentially become germinal center cells in secondary responses, while plasma cells are typically generated from IgG memory cells (13, 36).

The propensity for IgG memory cells to preferentially and rapidly differentiate to plasma cells has been attributed to increased intracellular signaling of the IgG BCR compared with the IgM BCR (30). There are however other cell-intrinsic factors that affect the differentiation potential of IgG1 memory cells, and the full diversity of the IgG1 memory population has only recently started to be appreciated (14, 37). Subsets of secondary lymphoid organ memory IgG1 cells that preferentially give rise to plasma cells or germinal center cells after activation in secondary responses were identified in mice (14, 37). These subsets, which we called pro-plasma cell (pro-PC) and pro-germinal center cells (pro-GC), respectively, express distinct transcriptional programs and respond to activation with different kinetics (14). Importantly, the pro-PC subset, which expresses CD80 and CD73, is responsible for the rapid generation of plasma

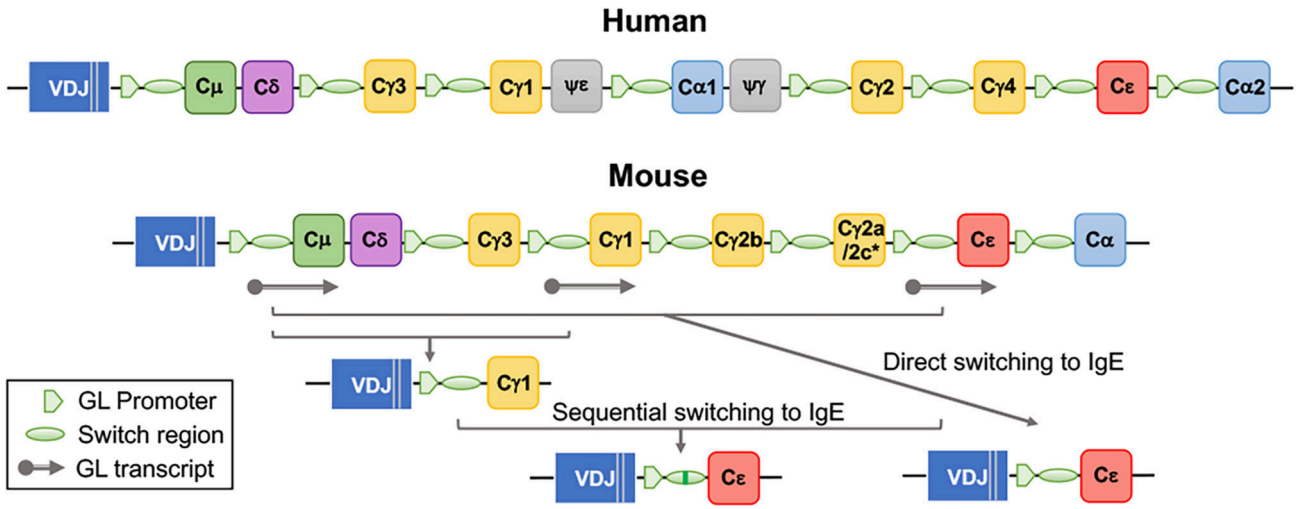

FIGURE 2 | Organization of immunoglobulin heavy chain constant region genes in human and mouse and generation of IgE by direct and sequential class switching. The gene encoding the constant region (C) of IgE, Cε, is localized at the 3' end of the locus, upstream of Cα2 in humans and of Cα in mice. *Mice of the BALB/c strain carry a Cγ2a gene, while mice of the C57/Bl strains carry the Cγ2c gene. Class switch recombination to IgE is initiated by pSTAT6 dependent production of the GL (germline) Cε transcript. Direct class switching occurs by recombination between Sμ and Sε regions. Sequential switching involves a two-step recombination, an initial recombination to IgG1, and a second recombination to IgE.

cells secreting high affinity antibodies in memory responses. Transcriptional analysis showed that pro-PC IgG1 memory cells express *Zbtb20*, a transcription factor that promotes plasma cell differentiation, while pro-GC IgG1 memory cells express *Bcl6*, *Foxp1*, *Foxo1,* and *Bach2*, transcription factors that inhibit plasma cell formation while promoting the germinal center fate (14). Thus, the differentiation potential of pro-PC and pro-GC memory cells is hardwired though the expression of distinct transcription factors. The specific roles of the IgG1 memory subsets in the generation of IgE plasma cells is discussed in section IgG1 Memory Cells are Precursors of IgE Plasma Cells. A lung resident memory B cell population which is phenotypically different from memory B cells of spleen and LN was recently described in mice infected with influenza virus. This tissue resident B memory population contributes to rapid local plasmablast differentiation following intranasal challenge (38).

Human memory B cell subsets have also been identified among unswitched (IgM^+) and switched B cells, but their correspondence to mouse memory B cell subsets is not yet clear. Human memory B cells that differ in CD27 expression have been described. $CD27^+$ human memory B cells have somatically mutated BCR genes, while $CD27^-$ memory B cells are mostly unmutated or carry a lower number of mutations than $CD27^+$ memory cells (39–42). The mutation frequency and replication history of memory B cells suggest that $CD27^+$ memory cells are derived from germinal center responses, while $CD27^-$ memory cells form outside germinal centers (40). Human memory cells expressing CD27 and CD80 can efficiently activate T cells and differentiate into plasma cells that secrete class-switched antibodies (43). Thus, in both human and mouse, CD80 expression marks memory B cells with the ability to become plasma cells. CD80 may be functionally important in this process, as CD80-deficient B cells produced fewer antigen-specific plasma cells in spleen and bone marrow after immunization (44).

The life span of memory B cells varies greatly. Some human memory B cells can be detected for decades, as in the case of smallpox-specific IgG memory cells (45). In other cases, such as in the B cell memory response to malaria merozoite (46) and to some epitopes of influenza virus (47), the memory B cell population declines quickly after infection. Though the factors that determine memory B cell longevity are still largely unknown, recent work in mice has shown that the frequency and affinity of antigen-specific naïve B cells play a role (48). The longevity of memory cells could be important in the context of allergy, as it may explain why individuals can remain allergic for years following their last encounter with allergen. However, this allergic memory could also be maintained by long-lived IgE plasma cells.

Long-Lived Plasma Cells

Much of our understanding of the dynamics of plasma cell generation derives from mouse studies. Primary immune responses lead to an initial wave of short-lived plasma cells (49, 50) that provide an important source of early, low-affinity antibodies. Additionally, in most primary responses, other activated B cells form germinal centers, where they proliferate extensively and undergo affinity maturation. The early germinal center primarily generates memory cells, with increasing plasma cell output over time (51).

The germinal center cells that will differentiate into plasma cells are among the pool of high affinity clones (12, 52, 53). Similarly, in memory responses, high affinity plasma cells rapidly differentiate within a week after challenge from clonal expansion of a subset of specialized high affinity memory cells (14). Newly formed plasma cells divide in the secondary lymphoid organs, then enter the circulation to migrate to the bone marrow,

where they complete the process of terminal differentiation into non-dividing, long-lived plasma cells that secrete large amounts of antibody (6, 7). Long-lived IgG plasma cells are predominantly located in the bone marrow, but they are also present in the spleen, albeit in lower numbers (7, 29). IgA plasma cells form mainly in the intestine and home to the intestinal lamina propria (54–56), though they also can be found in the bone marrow and the spleen (57). Plasma cells secrete immunoglobulin at rates estimated to reach 10,000 molecules per second (7).

The differentiation of B cells into plasma cells requires a profound cellular reprograming (58). The prototypical B cell pathways including BCR signaling, and antigen processing and presentation are downregulated; while pathways involved in protein synthesis, N-glycosylation, endoplasmic reticulum (ER) stress and the unfolded protein response are upregulated (59–62). The ER and Golgi systems expand substantially, and metabolic reprogramming supports the secretory demands of the plasma cell: lipid synthesis increases to accommodate organelle remodeling, while glucose uptake and oxidative phosphorylation are upregulated to fuel plasma cell function (61, 63, 64).

The longevity of plasma cells can span from a few days to decades. Since bone marrow plasma cells die rapidly *ex vivo*, plasma cell lifespan is likely determined by the anatomical microenvironment, notably the specialized bone marrow niche that is believed to sustain long-term plasma cell survival (65–67). In this niche, plasma cells physically interact with stromal cells and hematopoietic cells that secrete factors important for plasma cells retention and survival within the niche, including CXCL12, APRIL, BAFF, and IL-6. Bone marrow stromal cells support plasma cell survival *in vitro* through VLA4-VCAM interactions and IL-6 production (68). In the bone marrow, plasma cells localize adjacent to VCAM-1^+ stromal cells that produce CXCL12 (69). Plasma cells that lack CXCR4, the receptor for CXCL12, mis localize in the spleen, accumulate in circulation, and fail to home to the bone marrow (70). Among hematopoietic cells, eosinophils, basophils, and megakaryocytes contribute to plasma cell survival by producing APRIL and IL-6 (71–73). Plasma cells deficient in BCMA, the receptor for APRIL and BAFF, have impaired survival in the bone marrow (74), and both APRIL and BAFF support plasma cell survival *in vivo* (75). The evidence for reliance on other cell types strongly supports an important role for cell-extrinsic factors in plasma cell longevity.

It is unclear to what extent plasma cell longevity is also affected by cell-intrinsic factors. Several pro-survival genes in the *Bcl-2* family are expressed at higher levels in plasma cells than in other B cells, and plasma cell expression of the anti-apoptotic gene *Mcl-1* is required for survival beyond a few weeks *in vivo* (76). However, *Mcl-1* expression is itself regulated by BCMA (76), the receptor for APRIL and BAFF - both cell-extrinsic survival factors. Recent work has revealed metabolic differences between splenic plasma cells at day 7 post-immunization, which are enriched in short-lived plasma cells, compared with the more typically long-lived plasma cells in bone marrow (77). Bone marrow plasma cells were shown to uptake more glucose, import more pyruvate into mitochondria, and adapt better to bioenergetic pressure than splenic plasma cells, suggesting that these differences contribute to their long-term survival (77).

Long-lived plasma cells are an essential component of immunity whose function is to continuously secrete antibodies. Long-lived plasma cells originate from germinal center reactions, and home to bone marrow niches that support their survival. Questions remain on the immune conditions that allow differentiation of long-lived plasma cells, and the relative contribution of cell-intrinsic and niche factors to plasma cell survival and longevity. IgE plasma cells have not yet been thoroughly studied, and have only recently received more attention. They are discussed in detail for mice in section Most IgE Cells are Plasma Cells, and for humans in section Human IgE Cells.

THE IGE MEMORY RESPONSE IN MICE

There is strong evidence that IgE responses have memory. Secondary IgE responses to helminth infection and to immunization in mice are faster and of greater magnitude than the primary response (78, 79), which is typical of B cell memory. Consistent with B cell memory, the affinity of IgE antibodies and the frequency of high affinity mutations in IgE genes increase with repeated immunization (14, 80–83). Paradoxically, there are many hurdles for IgE memory: the IgE germinal center phase is exceptionally transient, and there is a paucity of bona fide IgE memory cells (14, 80, 81, 83).

A number of studies have provided strong evidence that the memory for IgE responses depends on IgG1 memory cells that class switch and differentiate to IgE plasma cells (14, 82, 84, 85). This mechanism compensates for the paucity of true IgE memory cells while at the same time imposing great stringency to IgE production in memory responses, as T cell help and high levels of IL-4 are required for *de novo* switching to IgE (84). The next sections will discuss the current knowledge of how IgE memory responses in mice are generated and maintained.

IgE Germinal Center Cells and the Missing IgE Memory Cells

The identification of IgE germinal center cells in mice has for a long time been hampered by the transient nature of this population, and by their very low expression of membrane IgE. The development of fluorescent protein IgE-reporter mice (81, 83), and improved labeling methods using the anti-IgE monoclonal antibody R1E4 (81, 84), which does not recognize IgE bound to cellular FcεRI or FcεRII (86, 87), have facilitated the functional analysis of live IgE-expressing cells.

IgE and IgG1 germinal center cells form early in primary responses (81, 83), coinciding with the peak of IL-4 production (88). Unlike IgG1 germinal center cells that persist from several weeks to months, IgE germinal center cells quickly disappear during the primary response, declining rapidly from a peak at day 10–12 post primary immunization, and are very scarce in

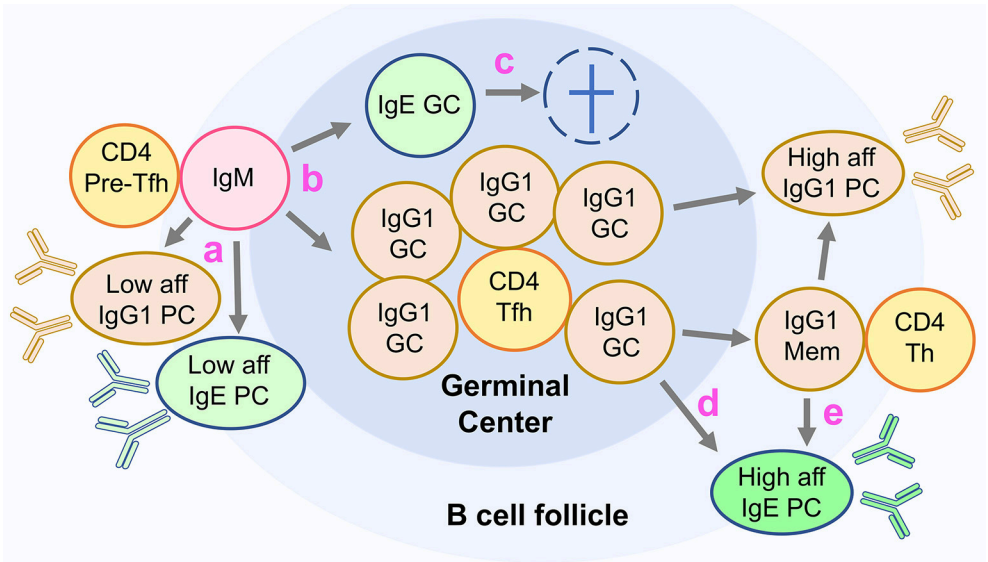

FIGURE 3 | Unconventional differentiation of IgE cells in mice. The figure depicts the current model of IgE cell differentiation in mice. (a) In primary responses, activated IgM expressing lymphocytes undergo class switch recombination to IgG1 and IgE producing a fast wave of low affinity short lived plasma cells (PC). (b) Some activated IgM cells switch to IgG1 or IgE and differentiate into germinal center cells (GC). (c) IgE GC cells are transient and do not generate IgE memory cells or high-affinity IgE PC. (d) High affinity IgE PC are generated in primary responses from IgG1 GC cells, and (e) in memory responses from IgG1 memory cells.

secondary responses accounting for <0.1% of $B220^{+}CD138^{-}$ cells (14, 81, 83) (**Figure 3**).

Compared to IgG1 germinal center cells, IgE germinal center cells have several deficits that impair their function: they have a two- to threefold higher rate of apoptosis, express the BCR at three- to fourfold lower levels and have overall reduced intracellular BCR signaling (81). In addition, IgE germinal center cells have decreased cell membrane expression of important co-stimulatory molecules CD21/35, OX-40L and ICOSL, and are markedly underrepresented in the light zone of the germinal center. While approximately one in three IgG1 germinal center cells are light zone cells, one in seven IgE germinal center cells are light zone cells (81). These findings suggest impaired IgE function in the light zone of the germinal center, where newly mutated germinal center cells typically test their BCR through interactions with FDC and Tfh cells (8).

We investigated if the low expression of the BCR in IgE germinal center cells was due to decreased amount of heavy chain transcripts. We found that the overall heavy chain transcript level was the same between IgG1 and IgE germinal center cells. However, there was a difference in the ratio of membrane to secreted IgE transcripts in IgE germinal center cells, with a predominance of secreted IgE transcripts (81). Three atypical weak polyadenylation signals (AGTAA, AAGAAA, and ATTAAA) are found downstream of IgE membrane exons in human and mouse IgE genes, while an optimal consensus AATAAA sequence signals the polyadenylation of secreted IgE transcripts (89). The weak polyadenylation signals for membrane IgE favor the production of secreted IgE in $B220^{+}$IgE cells (89) and likely in IgE germinal center cells (81).

The transient nature of IgE germinal center cells may be responsible for the paucity of true functional IgE memory cells in mice (14, 81, 83). It is also possible that IgE memory B cells form but die very rapidly without contributing to memory responses.

Most IgE Cells Are Plasma Cells

The predominance of the plasma cell fate is one of the hallmarks of the IgE response (80). Various factors may contribute to this: the transient and apoptotic IgE germinal center phase, the lack of IgE memory cells, and a tendency for IgE B cells to differentiate into plasma cells (**Figure 3**).

Intriguingly, recent work suggests that the very expression of the IgE BCR intrinsically drives plasma cell differentiation independently of antigen binding (30, 90–92). B cells expressing membrane IgE relocated the IgE BCR to lipid rafts, changed cell morphology, migrated less to CXCL12 and underwent increased apoptosis (91). Ectopic expression of membrane IgE in primary B cells *in vitro* resulted in increased autonomous BCR signaling that triggered plasma cell differentiation (90, 92). This increased BCR signaling could be attributed partly to a physical association of membrane IgE with CD19. Accordingly, IgE plasma cell differentiation *in vitro* was suppressed by reduced BCR signaling in CD19- and Blnk-deficient B cells. *In vivo*, reduced BCR signaling by CD19 haplo-insufficiency or Blnk deficiency increased the IgE germinal center cell population at the expense of IgE plasma cell population (83, 90). A different experimental approach compared B cells engineered to express the heavy chain of IgG1 (*Ighγ1*) or IgE (*Ighε*) from development (93). *Ighε/ε* mice had low numbers of mature B cells, and these B cells expressed BCR at low levels, similarly to what has been described for germinal center IgE cells. Low IgE BCR

expression and signaling may be responsible for the inability of IgE to promote normal B cell development. Augmenting BCR signaling through PTEN deletion rescued mature B cell numbers in the *Ighε/ε* mice. Thus, while overexpression of membrane IgE *in vitro* promotes plasma cell differentiation through increased tonic BCR signaling, both IgE germinal center cells and naïve B cells expressing membrane IgE have lower BCR density, decreased BCR signaling and are highly apoptotic. The *in vivo* conditions in which membrane IgE signaling promotes plasma cell differentiation remain undetermined, but membrane IgE expression is essential for IgE responses, as deletion of IgE membrane exons in mice leads to an almost complete absence of IgE production *in vivo* (78).

A distinct characteristic of mouse IgE plasma cells is that they express higher levels of membrane immunoglobulin than IgE germinal center cells (80, 81, 83) and IgG1 plasma cells. Whether membrane IgE is important for the survival of IgE plasma cells and whether it could have signaling function in mice is not known. BCR signaling function has been described for IgM and IgA plasma cells (94, 95), so it is plausible that such function could occur in IgE plasma cells.

Mouse IgE plasma cells are also unusual in that they accumulate in secondary lymphoid organs, where they may constitute one third or more of all plasma cells, and have a delayed accumulation in the bone marrow (80, 81, 83). When equal numbers of IgG1 and IgE plasma cells isolated from lymphoid organs of immunized mice were transferred intravenously to naïve mice, serum IgG1 titers were much higher than those of IgE. While transferred IgG1 plasma cells localized mostly to the bone marrow, IgE plasma cells could be found in the spleen and bone marrow (81). IgE plasma cells appear thus to be less efficient than IgG1 plasma cells in homing to the bone marrow, and whether directly related, in contributing to the circulating antibody pool. Increased IgE plasma cell apoptosis and decreased responsiveness to CXCL12 (91, 96) may be some of the factors involved in the poor bone marrow localization of IgE plasma cells.

The Life Span of IgE Plasma Cells

A very important question for the understanding of allergic responses is whether long-lived IgE plasma cells exist. Experimental evidence in mice has demonstrated the existence of long-lived and short-lived IgE antibody responses. Early experiments in the 1980s showed that IgE-secreting cells and IgE antibodies were present for up to a year after intraperitoneal immunization of mice and rats with purified protein in alum adjuvant. A large part of the IgE response remained after lethal X-ray irradiation, a treatment that depletes memory cells but not plasma cells (97–99). In a more recent study, mice immunized intraperitoneally with OVA in alum and then re-challenged by OVA inhalation had IgE, IgG, and IgA plasma cells in lung, spleen and bone marrow. Like their IgG and IgA counterparts, IgE plasma cells disappeared from the lung after termination of OVA inhalation, but persisted in spleen and BM until the end of the analysis at day 100 after immunization. Spleen and BM plasma cells were resistant to cyclophosphamide, a drug that depletes dividing plasmablasts but not plasma cells, suggesting that plasma cells in these compartments are long-lived (100).

Further evidence for the persistence of IgE antibodies and anaphylactic responses was obtained by prolonged treatment of peanut-immunized mice with anti-CD20 antibodies, which deplete naïve and memory B cells but not plasma cells. Serum IgE antibodies and IgE plasma cells were not affected by a 15-week treatment with anti-CD20 antibodies (101).

Treatment with the proteasome inhibitor bortezomib to deplete IgE plasma cells has provided more ambiguous results, in one case reducing circulating IgE and plasma cells but not allergy (102), and in another two studies, reducing IgE and suppressing allergic reactivity (103, 104). The variable results described in studies targeting plasma cells with bortezomib could be due to different sensitivities of plasma cells populations, and to an effect of plasma cells rebounding after plasma cell depletion (105). The persistence for several weeks of IgE bound to mast cells may also explain allergic reactivity in the absence of detectable serum IgE.

A recent study using a model of oral sensitization to peanut characterized the anaphylactic response and antigen-specific IgE and IgG1 levels during 15 months after sensitization (103). While peanut-specific IgG1 antibodies persisted during this period, specific IgE antibodies were undetectable at 6 months after sensitization, a time that coincided with a large decrease in the anaphylactic response. In this model, the authors calculated the half-life of IgG1 plasma cells to be 234 days and that of IgE plasma cells to be only a quarter of that, or 60 days. By transferring serum of sensitized mice to IgE-deficient naïve mice and measuring their anaphylactic response, they estimated that the half-life of IgE bound to mast cells was 67 days, compatible with previous findings (106).

Taken together, these studies indicate that IgE plasma cells can persist in mice from a few months to a year in the absence of a new allergen exposure. Although the precise determinants of plasma cell longevity in general are under debate (section Memory B Cells), the unique factors affecting IgE plasma cell longevity are even less clear. Recent efforts have focused on describing the lifespan of IgE plasma cells and identifying factors that impact the generation of IgE plasma cells.

The Role of IgG1 Memory B Cells as Precursors of IgE Plasma Cells

Sequencing of the switch (S) region of mouse and human IgE heavy chain genes showed that the junctional Sμ-Sε sequences often contained remnants of IgG switch regions (Sμ-Sγ-Sε), specifically Sγ1 in mice, and Sγ1 and Sγ4 in humans (25, 87, 107–109). The concept of sequential immunoglobulin class switching was thus established, whereby a B cell will undergo a switch from IgM to a downstream isotype, usually IgG (generating an Sμ-Sγ hybrid S region), followed by a second sequential switch, to IgE (generating a Sμ-Sγ-Sε hybrid S region) (**Figure 2**). The biological significance of sequential switching in IgE responses was only appreciated much later. As will be discussed below, we found that sequential switching from IgG1 to IgE is essential for the generation of high affinity IgE.

Our early studies of IgE cell biology showed a predominance of IgE plasma cells, a paucity of IgE germinal center cells, and an absence of IgE memory cells (80). Nevertheless, the presence of mutations in the BCR variable region indicated that IgE antibodies underwent affinity maturation. We proposed at the

time that maturation of the IgE antibody response occurred in a precursor IgG1 cell phase, and we showed that purified IgG1 cells could undergo class switching to IgE *in vivo* and *in vitro* (80). To determine if sequential switching was necessary to produce high affinity IgE, we analyzed the IgE response in mice genetically deficient in class switching to IgG1 (82). IgG1-deficient mice, in which IgE cells were generated by direct switching from IgM cells, produced IgE at levels comparable to wild type mice after repeated immunization, but were unable to efficiently generate affinity matured IgE (82). Furthermore, we showed that $IgM^{-}IgD^{-}B220^{+}$ switched B cells (containing germinal center and memory $B220^{+}$ $IgG1^{+}$ and IgE^{+} cells) generated a recall IgE response identical to IgE-depleted switched B cells (81). These findings demonstrate that $B220^{+}$ IgE cells do not contribute to the formation of high affinity IgE plasma cells and to the memory of IgE responses. Instead, these results indicate that these functions are contained in the IgG1 population (**Figure 3**).

The study of switch regions in DNA of IgE germinal center and IgE plasma cells shed light on the developmental origin of these populations. We found that IgE germinal center switch regions did not contain Sγ1 remnants while in contrast a substantial portion of IgE plasma cells did. The percentage of IgE plasma cell switch regions with Sγ1 remnants increased after repeated immunization to approximately 60%, and was highest in the bone marrow (81, 82). These results indicate that in mice, IgE germinal center cells differentiate directly from IgM cells, whereas a substantial number of IgE plasma cells originate from IgG1 cells (81). Since Sγ1 remnants may be lost in the second recombination process, the proportion of IgE plasma cells that derive from IgG1 precursors is likely to be even higher. These findings are consistent with the concept of IgE plasma cells deriving principally from IgG1 germinal center cells or from IgG1 memory cells, rather than from IgE germinal center cells.

The Voehringer group studied the B cell repertoire in murine helminth infection, and found considerable overlap between the repertoires of IgG1 and IgE cells, indicating a common precursor origin of these two isotypes (85). Furthermore, they show that an IgE memory response could be generated by adoptive transfer of IgG1 cells (85). These results demonstrate a fundamental role for IgG1-expressing cells in the memory of IgE responses.

The findings described above are consistent with previous observations that IL-4, a cytokine necessary for class switching to IgE, is required for the production of IgE but not IgG1 in secondary responses (110). This indicates that IgE production in memory responses involves *de novo* class switching to IgE, rather than the activation of supposed IgE memory cells.

We have more recently investigated the role of different subsets of IgG1 memory cells in the IgE memory response (14). As described above (see section Memory B Cells), we identified pro-PC and pro-GC subsets of IgG1 memory cells that preferentially differentiate into IgG1 plasma cells or IgG1 germinal center cells, respectively. We found that the pro-PC IgG1 memory subset give rise to IgE plasma cells secreting high affinity IgE antibodies capable of mediating anaphylaxis (**Figure 4**). Thus, the pro-PC IgG1 memory population contains a memory for allergic responses that under conditions involving T cell help and IL-4 signaling can be activated to generate IgE plasma cells by *de novo* class switching to IgE (14).

Interestingly, the pro-GC IgG1 memory subset also gave rise to IgE plasma cells, but this IgE response was delayed and produced IgE plasma cells secreting low affinity IgE (**Figure 4**). This is intriguing, as high affinity clones were also present among the pro-GC IgG1 memory population, albeit at an approximate two-fold lower frequency than in the pro-PC population, hinting at distinct mechanisms of clonal selection operating in these two memory subsets (14).

An important observation of this study is that the vast majority of IgE cells generated from both IgG1 memory cell subsets were IgE plasma cells, with almost no IgE germinal center or memory cells being produced. This is consistent with previous findings of Sγ1 remnants in the switch regions of IgE plasma cells but not in IgE germinal center cells, which led to the conclusion that IgE germinal center cells are generated directly from IgM cells, while sequential switching from IgG1 cells only generates IgE plasma cells (81).

The differentiation potential of IgG1 memory subsets manifests differently in IgG1 and IgE progeny. IgG1 pro-PC and IgG1 pro-GC memory subsets guide their fate into IgG1 plasma cells or IgG1 germinal center cells, respectively, in a largely pre-determined B cell autonomous way. In contrast, their differentiation into IgE plasma cells is likely determined by the expression of the new IgE BCR after class switching to IgE. It is possible that IgE BCR signaling contributes to the formation of IgE plasma cells (90, 92) from IgG1 memory cells regardless of precursor IgG1 subset.

In sum, several lines of evidence obtained by various groups strongly supports an essential role for IgG1 memory cells in the generation of IgE plasma cells.

THE HUMAN IGE RESPONSE

While increasing evidence has accumulated on the generation and memory of IgE responses in mice, comparably much less is known about human IgE cell differentiation and memory. Much of the current thinking has been influenced by the experimental studies in mice. The characteristics of human IgE plasma cells and whether bona fide human IgE memory cells exist remain largely unknown. Nevertheless, a number of studies of human IgE cells *in vivo* and *in vitro*, as well as several longitudinal studies on the development of IgE and IgG antibodies, provide insights into the mechanisms of human IgE cell differentiation and memory in atopic patients, and these will be discussed in the following sections.

Human IgE Cells

In humans, the extremely low frequency of IgE cells, together with the "false positives" which derive from the stable and prolonged binding of monomeric IgE to basophils and other cells in blood, greatly hindered the study of human IgE cells. Nevertheless, human IgE plasma cells were first identified in peripheral blood of atopic and normal subjects as IgE-secreting cells by ELISPOT (111), and then by flow cytometry analysis of $CD138^{+}$ purified cellular fractions (112). IgE plasma cell

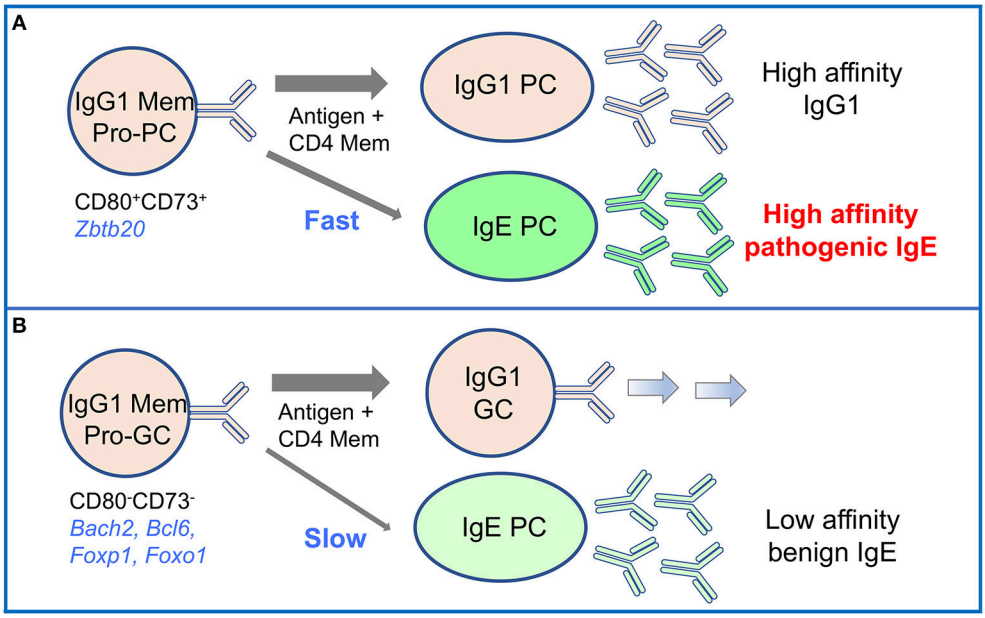

FIGURE 4 | High-affinity IgE is derived from the CD80$^+$ pro-PC subset of IgG1 memory cells in mouse memory responses. Two subsets of mouse IgG1 memory cells identified by differential expression of CD80 and CD73 have distinct differentiation potential upon activation. **(A)** CD80$^+$CD73$^+$ pro-plasma cell (Pro-PC) IgG1 memory cells generate with fast kinetics IgG1 PC and IgE PC enriched in high affinity clones. IgE antibodies derived in this pathway mediate anaphylactic responses. **(B)** CD80$^-$CD73$^-$ pro-germinal center (Pro-GC) IgG1 memory cells respond to activation with slower kinetics. They preferentially differentiate into IgG1 GC cells, and also generate a late response of low affinity non-pathogenic IgE PC.

frequency correlated with the concentration of serum IgE and with the amount of IgE produced spontaneously in cell culture. Interestingly, and in sharp contrast to mice, human IgE plasma cells were found to express low levels of membrane IgE (112). The differentiation of IgE plasma cells in the respiratory mucosa of allergic patients by direct and sequential switching was demonstrated through the identification and sequencing of switch products (113). IgE production has also been demonstrated in cultures containing respiratory mucosal explants (107, 113–115).

Although there is no direct evidence illustrating the process of human IgE cell differentiation *in vivo*, the origin of IgE cells can be partially inferred from the analysis of somatic mutations, switch recombination products, and antigen receptor repertoire, which may shed light on the memory cell subsets from which human IgE plasma cells differentiate.

Recently, the van Zelm group characterized IgE plasma cells and IgE memory cells in atopic and non-atopic subjects (116, 117). They analyzed the phenotype, replication history, and mutation rates of IgE cells in human blood and tonsil samples. They identified IgE plasma cells by CD19 and CD38 expression (CD19$^+$CD38^{hi}), but these IgE plasma cells did not express CD138. This description of IgE plasma cells contrasts with the one by Horst and collaborators described above (112). IgE plasma cells and IgE memory cells (characterized as CD19$^+$CD38^{dim}) were increased in the blood of children with atopic diseases (117). The frequency of somatic mutations in IgE plasma cells was similar to IgM plasma cells and lower than in IgG or IgA plasma cells. IgE memory cells could be further subdivided by CD27 expression. IgE$^+$CD27$^+$ memory cells had more mutations, higher replication history, and higher frequency of Sγ1 footprints in their switch regions than the IgE$^+$CD27$^-$ memory cells, indicating that IgE$^+$CD27$^+$ cells are more likely to have an IgG1 germinal center origin. Both IgE$^+$CD27$^+$ and IgE$^+$CD27$^-$ memory cells were atypical compared to other memory B cells: they expressed very low levels of the BCR signaling molecule CD79b (Igβ), and low levels of membrane immunoglobulin (116). Although these human IgE memory cells were atypical in their low BCR expression, they could differentiate into plasma cells *in vitro* (116). These findings suggest potential differences between mice and humans regarding the existence of IgE memory cells. However, given that recall responses of memory B cells depend on BCR recognition and signaling, it is unclear how adequately these abnormal IgE B cells can exert memory functions *in vivo*.

In contrast, and in support of IgE-expressing cells in humans being predominantly plasma cells, a recent study showed that circulating IgE-expressing cells in food allergic individuals were mostly plasmablasts with immature phenotype (118). Sequencing of the variable regions of single cell sorted IgE cells identified a clonal family with identical gene rearrangements in unrelated individuals. By cloning and expressing six IgE antibodies belonging to the one shared clonal family the authors showed that these antibodies had strikingly high affinity for peanut antigen Ara h2, with cross-reactivity to other peanut antigens (118). These studies propose important potential mechanisms of clonal

selection and affinity maturation of human IgE cells. Further work is necessary to determine the generality of these findings in atopic conditions and in healthy individuals.

To elucidate the ontogeny of human IgE cells, Ramadani and collaborators compared the ability of tonsillar B cell populations to generate IgE cells after stimulation with anti-CD40 antibodies and IL-4 (119, 120). Among tonsillar B cells, the IgE cells generated from germinal center cells had the highest frequency of Sγ remnants indicative of sequential switching (120), and these germinal center cells generated more IgE cells than naïve or memory cells. IgE plasmablasts and plasma cells, and IgE cells with characteristics of germinal center cells were identified in this culture system. Total tonsil B cells gave rise to IgE plasma cells by direct and sequential switching. Purified tonsil germinal center B cells generated IgE plasma cells by sequential switching, and these were the main source of IgE plasma cells. As in mice (84), more IgE cells than IgG1 cells had a plasma cell phenotype.

The membrane form of human and primate IgE, but not murine IgE, can be expressed as two different isoforms generated by alternative splicing (121). The long isoform contains an additional extracellular membrane-proximal domain (EMPD) of 52 amino acids. The long isoform was found to be transported to the plasma membrane at a slower rate than the short isoform (121), and to regulate survival and signaling in transfected mouse B cell lines (122). The two membrane isoforms of IgE have been detected *ex vivo* in human cells (30). Recent studies using retroviral transduction of a human B cell line demonstrated that the short isoform was abundant in the plasma membrane, while the long form localized mainly to the endoplasmic reticulum (ER) (123). Both isoforms of membrane IgE assembled with the BCR signaling complex Igα/Igβ, but intracellular signaling was diminished in cells expressing the long isoform. Interestingly, IgE plasma cells generated *in vitro* from tonsillar B cells downregulated the expression of the short isoform of membrane IgE while upregulating the expression of the EMPD-containing long isoform of IgE (119).

In summary, IgE plasma cells and IgE memory cells have been identified in human blood and tonsils, and germinal center-like IgE cells and IgE plasma cells have been generated *in vitro*. There is ample evidence that at least part of the human IgE cell population is generated through the sequential switching of IgG cells, and that the sequential switching and germinal center origins are associated. The existence of an *in vivo* functional population of human IgE memory remains unproved, while new data supports the predominance of the plasmablast phenotype among circulating IgE cells. Furthermore, it remains unclear whether a bona fide population of IgE germinal cells is present in humans, and whether it shares a similar pro-apoptotic phenotype as in mice.

Life-Span of Human Serum IgE Responses

Human IgE responses are diverse in magnitude, localization, and in the clinical symptoms that manifest. For example, IgE production and IgE-mediated mast cell reactivity may be mainly localized to the nasal mucosa, with low systemic impact. In other cases, widespread mast cell sensitization and systemic reactions may occur, as is the case with IgE-mediated anaphylaxis. Furthermore, IgE responses may be regulated periodically by variations in allergen exposure resulting in generation of short-lived IgE plasma cells, or may be sustained over time, either because of chronic exposure to perennial allergens, or due to long-lived IgE plasma cells. As discussed below, there is evidence for both short-lived and long-lived humoral IgE responses.

An example of seasonal variation in circulating IgE was shown in a 3-year follow up of a patient allergic to grass pollen. The seasonal increase in pollen count was immediately followed by a temporary serum increase of pollen-specific IgE and IgG4 (124). This finding demonstrates the existence of a memory response to pollen, and suggests that relatively short-lived IgE plasma cells periodically formed in the pollen season. Another study, in contrast, provides evidence of the existence of long-lived human IgE plasma cells. When patients that had been infected with filaria moved away from the endemic area and were treated with anti-helminthic therapy, they continued to have anti-filaria IgE antibodies several years after treatment, though at lower levels than before treatment (125). Newly acquired allergies to food and environmental allergens in patients that underwent allogeneic bone marrow transplantation (126–128) have been considered evidence of long-lived IgE plasma cells. While this could be the case, it is also important to consider that the bone marrow contains T and B memory cells, and these could have been responsible for the generation of allergen-specific IgE plasma cells in the transplanted individuals.

The contribution to serum IgE of long-lived IgE plasma cells vs. continuously generated short-lived IgE plasma cells could be evaluated if the production of new IgE plasma cells was inhibited. If, as suggested in some mouse and human studies, formation of new human IgE plasma cells in sensitized patients requires *de novo* class switching to IgE (rather than differentiation from true IgE memory cells), inhibiting class switching to IgE should greatly reduce the formation of new IgE plasma cells. Since class switching to IgE in humans depends on the cytokines IL-4 and IL-13, some inferences can be drawn from the treatment of atopic patients with dupilumab, an anti-IL-4Rα antibody that inhibits IL-4 and IL-13 signaling. Serum IgE levels were reduced by about 40–50% after 3–4 months of treatment of patients with asthma (129), atopic dermatitis (130), and chronic rhinitis (131). This suggests that at least part of the serum IgE pool is derived from continuous generation of new IgE plasma cells that survive only few months. As a proof that *de novo* class switching to IgE was prevented by blocking IL-4Rα signaling, patients with ongoing dupilumab treatment did not produce IgE to new vaccine antigens (132). While the dupilumab treatment suggests continuous *de novo* production of IgE plasma cells in atopic patients, it is important to point out that the reduction of serum IgE was only partial and serum IgE levels remained quite high at the end of the treatment. Thus, the existence of very long-lived IgE plasma cells in atopic patients cannot be excluded. It remains to be seen if IgE can be further reduced by longer dupilumab treatment, if allergen specific-IgE levels can decrease to the point of demonstrating prevention of allergen-driven mast cell degranulation, and how stable the changes in IgE levels are when treatment is discontinued.

The findings described above illustrate the variation in the persistence of serum IgE in different atopic conditions, which are compatible with both short-lived and long-lived human IgE plasma cells. As for other plasma cells, the microenvironment for generation and homing of IgE plasma cells may affect their long-term survival.

Is Human IgE Memory Contained in IgG Memory Cells?

Several lines of evidence are compatible with the notion that human IgG memory cells are the predominant precursors of IgE plasma cells, during allergic sensitization and in allergic memory responses. These findings derive from studies on the development and association of specific IgG and IgE responses in children and adults (**Table 2**), on the analysis of the switch-recombination history of IgE genes, and on the relatedness of the IgE and IgG immunoglobulin repertoires.

If antigen-specific human IgE is in fact generated from IgG precursors, antigen-specific IgG responses would precede and will be associated with antigen-specific IgE responses. In this context, it would be expected that IgE and IgG antibodies and plasma cells against the sensitizing allergen will be found in allergic individuals. This is in fact what has been found in several human studies. A longitudinal analysis of IgG and IgE antibodies to food (chicken ovalbumin, cow's milk β-lactoglobulin) and airborne allergens (mite and rye antigens) in children from 3 to 60 months of age detected specific IgG antibodies before the appearance of specific IgE antibodies (133). Another study followed a cohort of children at 6, 18 months, and 8 years of age. The authors found a positive correlation between IgE sensitization, clinical allergy, and high levels of specific IgG1 and IgG4 antibodies (23).

Based on cross-reactivities between airborne and food allergens, the Aalberse group hypothesized that an early IgG response against food allergens predisposes children to later IgE-sensitization to airborne allergens (19). The group found that at-risk children with high IgG against food allergens were more likely to develop IgE antibodies against airborne allergens. Recently the same group described detection of simultaneously occurring antigen-specific IgE and IgG against airborne allergens in allergic individuals (137).

Yet another study of allergic sensitization in children analyzed the correlation between IgA and IgG antibodies against wheat

TABLE 2 | Development of allergen-specific IgG antibodies is generally associated with allergen-specific IgE sensitization.

Study population age	Allergens	Findings	+ Association between IgG and IgE	References
3–60 months	Food allergens: chicken ovalbumin, cow's milk beta-lactoglobulin Airborne allergens: mite and rye.	Specific IgG antibodies were detected before the appearance of specific IgE antibodies.	Yes	(133)
3 months to 8 years	Food and airborne allergens	At-risk children with high IgG against food allergens, were more likely to develop IgE antibodies against airborne allergens.	Yes	(19)
6, 18 months, and 8 years	Food allergen: ovalbumin. Airborne allergens: pollen Bet v 1 and cat dander.	Positive correlation between IgE sensitization, clinical allergy and high levels of specific IgG1 and IgG4 antibodies.	Yes	(23)
1 and 6 years	Food and airborne allergens.	Increased IgA and IgG antibodies against gliadin or cow's milk b-lactoglobulin at age 1 were associated with IgE sensitization at age 6.	Yes	(134)
1–13 years	Food and airborne PR-10 family of allergens.	Birch-atopic children developed a strong and persistent IgG response that preceded the IgE response to PR-10 allergens. Non-atopic children developed weak and transient IgG antibody response not involving IgE.	Yes	(135)
2 and 7 years	91 purified allergens, food and airborne.	The prevalence and magnitude of allergen-specific IgG at age 2, was higher in IgE-sensitized children than in non-sensitized children at ages 2 and 7	Yes	(136)
Children and adult	Airborne allergens.	High specific IgG antibodies were found in subjects with a positive specific IgE response. Low levels of specific IgG antibodies were found in subjects with no IgE response.	Yes	(137)
2–14 years	Airborne allergens: mite and cat.	IgE sensitization to mite allergens was correlated with exposure to mites and high specific IgG and IgG4.	Yes	(138)*
		High exposure to cat allergens without IgE sensitization was associated with high specific IgG and IgG4.	No	
Adults	Bee venom	Most beekeepers, who are frequently exposed to bee venom, develop high specific IgG4 antibody responses even in the absence of IgE sensitization.	No	(139)*

** These manuscripts describe individuals with high allergen-specific IgG responses that are not associated with an IgE response.*

gliadin and cow's milk β-lactoglobulin at 1 year of age, and the development of IgE antibodies to food or inhaled allergens at age 6 (134). Increased IgA and IgG antibodies against gliadin or cow's milk β-lactoglobulin at age 1 were positively correlated with IgE sensitization in the child cohort at age 6. A recent longitudinal study of serum IgG and IgE antibody reactivity to the PR-10 family of allergens (which includes proteins in pollen and vegetable foods) was carried out in birch-allergic and non-atopic children from age 1–13 years (135). A weak and transient IgG antibody response, not involving IgE, was identified in non-atopic children. In contrast, birch-atopic children progressively developed a strong, and persistent IgG response that preceded an IgE atopic response to PR-10 allergens. The same group analyzed the correlation between IgG antibodies to a large panel of 91 purified allergens at age 2, and IgE sensitization at ages 2 and 7 (136). The authors found that both the prevalence and magnitude of allergen-specific IgG at age 2 was higher in IgE-sensitized children than in non-sensitized children at ages 2 and 7.

Thus, in general, high allergen-specific IgG responses during childhood precede or accompany IgE sensitization, and low IgG and IgA responses occur in non-allergic individuals (**Table 2**). There are some exceptions of high IgG/IgG4 responses without IgE sensitization in individuals exposed to high indoor levels of cat allergens (138), and in beekeepers repeatedly exposed to bee venom (139).

Importantly, the identification of Sγ remnants in human IgE switch regions has provided molecular validity for human IgG cells being the precursors of IgE plasma cells (107, 108, 113, 115). As discussed previously (see section Human IgE Cells), studies of the switch regions of human IgE cells isolated from peripheral blood (116, 117), or generated *in vitro* (119, 120), also identified Sγ remnants indicative of sequential switching. Consistent with the generation of human IgE cells by sequential switching, a high throughput DNA sequencing analysis of the IGH repertoire of allergic and healthy adults found that clonal lineages containing IgE members were predominantly related to IgG1 lineages (140). Sequential switching from IgG1 to isotypes other than IgE has also been described (40), but its biological significance is not yet known.

As we are proposing here that antigen-specific IgG cells are the precursors of pathogenic IgE in human allergy, we cannot ignore that IgG antibodies are also involved in protection from allergic reactions in patients that spontaneously outgrow allergies, and after successful immunotherapy (141). High levels of IgG antibodies, especially IgG4 antibodies, are found in sensitized patients that overcome allergies either spontaneously or after immunotherapy. IgG is believed to exert protection by sequestering the allergen and binding to inhibitory receptors, for example FcγRIIb in mast cells, as shown in mouse models (142). Other tolerance mechanisms are also involved in overcoming allergies, such the induction of $Foxp3^+$ regulatory T cells, IL-10 secreting B regulatory cells and Tr1 cells. This topic is not discussed here, but excellent reviews can be found elsewhere (143–145).

In the section above we discussed the evidence that allergen-specific IgG responses precede IgE-sensitization in humans, and lack of IgE sensitization is mainly associated with weak allergen-specific IgG and IgA responses. These observations, together with the molecular marks of sequential switching from IgG to IgE, and the relatedness of IgE and IgG repertoires in allergic patients, are consistent with a model whereby human pathogenic IgE responses to allergens are in large part generated from allergen-specific IgG precursors.

CONCLUDING REMARKS

We have accumulated considerable knowledge into the mechanics of the generation of IgE immune responses from studies in mice. These studies have demonstrated that mouse IgE cells follow a unique differentiation pathway characterized by an impaired germinal center phase, the predominance of the plasma cell phenotype, and a dependence on sequential switching to generate high affinity IgE. Furthermore, recent work has identified the subset of IgG1 memory cells that gives rise to high affinity IgE plasma cells.

While much less is known on the biology of human IgE cells, accumulating evidence suggests that the differentiation of human IgE cells is similar to that of mouse IgE cells. Several studies have shown that human IgE cells carry DNA footprints of sequential switching from IgG cells, and consistently, longitudinal studies in children found that allergen-specific IgG responses precede and are a risk factor for allergic sensitization. A few *in vivo* and *in vitro* studies have found that human IgE cells are predominantly plasma cells, and while circulating human IgE memory cells have been described, their function *in vivo* and their relevance for allergic disease is still unproven. Future studies utilizing the new cellular and genomic technologies, and improved humanized animal models, may shed new light on the origin, life span and unique characteristics of pathogenic IgE cells. These insights may help to design new therapies for allergic diseases.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Influences of Maternal Factors Over Offspring Allergies and the Application for Food Allergy

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The prevalence of food allergy has been steadily rising worldwide with the highest incidence noted among younger children, and increasingly recognized as a growing public concern. The first known ingestion of foods often causes allergic reaction, suggesting that sensitization of offspring with food allergens may occur during pregnancy and/or through breastfeeding. This creates a milieu that shapes the neonatal immune responses to these allergens. However, the effects of maternal allergen exposure and maternal sensitization with allergens on development of allergies in offspring remain controversial. This review discusses recent advances from human data in our understanding of how maternal factors, namely, food allergens, allergen-specific immunoglobulins, cytokines, genetics, and environmental factors transferred during pregnancy or breastfeeding influence offspring allergies and how such effects may be applicable to food allergy. Based on information obtained from mouse models of asthma and food allergy, the review also dissects the mechanisms by which maternal factors, including the impact of immune complexes, transforming growth factor-β, vitamin A, and regulatory T-cell responses, contribute to the induction of neonatal tolerance vs. development of allergic responses to maternally transferred allergens.

Keywords: allergen, asthma, breast milk, food allergy, immune complexes, immunoglobulins, *in utero*, environmental factors

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INTRODUCTION

The rise in the prevalence of allergic diseases such as asthma, pollinosis, and food allergy has continued worldwide for more than 50 years and has become a global public health concern, especially in young children. Sensitization rate to one or several allergens among school children are approaching 40 to 50% worldwide (1). Consistently, a recent cohort study identified 32% of children in southern Sweden suffered with at least one allergic disease and 33% of them develop multiple allergies (2).

Food allergy currently affects 5–8% of the United States population (3). The prevalence of food allergy in young children could grow as rapidly as 1% in a decade (4). Diagnosis of food allergy by food challenge or a combination of history of clinical symptoms and indication of food-specific immunoglobulin (Ig) E by skin prick tests or blood tests (5) provide compelling

data that the incidence of food allergy is increasing in both Western and developing countries (6). Such escalation in the prevalence of food allergy has been stated as the second wave of the allergy epidemic after asthma (6). Food allergy is adverse immune responses to normally harmless food allergens that result in clinical symptoms implicating the dermatologic, respiratory, gastrointestinal, cardiovascular, and neurologic systems. Eight major food allergens (milk, egg, peanut, tree nuts, wheat, soy, fish, and crustacean shellfish) (7) cause most of the serious food allergy reactions in the United States. Peanut is the most common food allergen in Europe and in the United States and its prevalence in young population under 18-year-old increased from 0.2% in 1997 to 1.1% in 2008 (8). Food allergy is the most common reasons for potentially life-threatening anaphylaxis in children. The numbers of visit to a large academic pediatric emergency department for food-induced anaphylaxis has been more than doubled from 2001 to 2006 (9). There are currently limited options of management and treatment for food allergy, including avoidance of causal allergic foods, treatment of accidental exposures with epinephrine, and allergen-specific immunotherapy (10), and additional therapeutic and preventive approaches are urgently needed.

The first known ingestion of foods often causes allergic reactions (11), suggesting that exposure of offspring to food allergens that shape neonatal immune system may occur during pregnancy and/or through breastfeeding. However, the effects of maternal factors on the development of food allergy in offspring are poorly understood. Epidemiological studies in humans have shown conflicting results regarding the role of maternal allergen exposure in the development of allergies in offspring. Past studies have identified an increased risk (12) or no association (13) of maternal peanut consumption with peanut sensitization in offspring. In contrast, maternal exposure and sensitization to food allergens could be protective of offspring from allergic diseases in humans and in mice (14–19). In this review, we highlight recent research advances in the understandings from mouse and human studies of protective and adverse impacts of maternal factors in the development of allergies and food allergy in children with a focus on maternal factors including allergens, immunoglobulins (Igs), immune complexes (IC), cytokines transferred from mothers to offspring *in utero* and via breast milk, together with genetic and environmental factors that could further facilitate the neonatal immune responses to allergens.

MATERNAL PROTECTIVE INFLUENCES OVER OFFSPRING ALLERGY

Human Studies

Maternal allergen consumption during their pregnancy and breastfeeding has been thought to control allergen sensitization in offspring, because first contact to food allergens could occur *in utero* as major food allergens could appear in amniotic fluid in an intact form (20). Contrarily, maternal nutrition status, allergens, and Igs, transferred *in utero* and via breast milk may prevent allergic sensitization in children. Two decades ago, United Kingdom Government's Chief Medical Officer's Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) recommended that atopic mothers should avoid consumption of peanut and peanut products during pregnancy and breastfeeding to prevent peanut allergy in offspring. Following this recommendation, however, the prevalence of peanut allergy in school-age children increased and even resulted in the highest prevalence of peanut allergy in 4- to 5-year-old children (21). These data indicate no significant preventive effect by maternal allergen avoidance. Further, maternal dietary restriction during pregnancy or breastfeeding that aimed to prevent offspring allergy did not show a significant protective effect, instead, resulted in a lower gestational weight gain or adverse effects in maternal nutrition and fetal growth (22, 23). More recent studies have implied that the effect of maternal diet should be considered together with postnatal introduction of food in offspring (24–26). These studies underscore the requirement of alternative strategies rather than maternal dietary antigen avoidance for the prevention of food allergy (**Table 1**). In this section, we focus on the effects of maternal nutrition *in utero* and via breastfeeding on prevention of allergies in children.

In utero Factors

Food allergen consumption

Reducing the risk of allergy by dietary means is a logical response to the increase in food allergy and other allergic diseases. In contrast to maternal allergen avoidance, prenatal consumption of potentially allergenic foods has been shown to prevent allergic sensitization in children. A study enrolled 6,288 children in Finland showed an association between high ingestion of milk products during pregnancy and a lower risk of cow's milk allergy in children [odds ratio (OR), 0.56] (27). The preventive effects were observed in children of non-allergic mother (OR, 0.30). Maternal ingestion of milk products was correlated with levels of beta-casein-specific IgA in cord blood in children without cow's milk allergy. Consequently, the study suggested that maternal milk ingestion during pregnancy exhibits tolerogenic effects *in utero* especially in non-allergic mothers. In a recent prospective study with 8,205 children between 10- and 14-year-old, the prevalence of peanut or tree nuts allergy in offspring was lower in children of non-allergic mothers who ingested at least five servings of peanut/tree nuts per week during pregnancy (OR, 0.31) (15). However, there was no association of maternal consumption of peanut/tree nuts during pregnancy and the risk of peanut/tree nuts allergy in offspring of mothers who were allergic to peanut/tree nuts, indicating that this preventive effect may be operative in non-allergic mothers but not in allergic mothers (15). Another cohort study in United States enrolled 1,277 mother-child pairs reported that maternal diet during pregnancy was associated with decreased allergy and asthma in mid-childhood (mean age, 7.9-year-old) (14). Higher maternal consumption of peanut during the first trimester was associated

Abbreviations: AIT, Allergen-specific immunotherapy; aRR, Adjusted rate ratio; CT, cholera toxin; DC, dendritic cell; FcRn, neonatal Fc receptor; Foxp3, forkhead box p3; IC, immunecomplex; IFN, interferon; Ig, immunoglobulin; IL, interleukin; i.p., intraperitoneal; mMCP1, mouse mast cell protease I; OR, odds ratio; OVA, ovalbumin; PUFAs, polyunsaturated fatty acids; sIgA, secretary IgA; TGF-β, tumor growth factor β; Th, helper T cell; Treg, regulatory T cell; WT, wild type.

TABLE 1 | Maternal and offspring food consumption and the outcomes in offspring allergy in human cohort studies.

Readout	Population/size	Dose/duration/timing	Association/outcome	Effects	References
IN UTERO					
Cow's milk allergy in children	6,288 children	During pregnancy	Association of high consumption of milk products during pregnancy with a lower risk of cow's milk allergy in offspring of non-allergic mothers	Protective	(27)
Peanut or Tree nuts allergy in offspring	8,205 children (10 to 14 years of age)	During pregnancy	· Association of maternal consumption of at least five servings of peanut/tree nuts per week with a lower prevalence of peanut or tree nut allergy in offspring of non-allergic mothers	· Protective (non-allergic mothers)	(15)
			· No association of maternal consumption of peanut/tree nuts with the prevalence of peanut or tree nuts allergy in offspring of peanut/tree nuts-allergic mothers	· No association (allergic mothers)	
Allergy and asthma in mid-childhood	1,277 mother-child pairs (7.93 ± 0.82 years of age)	During pregnancy	Association of higher maternal consumption of (1) peanut during the first trimester with a reduced risk of peanut allergic reaction, (2) milk during the first trimester with reduced asthma and allergic rhinitis, and (3) wheat during the second trimester with reduced atopic dermatitis in offspring	Protective	(14)
Peanut sensitization in offspring	503 infants (3 to 15 months of age) with likely milk or egg allergy without previous diagnosis of peanut allergy	· The first or second trimester of pregnancy or during breastfeeding	· No significant effect of maternal peanut consumption on offspring peanut sensitization	· No association	(12)
		· The third trimester of pregnancy	· A dose-dependent association of maternal peanut consumption during the third trimester of pregnancy with peanut sensitization in offspring	· Allergic	
		· Never breastfed with frequent consumption of peanut during pregnancy	· A strong association of maternal peanut consumption with peanut sensitization in offspring	· Allergic	
Peanut allergy	13,971 children of non-atopic and atopic mothers (1 to 18 months of age)	During pregnancy	No association of maternal peanut consumption during pregnancy with challenge-proven peanut allergy in preschool children	No association	(13)
Maternal avoidance and avoidance of solid and allergic food in childhood	288 infants of atopic parents (4, 12, and 24 months of age)	· Maternal food avoidance during the third trimester of pregnancy and breastfeeding period	· Association of maternal food avoidance with a lower cumulative prevalence of atopy in offspring at 12 months, occurring from reduced food-associated atopic dermatitis, urticaria, and/or gastrointestinal disease, as compared to offspring of mothers with unrestricted diets	· Protective	(28)
		· Offspring avoidance of solid food for 6 months, avoidance of cow's milk, corn, citrus, and wheat for 12 months, and avoidance of egg, peanut, and fish for 24 months	· Association of offspring food avoidance with a significant suppression of the prevalence of food allergy and milk sensitization before 2 years of age.	· Protective (before 2 years of age)	(29)
			· No association of offspring food avoidance with the prevalence in food allergy, atopic dermatitis, allergic rhinitis, asthma, any atopic disease, and serum IgE levels at 7 years of age	· No association (at 7 years of age)	

(Continued)

TABLE 1 | Continued

Readout	Population/size	Dose/duration/timing	Association/outcome	Effects	References
BREASTFEEDING					
Peanut sensitization at 7 years of age	342 infants (7 years of age)	During breastfeeding and direct peanut introduction to infants	· Lowest prevalence of peanut sensitization in infants whose mothers consumed peanut while breastfeeding with an early direct introduction of peanut before 12 months of age	· Protective (infants breastfed with early introduction)	(26)
			· Increased risk of peanut sensitization in infants whose mothers consumed peanut while breastfeeding with delayed introduction of peanut beyond 12 months of age, or maternal peanut avoidance with an early direct introduction of peanut by 12 months of age	· Allergic (infants breastfed with delayed introduction)	
Milk sensitization	186 children (0 to 4 years of age)	Breastfeeding more than 6 months	Exclusive or partial breastfeeding for more than 6 months reduced milk sensitization in children at 1 and 1.5 years of age as compared to formula-fed children.	Protective	(30)
Food allergy	649 children with challenge-proven food allergy (5 to 214 months of age)	Direct breastfeeding or bottle-feeding	· Every additional month of breastfeeding decreased a risk for food allergy by ∼4% per month. · No significant association between the prevalence of food allergy and direct breastfeeding vs. bottle-feeding	Protective	(31)
Childhood asthma	3,296 children (3 years of age)	· Direct breastfeeding	· Direct breastfeeding was more protective against childhood asthma at 3 years of age as compared to formula-feeding.	· Protective	(32)
		· Indirect breastfeeding	· Indirect breastfeeding (breastfeeding with some expressed breast milk) provided an intermediate protection as compared to direct breastfeeding.	· Protective	
Offspring wheezing	2,773 infants (3, 6, 12 months of age)	Breastfeeding with maternal asthma	· Offspring wheezing was reduced by 62% with exclusive breastfeeding and by 37% with partial breastfeeding supplemented with complementary foods as compared to infants with no breastfeeding.	· Protective (exclusive and partial breastfeeding supplemented with foods)	(33)
			· Offspring wheezing was not protected by partial breastfeeding supplemented with formula.	· No association (partial breastfeeding with formula)	
EARLY INTRODUCTION					
Peanut allergy (LEAP)	834 infants (4 to 10 months of age) at high risk for allergy	Food introduction between 4 and 10 months of age	· Among the infants initially negative for peanut skin-prick test, the prevalence of peanut allergy at 60 months of age was lower in the consumption group than those in the avoidance group.	· Protective (skin-prick test negative infants)	(34, 35)
			· Among the infants initially positive for peanut skin-prick test, the prevalence of peanut allergy was lower in the consumption group than those in the avoidance group.	· Protective (skin-prick test positive infants)	

(Continued)

TABLE 1 | Continued

Readout	Population/size	Dose/duration/timing	Association/outcome	Effects	References
Food allergy (EAT)	1,303 infants (3 months of age)	Food introduction before 6 months of age	· Multiple allergenic foods can be safely introduced into infant diet without adverse influence on breastfeeding. · Significantly lower prevalence of any food allergy in the early introduction group in families that closely adhered to the challenging protocol	· Safe early introduction of allergenic foods · Protective	(36)

EAT, Enquiring About Tolerance; LEAP, Learning Early About Peanut Allergy.

with 47% reduced odds of peanut allergen reaction (OR, 0.53). Higher maternal milk ingestion during the first trimester was also associated with reduced risk of asthma (OR, 0.83) and allergic rhinitis (OR, 0.85). Maternal consumption of wheat during the second trimester was associated with reduced risk of atopic dermatitis (OR, 0.64). These results indicate that early encounter with food allergens through maternal diet during the critical period of fetal development during the first trimester may lead to tolerance rather than sensitization. Collectively, these studies suggest that maternal allergen consumption during pregnancy could be beneficial to prevent allergic sensitization in offspring.

Allergen-specific Igs

Placental transfer of maternal Igs is known to provide a protective passive immunity to the fetus against infection (37). The selective transport of maternal IgG to the fetus during pregnancy is mediated by the neonatal Fc receptor (FcRn) expressed in placental syncytiotrophoblast (38). Allergen-specific immunotherapy (AIT) appears safe in pregnant women (39) and placental transfer of allergen-specific IgG1 and IgG4 antibodies, but not IgE, from AIT-treated mothers to offspring has been shown (40). Consistently, children of AIT-treated mothers during pregnancy exhibited high levels of IgG antibodies and fewer positive skin prick test as compared with children of untreated mothers (41). Increased levels of cord blood IgG antibodies to cat and birch were associated with less atopic symptoms in the children of allergic mothers during the first 8 years of life (42). These results suggest that maternal allergen-specific IgG antibodies may reduce the development of offspring atopy, but further data are required regarding a potential protective role of placentally transferred allergen-specific IgG toward allergic diseases, particularly food allergy in offspring.

Microbiota

Over the last decade, the importance of commensal bacteria in the host immune responses has been increasingly recognized, particularly bacteria colonizing the gastrointestinal tract. The composition of intestinal microbiota in newborn infants is dependent on many factors, such as type of maternal deliveries. Cesarean delivery has been associated with the incidence of atopy and allergic diseases in children, including atopic dermatitis, rhinitis, asthma, and eosinophilic esophagitis (43–46). Through vaginal delivery, offspring ingest maternal vaginal and colonic microbiota with passage through the birth canal, which probably represents the most important phase of initial gut colonization. This exposure of newborn infants to maternal bacteria results in stimulation of toll-like receptor (TLR) and production of interleukin (IL)-12 and interferon (IFN)-γ, which promote the differentiation of naïve helper T (Th) cells into Th1 effector cells that secrete IFN-γ (44, 47). Cesarean section was associated with reduced pro-inflammatory cytokine responses to TLR1/2 stimulation, followed by an increased abundance of bacterial colonization in the airway during late infancy, therefore increasing the risk of infantile wheezing (48). Levels of transforming growth factor (TGF)-β1 were lower in newborns following cesarean section (49). Hence, newborns born through vaginal delivery and exposed to higher levels of TGF-β1 may exhibit more protection toward atopy at later life, potentially reflecting the capacities of TGF-β1 to skew the production of IgE toward non-inflammatory isotypes IgG4 and IgA (50). Additional long-term studies of investigating maternal microbiota and its relationship with infant microbiota, as well as the development of infant food allergy are needed.

Dietary supplements

Development of human immune system begins *in utero* and continues after birth (51). Nutrition influences the global health and disease of children in early life. During the last decade, many examples showed prevention of allergic sensitization in children by modifying maternal diet during pregnancy (52, 53), indicating an *in utero* programming of allergic susceptibility in offspring. Increased levels of 25-hydroxyvitamin D (25(OH)D) in cord blood have been inversely associated with the risk of asthma and wheeze during childhood (54). Rationally, the association of maternal vitamin D supplementation with the development of atopic diseases in offspring has been investigated. In Vitamin D Antenatal Asthma Reduction Trial (VDAART), 806 women between the gestational ages of 10 to 18 weeks were supplemented with daily vitamin D for 22 to 30 weeks (55). Maternal supplementation with high-dose (4,000 IU/d) vitamin D reduced the risk of asthma and recurrent wheezing in offspring by 3 years of age (56, 57). In Copenhagen Prospective Studies on Asthma in Childhood in 2010 (COPSAC2010), 623 pregnant women were administered daily 2,400 IU/d vitamin D3 from the gestational ages of 24 week to 1 week postpartum (58). Combined analysis of these two studies demonstrated 25% reduced risk

of asthma and recurrent wheeze at 0–3 years of age (adjusted OR, 0.74; $p = 0.02$) by maternal vitamin D supplementation (59). Of note, high concentration of serum maternal 25(OH)D was associated with an increased incidence of allergic airway disease in offspring at 20–25 years of age (60). With regard to food allergy, vitamin D insufficiency early in life has been associated with the risk of food allergy (61). In addition, maternal prenatal vitamin D supplementation was inversely associated with the development of food allergy, especially in infants with polymorphisms that increases the biological availability of serum vitamin D (62). These heterogeneous findings may reflect the importance of context, timing, and dose of vitamin D in the actions on the offspring allergies and potential differences in the mechanisms of asthma and food allergy.

Early exposure of omega 6 (n-6) and omega 3 (n-3) polyunsaturated fatty acids (PUFAs), commonly found in many nuts, seeds, and vegetable oils, during pregnancy shows different capacities to alter the incidence of atopic sensitization and allergy in children (63). Arachidonic acid (20:4, n-6) is a catabolite of linoleic acid (18:2, n-6) and a main substrate for eicosanoids, which are a family of lipid mediators that induce inflammation (63, 64). Higher consumption of n-3, such as docosahexaenoic acid and eicosapentaenoic acid primarily found in certain fish, decreased the levels of inflammatory eicosanoids from arachidonic acid and increased the levels of alternative eicosanoids that have anti-inflammatory properties (63). Maternal intake of oily fish containing higher docosahexaenoic acid and eicosapentaenoic acid as well as fish oil supplements showed protective effects on wheeze and asthma in offspring (65). Similarly, maternal fish oil supplementation reduced the severity of atopic eczema and the risk of egg sensitization in 1-year-old offspring (66). High maternal intake of α-linoleic acid (18:3), another n-3 found in plant sources such as nuts and seeds, was associated with decreased risk of wheeze in Japanese children at 16- to 24-month-old (67). Taken together, maternal intake of n-3 PUFAs may have beneficial effects on preventing atopic disorders in offspring, including wheeze and asthma (63), although its preventive effect against food allergy is not clear (68). Whether these results reflect a generally healthier diet remains to be determined. The role of n-6 in offspring allergy will be discussed in section Dietary Supplements in Human Studies for maternal allergic influences.

Breastfeeding

Food allergen consumption

The recent clinical report by American Association of Pediatrics (AAP) proposes that no conclusions can be drawn regarding the effects of breastfeeding on preventing or delaying the development of food allergies in offspring (69), however, the report also recognizes that there is now evidence that early peanut introduction may prevent peanut allergy, as discussed below. The recent groundbreaking Learning Early About Peanut Allergy (LEAP) trial in the UK showed that introducing peanuts to infants at high risk for allergy at 4 to 10 months of age reduces the incidence of peanut allergy (34). The study recruited 834 infants with mild eczema with no egg allergy and infants with severe eczema, egg allergy, or both and subdivided based on peanut wheal responses by skin prick test into 0-, 1- to 4-, and >4-mm groups (35). All infants with mild eczema with no egg allergy showed no peanut-specific IgE sensitization, but 91% of the group with >4-mm peanut wheal responses exhibited peanut-specific IgE sensitization. The infants at the high risk for peanut allergy were randomly assigned to ingest or avoid peanut until 60 months of age ($N = 640$). Among the infants who were initially negative for skin-prick test ($N = 530$), the prevalence of peanut allergy at 60 months of age was considerably lower in the consumption group (1.9%) than those in the avoidance group (13.7%). Among the infants who were initially positive for skin-prick test ($N = 98$), the prevalence of peanut allergy was 10.6% and 35.3% in the consumption group and in the avoidance group, respectively ($p = 0.004$). Interestingly, levels of peanut-specific IgG4 were increased in the consumption group whereas levels of peanut-specific IgE were increased in the avoidance group. The study concluded that early introduction of peanut reduced the development of peanut allergy in high-risk children and that early peanut introduction does not prevent other allergies (asthma, seasonal rhinoconjunctivitis, perennial rhinoconjunctivitis, and eczema), sensitization to other food allergens (egg, milk) and aeroallergens, or reported allergic reactions to tree nuts and sesame (70). A follow-up Enquiring About Tolerance (EAT) trial in the UK with 1,303 infants demonstrated that early introduction of 6 allergenic foods [cow's milk, peanut, hard-boiled hen's egg, sesame, whitefish (cod), and wheat] was achievable by 6 months of age and did not affect breastfeeding (36). The study recruited exclusively breast-fed infants from 3 months of age and randomly assigned to standard introduction group (SIG) or early introduction group (EIG). The EIG was introduced 6 allergenic foods alongside continuous breastfeeding until at least 6 months of age. The study showed multiple allergenic foods can be safely introduced into infant diet without adverse influence on breastfeeding. The results failed to show better efficacy of early introduction in the prevention of food allergy to one or more of the 6 intervention foods: food allergy developed in 7.1 and 5.6% of the participants in the SIG and in the EIG, respectively ($p = 0.32$). However, in families that closely adhered to the challenging protocol, the prevalence of any food allergy was considerably lower in the EIG than in the SIG (2.4 vs. 7.3%, $p = 0.01$), as was the incidence of peanut allergy (0 vs. 2.5%, $p = 0.003$) and egg allergy (1.4 vs. 5.5%, $p = 0.009$). There was no significant effect with respect to the prevalence of allergy to milk, sesame, fish, or wheat. These studies suggest that the early introduction of all 6 allergic foods, although not easily achieved, may have beneficial effects in preventing food-allergen sensitization in high-risk children.

In addition to early direct food introduction to children, maternal consumption of potentially allergic foods during breastfeeding has been suggested to prevent food sensitization in offspring. A Canadian cohort study with 342 infants analyzed the association of maternal peanut consumption while breastfeeding, timing of direct peanut introduction to infants, and a combination of both, with peanut sensitization at 7 years of age (26). Breastfeeding and peanut consumption by mothers and infants were captured by repeated questionnaires during infancy. The lowest prevalence of sensitization with peanut was observed

in infants whose mothers consumed peanut while breastfeeding and who were directly introduced peanut before 12 months of age (1.7%). Maternal peanut consumption with delayed introduction of peanut to infants beyond 12 months of age or maternal peanut avoidance with a direct introduction of peanut by 12 months of age was associated with an increased risk of peanut sensitization ($p = 0.003$) (15.1 and 17.6%, respectively). These results suggest that a combination, but not alone, of maternal peanut consumption while breastfeeding and direct introduction of peanut in the first year of life provides a significant protection from peanut sensitization. Although the readout was peanut sensitization and not challenge-proven peanut allergy, this study raises an important point that the combination of maternal and offspring factors together during breastfeeding period plays a critical role in the prevention of food allergy in offspring.

Allergen, allergen-specific Igs, and cytokine

Maternal food allergen consumption results in an appearance of allergen in breast milk, though not always. Ara h 2 and Ara h 6 are detectable in breast milk (71). Ara h 6 appeared in breast milk from non-atopic peanut-tolerant mothers as early as 10 min after peanut ingestion and continued being detected over a 24-h period (72). IC consisting of allergen and allergen-specific IgG and IgA were present in breast milk. Such human milk containing peanut allergens and IC induced a partial oral tolerance in peanut-sensitized young mice (72), indicating a protective role of milk-born allergen in reducing disease susceptibility in offspring. Microarray technology revealed that allergen-specific IgG and IgE antibodies are transmitted from maternal blood into breast milk and that breast milk IgG pattern mirrored the profile of IgG reactivity in maternal blood (73), which suggest a possibility that the transmission of allergen-specific Igs from mothers to offspring via milk may affect the development of allergy in neonates. Our recent study demonstrated that allergen-specific IgG in milk may play a crucial role in protection of offspring from food allergy as IC. Our study showed that ovalbumin (OVA)-specific IgG4 was detectable in 10 subjects among 16 breast milk samples from non-atopic mothers. OVA-IgG4-IC were detectable in 8 of 10 mothers who had OVA-IgG4 (74). Importantly, supplementation of humanized mice expressing human FcRn constitutively with human breast milk containing OVA-IgG-IC resulted in protection of those mice against food allergic responses, suggesting a critical role of maternal IgG-IC in breast milk in the induction of tolerance against food allergy. These results, together with our mouse studies as discussed below (section Breastfeeding in Mouse Studies for maternal protective influences), imply that the concept of IgG-IC contributing to protection against food allergy may extend to humans (74).

Maternal cow's milk avoidance was associated with lower levels of milk allergen-specific IgA and IgG4 antibodies and the development of cow's milk allergy in infants (75). High concentration of milk allergen-specific IgA in breast milk significantly reduced transcytosis of milk allergen through gut lumen, suggesting that IgA may prevents excessive, uncontrolled food antigen uptake in the gut lumen and thus in the prevention of cow's milk allergy. The Prediction of Allergies in Taiwanese Children (PATCH) birth cohort study assessed levels of secretary IgA (sIgA) in colostrum and stool from infants within 5 days after birth and at 2 and 4 months of age (76). sIgA concentration in colostrum was significantly higher in allergic mothers than those in non-allergic mothers ($p = 0.01$). Fecal sIgA concentration in breastfed infants was significantly higher in those in formula-fed infants ($p < 0.05$). The association of breastfeeding with the increased levels of fecal sIgA in infants may suggest potential protective effects in early life.

A systematic review suggests a positive association between concentration of TGF-β1 or TGF-β2 in breast milk and a reduction in IgE titer and atopic inflammation in infancy and early childhood (77). In contrast, a prospective study with 398 mothers demonstrated an association of higher eczema risk with higher concentration of TGF-β2 in breast milk (OR, 1.04) at 6 months of age (78). Interestingly, detectable levels of IL-13 in colostrum were associated with lower parental-reported food allergy (OR, 0.10) at 6 months of age (78). High levels of IL-1β, IL-17, and C-C motif chemokine ligand (CCL) 17 (TARC) and low levels of C-X-C motif chemokine ligand (CXCL) 1 and thymic stromal lymphopoietin (TSLP) in breast milk were associated with a low risk of eczema at 0–3 years of age (79). Although the mechanisms are unknown and further large clinical studies are needed to confirm these results, allergens, IgG, IgA, and regulatory cytokines such as TGF-β in breast milk likely orchestrate to develop and maintain appropriate neonatal immune responses and may prevent adverse immunological outcomes in infants.

Breastfeeding duration, feeding modes, and maternal allergy

Although the beneficial effects of breastfeeding on global health of offspring are recognized, the effects of breastfeeding on the development of allergic diseases in offspring is controversial and remains largely elusive. Regarding the potential beneficial effects of breastfeeding duration on preventing allergies in children has been well-summarized in a recent review by American Academy of Pediatrics (69), although the available human data are still not sufficient to draw firm conclusions regarding various aspects of allergy prevention. Briefly, there is evidence that exclusive breastfeeding for 3 to 4 months decreases the development of eczema before 2 years of age, but there are no short- or long-term benefits of exclusive breastfeeding beyond 3 to 4 months for prevention of atopic diseases (69). Any duration of breastfeeding mode than 3 to 4 months is protective toward wheezing in the first 2 years of life (69). Some evidence suggests that longer duration of any mode of breastfeeding, including partial and exclusive breastfeeding, protects toward asthma even after 5 years of age (69). The role of breastfeeding duration in food allergies is much less clear. A cohort study in Taiwan enrolled 186 children demonstrated that exclusive or partial breastfeeding for longer than 6 months reduced milk sensitization in children at 1 and 1.5 years of age (30) compared to formula-fed children (OR, 0.37). A retrospective cohort study including 649 children with challenge-proven food allergy (31) demonstrated that every additional month of breastfeeding decreased a risk for food allergy by ∼4% per month (OR, 0.96; $p = 0.02$). Within breastfed children, there was no significant association between the incidence of food allergy and direct breastfeeding

vs. bottle-feeding ($p = 0.27$). Collectively, these studies indicate the association of prolonged breastfeeding with reduced risk of allergic sensitization and food allergy. Regarding feeding modes, a prospective birth cohort study in Canada with 3,296 children examined the effects of infant feeding modes on childhood asthma (32). Among direct breastfeeding alone, breastfeeding with some expressed breast milk, breast milk plus formula, or formula alone, direct breastfeeding was more protective against childhood asthma at 3 years of age as compared to formula-feeding. Indirect breast milk by other modes of infant feeding conferred intermediate protection. The differences in the preventive effects of direct breastfeeding vs. expressed breast milk may reflect a possible alternation of milk factors such as immune cells, cytokines, and microbiota. Maternal allergy may influence the effects of breastfeeding on allergic sensitization in children. A Canadian study that included 2,773 infants compared the effects of breastfeeding on offspring wheezing in the presence or the absence of maternal asthma (33). In the presence of maternal asthma, development of wheezing in infants was reduced by 62% with exclusive breastfeeding [adjusted rate ratio (aRR), 0.38] and by 37% with partial breastfeeding (supplemented with solid foods; aRR, 0.38) as compared to infants with no breastfeeding. Partial breastfeeding supplemented with formula was not protective for wheezing in infants (aRR, 0.89). Importantly, such association was not significant in the absence of maternal asthma. The role of breastfeeding duration, feeding modes, and maternal allergies in the development of offspring food allergy needs intensive future studies, however, these results above may favor the concept that longer period of breastfeeding confers protection against allergic diseases in infants.

Perspective

Maternal diet is not only an important factor for growth of fetus but also likely shapes neonatal immune responses and determines the susceptibilities of offspring to infectious diseases and allergies (80). Nutrition, allergens, Igs, cytokines, and also immune cells are transferred from mothers to neonates via placenta and during breastfeeding and may influence offspring allergy (**Table 2**). Direct breastfeeding may be beneficial to efficiently transfer protective factors such as microbiota from mothers to offspring. Human studies show conflicting results and whether and how maternal factors transferred *in utero*, breastfeeding, or both, exert preventive effects toward allergies in children (**Tables 1**, **2**). Future cohort study and meta-analysis with systematic data of allergen consumption (maternal and offspring), immune active milk components together with environmental and genetic factors are needed to answer those questions.

Mouse Studies

Substantial numbers of mouse studies have reported preventive effects of maternal factors on development of allergic inflammation in offspring such as allergic airway inflammation and food allergic responses (**Table 3**). Offspring of dams sensitized with allergen or offspring nursed by allergen-sensitized or -exposed dams during breastfeeding show protection from the development of antigen-specific IgE and allergic inflammation following sensitization with allergen (74, 119). Many of these studies focus on the role of maternally transferred allergens and IgG via placenta and breast milk to offspring.

Preconceptional and *in utero* Factors

Allergen, allergen-specific Igs, and cytokine

Several studies reported preventive effects on allergic sensitization and allergic inflammation in offspring born from dams sensitized with or exposed to allergen. Oral administration of wild-type (WT) dams with OVA during pregnancy has been shown to decrease allergen sensitization in offspring (99). Oral administration of dams with high-dose OVA (9 mg) during the first week of pregnancy resulted in a suppression of OVA-specific IgE levels in 3-day-old, but not in 25-day-old, offspring following *intraperitoneal* (*i.p.*) sensitization with OVA/alum and *i.p.* OVA injection (99). Oral administration of dams with low-dose OVA (1.5 mg) during pregnancy resulted in a sustained inhibition of OVA-specific IgE levels both in 3- and 25-day-old offspring. Fifty-seven percent of $CD4^+$ T cells from offspring of OVA-fed DO11.10 dams (transgenic mice bearing OVA-specific T-cell receptor) proceeded into S phase 72-h after *in vitro* stimulation with OVA. In contrast, the majority of $CD4^+$ T cells from offspring of unexposed DO11.10 dams were arrested in G_0/G_1 phase, suggesting that offspring T-cells are primed *in utero* by oral administration of dams with allergen. It is noteworthy that OVA was undetectable in amniotic fluid, placental serum, or breast milk from OVA-fed dams during pregnancy. In contrast, when dams were orally administered during the first week of breastfeeding, OVA was detectable in milk from OVA-fed dams in proportion to the dam's intake dose. Levels of maternal allergen-specific Igs were not examined in this study. These results suggest that oral exposure of dams to low-dose allergen during pregnancy may result in T-cell priming to allergen *in utero*, although whether maternal Igs are involved in this setting is not clear. Intranasal exposure of WT dams with OVA before conception resulted in the induction of tolerance in offspring toward allergic airway inflammation as well as allergen-specific Th2 responses following *i.p.* sensitization with OVA and alum and intranasal OVA challenge (100). Interestingly, the preventive effects against allergic inflammation were completely abolished in B-cell deficient μMT dams. The study proposes that OVA-IgG1-IC bind to CD16 and CD32 Fcγ receptors (FcγR) on dendritic cells (DCs) and promote an efficient allergen-priming by DCs to promote differentiation of forkhead box p3 $(Foxp3)^+$ regulatory T cells (Treg) *in utero*, suggesting a role of allergen-IgG-IC in the induction of neonatal tolerance during pregnancy. More recent study demonstrated that anti-IgE antibody treatment of pregnant dams prevents allergen-specific IgE production in offspring following allergen sensitization (120).

Effects of maternal IFN-γ on development of offspring asthma were investigated in dams treated with IFN-γ (101). Dams were *i.p.* injected with IFN-γ during pregnancy, then offspring were fostered after birth and nursed by normal dams untreated with IFN-γ (IFN/Nor). Conversely, offspring of naïve dams were fostered and nursed by dams treated with IFN-γ (Nor/IFN) or untreated normal dams (Nor/Nor). Following OVA inhalation, both IFN/Nor and Nor/IFN offspring showed

TABLE 2 | Maternal protective and risk factors for offspring allergy in human studies.

Factors	Association/outcome	Effects	References
PRENATAL MATERNAL HEALTH AND ENVIRONMENT			
Maternal iron concentration	Maternal serum concentration of ferritin status during the first-trimester of pregnancy was inversely correlated with risk of childhood wheeze and impaired lung function.	Protective (childhood wheeze)	(81)
Dexamethasone	Prenatal exposure of preterm infants to dexamethasone increased their susceptibility to allergic diseases, particularly asthma and allergic rhinitis.	Allergic (asthma, allergic rhinitis)	(82)
H2 blocker	Maternal exposure to H2 blockers or proton pump inhibitors during pregnancy increased the risk of developing asthma.	Allergic (asthma)	(83, 84)
Preeclampsia, pre-pregnancy overweight, and obesity	Preeclampsia, maternal pre-pregnancy overweight, and maternal obesity were associated with an increased risk of wheezing in offspring.	Allergic (wheezing)	(85)
Phthalate	· High levels of maternal exposure to phthalate during pregnancy reduced the number of Treg cells in cord blood as well as in children at 2 years of age.	· Allergic (atopic dermatitis)	(86)
	· Prenatal high concentrations of phthalate metabolites in maternal urine were significantly associated with a higher risk for atopic dermatitis in children until 3 years of age.	· Allergic (atopic dermatitis)	
Maternal adverse life events	Prenatal maternal adverse life events were suggested to link an increased risk for the development of atopic disorders in offspring.	Allergic (asthma, eczema)	(87)
Vaginal delivery	Protection toward atopy at later life, potentially reflecting the capacities of TGF-β1 to skew the production of IgE toward non-inflammatory isotypes IgG4 and IgA	Protective (atopy)	(49, 50)
Cesarean section	· Associated with decreased pro-inflammatory cytokine responses to TLR1/2 stimulation, followed by a higher abundance of bacterial colonization in the airway during late infancy, and increasing the risk of infantile wheezing	· Allergic (wheezing)	(48)
	· Lower levels of TGF-β1 in newborns	· Allergic (atopy)	(49)
Vitamin D	· 25% reduced risk of asthma and recurrent wheeze at 0–3 years of age by prenatal vitamin D supplementation based on combined analysis of VDAAT and COPSAC2010	· Protective (asthma, wheezing)	(59)
	· Maternal prenatal vitamin D supplementation was inversely associated with the development of food allergy.	· Protective (food allergy)	(62)
	· Prenatal high concentration of serum maternal 25(OH)D was associated with an increased incidence of allergic airway disease in offspring at 20 to 25 years of age.	· Allergic (allergic airway disease)	(60)
Antibiotics	Both prenatal and child's use of antibiotics were associated with an increased risk of cow's milk allergy.	Allergic (cow's milk allergy)	(88)
MATERNAL PUFAS			
Fish oil, docosahexaenoic acid, and eicosapentaenoic acid	· Prenatal intake of oily fish containing higher docosahexaenoic acid and eicosapentaenoic acid as well as fish oil supplements showed protective effects on wheeze and asthma in offspring.	· Protective (wheezing, asthma)	(65)
	· Prenatal fish oil supplementation reduced the severity of atopic eczema and the risk of egg sensitization in one-year-old offspring.	· Protective (atopic eczema, egg sensitization)	(66)
α-linoleic acid	High prenatal intake of α-linoleic acid was associated with a reduced risk of wheeze in Japanese children at 16 to 24 months of age.	Protective (wheezing)	(67)
n-6 PUFA	Higher prenatal consumption of n-6 than n-3 during pregnancy was associated with higher risk of rhinoconjunctivitis in offspring at 5 years of age.	Allergic (rhinoconjunctivitis)	(89)
MATERNAL ALLERGY			
Cytokine	Prenatal cytokine production (cord-blood concentrations of IL-4, IFN-γ, and tumor necrosis factor) was associated with the development of atopy and asthma at 6 years of age.	Allergic (atopy and asthma)	(90)

(Continued)

TABLE 2 | Continued

Factors	Association/outcome	Effects	References
Allergen sensitization	· Maternal asthma status did not confer additional risks for asthma or complications to black women and their infants, while maternal asthma status increased risk for both asthma and many complications to white and Hispanic.	· No association in black women (asthma) · Allergic in white and Hispanic women (asthma)	(91)
	· Maternal sensitization to allergens was associated with reduced IFN-γ production from stimulated maternal peripheral blood mononuclear cells and elevated production of IL-13 in cord blood mononuclear cells from offspring.	· Potentially allergic (Th2 responses)	(92)
	· Children from mothers with mild uncontrolled asthma, moderate-to-severe controlled asthma, and moderate-to-severe uncontrolled asthma showed higher prevalence of early-onset persistent asthma than those of mothers with mild controlled asthma.	· Allergic (asthma)	(93)
	· Maternal asthma was a greater risk for offspring asthma than paternal asthma.	· Allergic (asthma)	(94)
	· Maternal, but not paternal, total IgE levels correlated with elevated infant IgE levels and infant eczema.	· Allergic (IgE levels, eczema)	(95)
	· Maternal asthma status did not affect the microbial populations in children.	· No association (microbiota)	(96)
BREAST MILK FACTORS			
IgA in breast milk	· High concentration of milk allergen-specific IgA in breast milk reduced transcytosis of milk allergen through gut lumen.	· Protective (cow's milk allergy)	(75)
	· sIgA concentration in colostrum was higher in allergic mothers than those in nonallergic mothers. · Fecal sIgA concentration in breastfed infants was significantly higher in those in formula-fed infants regardless of maternal allergy status.	· Potentially protective (sIgA levels in colostrum) · Potentially protective (fecal sIgA levels in infant)	(76)
Maternal IgG transfer *in utero* and in breast milk	· Children of allergen-specific immunotherapy-treated mothers during pregnancy exhibited high levels of IgG antibodies and fewer positive skin prick test as compared with children of untreated mothers.	· Protective (grass allergy)	(41)
	· High levels of cord blood IgG antibodies to cat and birch were associated with less atopic symptoms in the children of allergic mothers during the first 8 years of life.	· Protective (atopy)	(42)
Allergens and IC in breast milk	· Human milk containing peanut allergens and IC induced a partial oral tolerance in peanut-sensitized young mice.	· Protective (food allergy in mice)	(72)
	· Allergen-specific IgG and IgE antibodies were transmitted from maternal blood into breast milk and that breast milk IgG pattern mirrored the profile of IgG reactivity in maternal blood.	· Offspring outcome unknown	(73)
	· Human breast milk containing OVA-IgG-IC reduced food allergic responses in mice.	· Protective (food allergy in mice)	(74)
Cytokine in breast milk	· Concentration of TGF-β1 or TGF-β2 in breast milk was associated with a reduction in IgE titer and atopic inflammation in infancy and early childhood.	· Protective (IgA levels, atopy)	(77)
	· Levels of IL-13 in colostrum were associated with lower parental-reported food allergy at 6 months of age.	· Protective (food allergy)	(78)
	· High levels of IL-1β, IL-17, and TARC and low levels of CXCL1 and TSLP in breast milk were associated with a low risk of eczema at 0 to 3 years of age.	· Protective (eczema)	(79)
	· A prospective study with 398 mothers demonstrated an association of higher eczema risk with higher concentration of TGF-β2 in breast milk at 6 months of age.	· Allergic (eczema)	(78)
	· Breast milk of allergic mothers contains higher levels of IL-4, RANTES, and IL-8 as compared to those of non-allergic mothers.	· Potentially Allergic (offspring outcome unknown)	(97)
	· Levels of IL-1β, IL-10, and β-lactoglobulin-specific IgA were lower in breast milk from mothers of infants with cow's milk allergy than in breast milk from mothers of infants without cow's milk allergy.	· Allergic (cow's milk allergy)	(98)

25(OH)D, 25-hydroxyvitamin D; COPSAC, Copenhagen Prospective Studies on Asthma in Childhood; IC, immunecomplex; IFN, interferon; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; PUFAs, polyunsaturated fatty acids; RANTES, regulated on activation, normal T cell expressed and secreted; sIgA, secretary IgA; TARK, C-C motif chemokine ligand (CCL) 17; TGF-β, tumor growth factor β; TLR, toll-like receptor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin; VDAART, vitamin D antenatal asthma reduction trial.

TABLE 3 | Maternal protective and risk factors for offspring allergy in mouse models.

Responsible factors	Strains	Treatment to dam	Dose/timing	Effects	References
IN UTERO					
Allergen	BALB/c, DO11.10	· Oral administration	· High-dose OVA (9 mg) during the first week of pregnancy	· Suppression of OVA-specific IgE levels in 3-day-old, but not in 25-day-old, BALB/c offspring	(99)
		· Oral administration	· Low-dose OVA (1.5 mg) during pregnancy · Low-dose OVA (1.5 mg) during pregnancy	· Sustained inhibition of OVA-specific IgE levels both in 3- and 25-day-old BALB/c offspring · *In utero* priming of offspring DO11.10-derived T cells	
· B cells · Allergen-IgG-IC	C57BL/6, Mature B-cell-deficient μMT	Intranasal exposure	500 μg OVA before conception	· Induction of tolerance in C57BL/6 offspring against allergic airway inflammation · Abolished preventive effects against allergic airway inflammation in μMT · OVA-IgG1-IC bound to FcγR and promoted differentiation of $Foxp3^+$ Treg.	(100)
Maternal IFN-γ	CD1	*i.p.* injection with IFN-γ	100 IU of recombinant IFN-γ during pregnancy	Maternal IFN-γ induced stronger protective effects when prenatally transferred *in utero* than when postnatally transferred via breast milk against allergic airway inflammation in offspring.	(101)
Maternal IgG and offspring IFN-γ	BALB/c, C57BL/6J, FcRn-deficient B6.129X1-*Fcgr*^{ttm1Dcr}	Oral administration	100 mg OVA before conception	· *In utero* IgG transfer decreased the disease severity in BALB/c offspring. · Offspring of FcRn-deficient dam nursed by OVA-fed C57BL/6 WT dams showed protective effects against OVA-induced asthma-like symptom. · Anti-IFN-γ treatment of offspring during sensitization and challenge with allergen abolished the protection.	(102)
Maternal TLR signaling and offspring IFN-γ	BALB/c, C57BL/6 *Tlr2/3/4/7/9*$^{-/-}$	Intranasal applications of freeze-dried *A. lwoffii* F78 suspended in PBS	10^8 CFU of *A. lwoffii* F78 during prenatal period	· IFN-γ-dependent prevention of experimental asthma in BALB/c offspring · A significant reduction in acetylation levels of histone 4 of the *Il4* promoter after OVA challenge · The preventive effect was completely abolished in heterozygous offspring of *A. lwoffii* F78-treated *Tlr2/3/4/7/9*$^{-/-}$ dam.	(103, 104)
Maternal Th1-biased immunity	C57BL/6J	OVA with incomplete Freunt's adjuvant (*s.c.*) or alum (*i.p.*)	Preconceptionally immunized with 25 μg OVA with incomplete Freunt's adjuvant or 2 mg alum	· Offspring of dams with Th1-biased immunity showed reduced levels of OVA-specific IgE and airway eosinophilia compared with offspring of dams with Th2-biased immunity or naïve dams. · Suppression of allergic airway inflammation in offspring from Th1-biased dams was antigen-specific.	(105)
Short-chain fatty acid, especially acetate	C57BL/6	Fed with high-fiber diet or drinking water with acetate or propionate	· High-fiber diet: SF11-025 · 200 mM acetate or propionate	· Offspring of dams fed high-fiber diet or acetate failed to develop allergic airway disease. · Dams showed significant changes in microbiota composition, which was accompanied by an increase in high-acetate-producing Bacteroidetes and short-chain fatty acids in their feces and serum.	(106)
IL-4 signaling in asthmatic dams	BALB/c	Immunized with OVA/alum and exposed to OVA aerosol	Preconceptionally immunized with 5 μg OVA/1 mg alum and exposed to 3% (w/v) OVA aerosol during pregnancy	· Offspring of OVA-dams, but not of unsensitized dams, exhibited allergic airway inflammation in response to suboptimal sensitization with OVA/alum and OVA aerosol. · This model of maternal transmission of asthma susceptibility to offspring was not allergen-specific.	(107)
Maternal allergen-specific T cells	BALB/c	Adoptive transfer of DO11.10-derived T cells to naive dams	5×10^6 DO11.10 T cells/mouse *i.p.* at 3 days before mating	· Higher responsiveness of offspring to suboptimal challenge of OVA · Donor T cells were identified in spleen and placenta of the recipient female dams but were not detectable in fetal tissues.	(108)

(Continued)

TABLE 3 | Continued

Responsible factors	Strains	Treatment to dam	Dose/timing	Effects	References
CD25 and GITR	BALB/c		· Depletion of $CD25^{high}$ T cell population in 4-day-old newborn mice before induction of airway inflammation · Anti-GITR antibody treatment before induction of airway inflammation with suboptimal sensitization protocol · Anti-GITR antibody treatment after induction of airway inflammation	· Increased susceptibility to allergic airway inflammation in normal offspring · Substantial reduction in asthma symptoms in offspring of allergic dams · Significant attenuation of the susceptibilities to allergic airway inflammation in offspring of both naïve and asthmatic dams · Enhanced asthma-like allergic inflammation in normal offspring · Slight deterioration of allergic airway inflammation in the offspring of asthmatic dams	(109)
Skin barrier impairment	Offspring of male *Flg*$^{ft/ft}$/*Tmem79*$^{ma/ma}$ and female C57BL/6	*i.p.* sensitization with OVA/alum and exposure to OVA aerosols (C57BL/6 WT dam)	5 μg OVA/1 mg alum and exposed to 3% (w/v) OVA aerosol before mating with unsensitized flaky tail male mice	· Increase in food allergen sensitization, serum mMCP1, and anaphylaxis to food allergen sensitization · Associated with higher levels of transcripts for CCL11, TSLP and IL-33 in skin, and TSLP in jejunum	(110)
BREASTFEEDING					
Allergen in breast milk	BALB/c	Airborne allergen exposure	0.5% OVA every other day during lactation	· Intact OVA and its degraded products were detectable in breast milk. · Offspring of OVA-exposed dams showed a significant protection against allergic airway inflammation, production of antigen-specific IgE, IgG1, and IgA antibodies, allergen-specific secretion of Th2 cytokines and IL-10 compared to offspring of unexposed dams. · The decrease in the disease susceptibility in offspring was independent on maternal Igs or IL-10.	(111)
Maternal TGF-β during breastfeeding and infant IFN-γ	BALB/c	Oral administration	2 mg OVA three times a week in the first, second, and third week of or throughout breastfeeding	· Oral tolerance induction in offspring were completely abolished in those nursed by dams treated with anti-TGF-β monoclonal antibodies. · Oral tolerance induction in offspring of OVA-fed dams was insufficient in 1-week-old neonates, accompanied by a reduction in gut barrier, retinaldehyde dehydrogenase expression by mesenteric lymph node $CD103^{+}$ neonatal DCs. · One-week-old neonates were refractory to oral tolerance elicited by maternal allergen transfer via milk due to a physiological vitamin A deficiency. · Vitamin A supplementation rescued these neonatal defects and induced efficient tolerance in 1-week-old mice. · Oral tolerance was also dependent on offspring IFN-γ.	(111, 112)
IgG-IC	BALB/c	· OVA/alum *i.p.* · Exposure of OVA aerosols	· 10 μg OVA/2 mg alum, preconceptionally · 0.3% OVA during breastfeeding	· Induced a long-lasting allergen-specific protection from asthma. · OVA-IgG-IC transferred into offspring by FcRn was associated with the differentiation of $Foxp3^{+}$ Treg cells in offspring. · OVA-specific DO11.10-derived T cells co-cultured with bone marrow-derived DCs proliferated and upregulated Foxp3 expression *in vitro* in the presence of breast milk from OVA-exposed dams or · OVA-IgG-IC purified from breast milk. · OVA-IgG-IC induced the proliferation of DO11.10-derived T cells at least 100-fold more efficiently than OVA alone. · Milk IC-mediated tolerance was more profound than the tolerance induced by milk-borne free allergen and did not require TGF-β, IgA, or FcγRIIb.	(18)

(Continued)

TABLE 3 | Continued

Responsible factors	Strains	Treatment to dam	Dose/timing	Effects	References
IgG1 and FcRn in offspring	B-cell deficient *Igh6*^{tm1Cgn}, C57BL6/J, FcRn-deficient *Fcgrt*$^{-/-}$	· *i.p.* injection with OVA/alum · OVA aerosol	· 25 or 8 μg OVA/2 mg alum before mating · 1% OVA dairy before mating and during embryonic days 11–17 of pregnancy	· Offspring nursed by B-cell deficient *Igh6*^{tm1Cgn} mice failed to show protection against OVA-specific IgE production and OVA-induced allergic airway inflammation. · *Fcgrt*$^{-/-}$ offspring nursed by OVA-sensitized WT dams exhibited 1/1,000 to 1/10,000 lower levels of maternal OVA-specific IgG1 than those in similarly nursed *Fcgrt*$^{+/-}$ or WT offspring and failed to show protection against OVA-specific IgE production and allergic airway inflammation.	(113, 114)
Amount of allergen directly taken by infant	C3H/HeJ	Peanut exposure by gavage	10 mg/mouse ground peanut three times a week for 4 weeks prior conception or preconceptionally and during pregnancy and breastfeeding	· No influence of maternal peanuts exposure during pregnancy on offspring peanut sensitization · Enhanced peanut-allergen uptake by CD11c^{+} DCs in Peyer's patch subepithelial dome in coadministration of peanut extract with breast milk from allergic dams as compared to coadministration of that from naïve dams · Oral administration of small dose (1 mg) of peanut extract to offspring with breast milk from sensitized or naïve dams during post-weaning period failed to enhance the induction of tolerance compared to those orally administered peanut extract alone in offspring.	(115)
Low dose allergen exposure during pregnancy and lactation	C3H/HeJ	Oral peanut/CT exposure by gavage	· 10 mg/mouse ground peanut/20 μg/mouse CT weekly and peanut exposure (50 mg/mouse) preconceptionally · 10 mg/mouse ground peanut/20 μg/mouse CT preconceptionally and during pregnancy and breastfeeding	· Offspring of dams preconceptionally sensitized with peanut/CT exhibited maternal IgG1-mediated anaphylaxis in response to oral peanut challenge. · Offspring were protected from anaphylaxis following first peanut challenge as well as active peanut/CT sensitization and oral peanut challenge.	(17)
Allergens and allergen-specific immunoglobulins	BALB/c	· *i.p.* injection of OVA/alum · Oral administration of OVA	· 50 μg OVA/1.3 mg alum twice at 2-week interval periconceptionally · 1% OVA in drinking water during breastfeeding for 2 weeks	· Offspring of OVA-sensitized and challenged dams showed the most significant reduction in food allergic responses, as indicated by a decrease in levels of diarrhea occurrence, OVA-specific IgE, *Il4* mRNA expression, and numbers of mucosal mast cells in proximal colon compared to those in offspring from naïve dams. · Offspring from dams unsensitized and challenged with OVA exhibited a partial but a significant reduction in food allergic responses compared to those from naïve dams. · Offspring from OVA-sensitized dams without oral OVA challenge showed comparable food allergic responses to offspring of naïve dams.	(116)
Oral TGF-β supplementation after weaning	BALB/c	· *i.p.* injection of OVA/alum · Oral administration of OVA	· 10 μg OVA/ 2 mg alum. twice at 1-week interval at 2 days before mating · 2 mg OVA by gavage three times a week during lactation	· Offspring of naïve dams nursed by OVA-sensitized dams showed a significant reduction in levels of diarrhea score, serum mMCP1, and OVA-specific IgE antibodies compared to naïve offspring nursed by naïve dams. · Breastfeeding by OVA-dams decreased the frequencies of allergic diarrhea in response to multiple oral OVA challenges in 6-week-old, but not in 13-week-old offspring. · Supplementation with TGF-β after weaning till 12-week prolonged protection against diarrhea and improved gut barrier in 13-week-old mice breastfed by OVA-dams.	(117)

(Continued)

TABLE 3 | Continued

Responsible factors	Strains	Treatment to dam	Dose/timing	Effects	References
Maternal OVA IgG-IC and offspring FcRn	BALB/c, FcRn-deficient *Fcgrt*$^{-/-}$	· Epicutaneous sensitization	· 100 μg OVA preconceptionally and during pregnancy and breastfeeding	· Maternal allergen sensitization through skin prevented food anaphylaxis, allergen-specific IgE production, serum IL-4, serum mMCP1, and intestinal mast cell expansion in BALB/c offspring. · The protective effects of food allergy by OVA-sensitized dam were abolished in *Fcgrt*$^{-/-}$.	(74)
		· *i.p.* injection of OVA-IgG1-IC	· 100 μg OVA-IgG1-IC once weekly for 3 weeks during breastfeeding	· Offspring of naïve dams supplemented with OVA-IgG1-IC showed increase of serum OVA-IgG1-IC levels, OVA-specific Treg in mesenteric lymph node at weaning, and protection against food allergy.	
Th2-biased epigenetic alteration	C3H/HeJ	Intragastric	10 mg peanut/20 μg CT weekly for 5 weeks and 200 mg peanut at 6 weeks before conception	Offspring of dams sensitized and challenged with peanut exhibited increased food allergic responses following suboptimal dose of oral peanut/CT sensitization and peanut challenge, associated with hypomethylation at CpG sites of *Il4* gene promoter.	(118)

A. lwoffii, Acinetobacter lwoffii; CCL, C-C motif chemokine ligand; CFU, colony forming unit; CT, cholera toxin; DC, dendritic cell; FcγR, Fc gamma receptor; FcRn, neonatal Fc receptor; Foxp3, forkhead box p3; GITR, glucocorticoid-induced tumor necrosis factor receptor-family related receptor; IC, immunecomplex; IFN, interferon; Ig, immunoglobulin; IL, interleukin; i.p., intraperitoneal; IU, international unit; mMCP1, mouse mast cell protease I; OVA, ovalbumin; PBS, phosphate buffered saline; s.c., subcutaneous; TGF-β, tumor growth factor β; Th, helper T cell; TLR, toll-like receptor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin; WT, wild type.

reduced numbers of eosinophils in bronchoalveolar lavage fluid, but only IFN/Nor offspring showed almost normal lung histology. IFN/Nor offspring also showed amelioration of passive cutaneous anaphylaxis. These results suggest that maternal IFN-γ induces stronger protective effects when prenatally transferred *in utero* than when postnatally transferred via breast milk against allergic airway inflammation in offspring.

The importance of IFN-γ in the protection of offspring against allergic inflammation was proposed in another study that demonstrated that transfer of maternal IgG to the offspring induced offspring tolerance against asthma-like phenotype (102). Offspring of BALB/c WT dams orally administered OVA before conception were protected from development of allergic airway inflammation induced by *i.p.* sensitization and intranasal challenge with OVA. Offspring of OVA-fed dams nursed by naïve dams were also protected, suggesting the role of *in utero* IgG transfer in decreasing the disease severity in offspring. Offspring of FcRn-deficient dams, unable to transport IgG through placenta, nursed by OVA-fed WT dams showed protective effects against OVA-induced asthma-like symptoms, indicating the contribution of IgG transfer also via breast milk to protection of offspring. Anti-IFN-γ treatment of offspring during sensitization and challenge with allergen abolished the protection. This study suggests that this maternal IgG-mediated suppression of allergic airway inflammation is dependent on offspring IFN-γ, indicating the synergistic effects of maternal-offspring factors in shaping neonatal responses to allergic diseases. How OVA administration to dams through usually tolerizing oral route results in generation of allergen-specific IgG was not clear in this study.

Exposure of dams with farm-derived, gram-negative bacterium *Acinetobacter lwoffii* F78 during prenatal period resulted in prevention of experimental asthma in their offspring, which was dependent on maternal TLR signaling (103). The follow-up study demonstrated that the preventive effects were dependent on offspring IFN-γ (104) and that offspring from dams exposed to *A. lwoffii* F78 showed a significant reduction in acetylation level of histone 4, a marker for an open chromatin structure, of the *Il4* promoter after OVA challenge. Since bacteria were not detectable in offspring, these data imply that prenatal exposure of dams to certain microbiota exerts protective effects in offspring against allergic responses through epigenetic regulation of cytokines.

One of the earlier studies compared the effects of maternal factors from dams with Th1 immune responses vs. dams with Th2 immune responses on allergic airway inflammation in offspring. Dams were preconceptionally immunized with OVA and complete Freund's adjuvant *subcutaneously* followed by a booster of OVA plus incomplete Freunt's adjuvant *i.p.* to induce Th1-biased immunity, or immunized with OVA and alum *i.p.* to induce Th2-biased immunity. Immunized dams were exposed to OVA aerosol before pregnancy then received recall OVA aerosol during pregnancy. Offspring of dams with Th1-biased immunity showed reduced levels of OVA-specific IgE and airway eosinophilia in response to immunization with OVA and alum (OVA/alum) and challenge with OVA aerosol compared with offspring of dams with Th2-biased immunity or naïve dams. Offspring of dams with Th1-biased immunity to bovine serum albumin did not show protection against development of OVA-specific IgE or OVA-induced airway inflammation, suggesting that the suppression of allergic airway inflammation in offspring from Th1-biased dams is antigen-specific. Although offspring of Th2-biased dams failed to show protection against OVA-induced airway inflammation, these offspring showed a trend toward lower levels of OVA-specific IgE as compared to offspring of naive dams (105). As Th1-biased dams were challenged with allergen before and during pregnancy but not during breastfeeding, these data suggest that maternal transfer of allergen and allergen-specific Igs *in utero* in the context of Th1

immune responses results in the resistance of offspring to allergic airway inflammation. It is possible that allergen-specific Igs continued to be transferred from Th1-biased dams to offspring through breast milk, and that these antibodies also contribute to prevent allergic inflammation in offspring, as shown in their follow-up study (113) as discussed in section Breastfeeding in Mouse Studies for maternal protective influences.

Microbiota

Animal models have evidently shown that the intestinal dysbiosis confers susceptibility to food allergy (121) and that certain bacteria such as *Clostridia* species that are protective from food allergy (122–124). Germ-free mice colonized with feces from healthy infants, but not from cow's milk allergic infants, protected against anaphylactic responses to a cow's milk allergen and that *Anaerostipes caccae*, a non-spore-forming, butyrate producing *Clostridial* species was critical for the protection against food allergy (123). Bacteriotherapy with *Clostridiales* species, impacted by dysbiosis in human infants with food allergy, suppressed food allergy in mice via MyD88/ROR-γt^+ Treg cell pathway (124), indicating that microbiota critically regulate food allergy. A study reported that progeny of dams fed with high-fiber diet during pregnancy showed suppression of allergic airway inflammation (106). Dams fed with high-fiber diet showed significant changes in microbiota composition, which is accompanied by an increase in high-acetate-producing *Bacteroidetes* and short-chain fatty acids in their feces and serum. The increase in short-chain fatty acid, especially acetate, in dams during pregnancy induced Treg in offspring mediated by enhancing acetylation of Foxp3 promoter through inhibition of histone deacetylase 9 *in utero*. These reports suggest that maternal microbiota influences the susceptibility of offspring to allergies through epigenetic modification *in utero*.

Collectively, these observations indicate that maternal environment and maternal factors directly or indirectly affect offspring immune system during pregnancy likely contribute to the induction of tolerance. Potential preventive effects of *in utero* factors on offspring food allergy have been much less studied as compared to asthma models. Our study shows that offspring born from allergen-sensitized dams nursed by naïve dams exhibited a trend toward protection against food allergy (74), suggesting that *in utero* factors such as IC contribute to the reduced disease susceptibility, however, breastfeeding is essential to induce optimal tolerance induction. Investigations of the timing (prenatal, postnatal, or throughout), factors (allergen, Igs, IC, cytokine milieu, and microbiota), and dose of those factors in the interplay of pathogenic vs. regulatory responses in allergic diseases in offspring will be needed to understand this complex relationship.

Breastfeeding

In mouse studies, breastfeeding has been shown to be critical in the induction of tolerance toward allergic sensitization in postnatal period (74, 119). Similar to *in utero* period, maternal factors such as allergen, Igs, IC, and TGF-β in breast milk induce allergen-specific tolerance against allergic sensitization and inflammation. Maternal immune cells are present in breast milk and are transferred into offspring tissues (125). These factors may act individually or in synergy to prevent development of allergy in offspring through multiple mechanisms.

Allergen, allergen-specific Igs, and cytokine

Tolerance induction by allergen in milk has been the subject of many studies that use experimental asthma models. Aerosol OVA exposure of WT dams during lactation resulted in an allergen transfer to offspring via milk and the induction of allergen-specific tolerance toward allergic airway inflammation (111). Intact OVA and its degraded products were detected in breast milk from OVA-exposed dams during breastfeeding with the concentration in the similar range as those in human breast milk after consumption of egg or cow's milk (180 ± 20 ng/ml) (111). Offspring of OVA-exposed dams showed a significant protection against allergic airway inflammation, production of antigen-specific IgE, IgG1, and IgA antibodies, allergen-specific secretion of Th2 cytokines and IL-10 compared to offspring of unexposed dams. The decrease in the disease susceptibility in offspring was independent on maternal Igs or IL-10. Given that dams were *not sensitized* with allergen, i.e., in the absence of maternal allergen-specific Igs, it is not surprising that maternal Igs were not required for the induction of tolerance in this system. In contrast, the suppressive effects in offspring were completely abolished in those nursed by dams treated with anti-TGF-β monoclonal antibodies, indicating that maternal TGF-β during breastfeeding are required for the induction of tolerance. In mice, 1-week-old neonates were refractory to oral tolerance induced by maternal allergen transfer through milk due to a physiological vitamin A deficiency (112). Unsensitized dams were given OVA in the first, second, or third week of or throughout lactation and tolerance induction in offspring to OVA-induced allergic airway inflammation was examined. Oral tolerance induction in offspring of OVA-fed dams was insufficient in 1-week-old neonates, accompanied by a reduction in gut barrier, the expression of retinaldehyde dehydrogenase by mesenteric lymph node $CD103^+$ neonatal DCs leading to inefficient T-cell priming, and serum retinol levels as compared to those in adult mice. Supplementation with vitamin A rescued these defects in neonates and induced efficient tolerance in 1-week-old mice. Oral tolerance was also dependent on offspring IFN-γ. These data may suggest maternal allergen transfer through milk together with vitamin A supplementation as possible interventions for prevention of allergy in the neonates (112). The same group next demonstrated that breastfeeding by preconceptionally sensitized WT dams with OVA/alum *i.p.* exposed to OVA aerosols during breastfeeding induced a long-lasting allergen-specific protection toward asthma. Dams sensitized and exposed to OVA exhibited OVA-IgG-IC in breast milk (18). Eighty-five percent of OVA in breast milk formed OVA-IgG-IC (18). OVA-IgG-IC transferred into offspring by FcRn was associated with the differentiation of $Foxp3^+$ Treg cells in offspring. OVA-specific DO11.10-derived T cells co-cultured with bone marrow-derived DCs proliferated and upregulated Foxp3 expression *in vitro* with breast milk from OVA-exposed dams or OVA-IgG-IC purified from breast milk. OVA-IgG-IC promoted the proliferation of DO11.10-derived T cells at least 100-fold more efficiently than OVA alone. Milk

IC-mediated tolerance was more profound than the tolerance induced by milk-borne free allergen and did not require TGF-β, IgA, or FcγRIIb (18). These studies suggest that milk-borne allergen protects offspring against asthma, and allergen as IC induces more efficient tolerance in neonates independently of TGF-β. Consistently, earlier studies indicated that when dams are sensitized with allergen, breast milk-induced tolerance in offspring require maternal B cell and offspring FcRn (105, 113, 114). Offspring nursed by B-cell deficient *Igh6*^{tm1cgn} mice failed to show protection against OVA-specific IgE production and OVA-induced allergic airway inflammation (113). The same group demonstrated that FcRn$^{-/-}$ offspring nursed by OVA-sensitized WT dams exhibited 1/1,000 to 1/10,000 lower levels of maternal OVA-specific IgG1 than those in similarly nursed FcRn$^{+/-}$ or WT offspring (114) and failed to show protection against OVA-specific IgE production and allergic airway inflammation. Collectively, these studies imply that allergen, IgG, and IC in breast milk as well as offspring FcRn are crucial for the induction of tolerance in allergic airway inflammation in offspring.

Regarding food allergy, maternal effects on protection of offspring toward food allergy were investigated in several mouse models. C3H/HeJ dams (lack a functional TLR4 that recognizes lipopolysaccharide) preconceptionally exposed to peanut exhibited variable levels of maternal Igs in sera and breast milk, though, maternal preconceptional peanut exposure or also during pregnancy and breastfeeding showed no influence on offspring peanut allergy induced by oral immunization with peanut and cholera toxin (CT) and oral plus *i.p.* peanut challenge (115). The efficacy of a direct oral tolerance induction in offspring was similar in offspring of dams exposed to peanut and in offspring of dams that did not consume peanut. Oral administration of crude peanut extract together with breast milk of peanut exposed dams onto 5-week-old offspring induced an enhanced peanut-allergen uptake by CD11c^{+} DCs in Peyer's patch subepithelial dome as compared to coadministration of peanut extract with breast milk from naïve dams. However, oral administration of small dose (1 mg) of peanut extract to offspring with breast milk from sensitized or naïve dams during post-weaning period failed to enhance the induction of tolerance compared to those orally administered peanut extract alone in offspring. The authors suggest that dose of dietary allergens contained in breast milk may not be adequate to induce oral tolerance in their system. These results support the recent recommendations of no restrictions in diet for pregnant and breastfeeding mothers. Human milk containing peanut allergens induced partial protection in young mice following peanut sensitization (72), supporting a protective role of milk-born allergens in reducing offspring disease susceptibility. A more robust immunization of C3H/HeJ dams with oral peanut/CT and peanut exposure during pregnancy and lactation resulted in reduction of peanut allergy in offspring (17). Offspring of dams preconceptionally sensitized with peanut/CT exhibited maternal IgG1-mediated anaphylaxis following oral peanut challenge (first exposure). The anaphylaxis reaction was also dependent on platelet activation factor, but not IgE, as maternal IgE was undetectable in offspring. Remarkably, when sensitized dams received peanut/CT also during pregnancy and lactation, offspring showed protection against anaphylaxis following first peanut challenge as well as direct peanut/CT sensitization and oral peanut challenge. These findings suggest that maternal immune responses during pregnancy and lactation regulate the reduction of disease susceptibility in offspring.

Another study compared the susceptibility to food allergic responses of offspring from OVA/alum-sensitized vs. unsensitized dams with and without maternal oral OVA challenge during breastfeeding (116). Offspring of OVA-sensitized and challenged dams showed the most significant reduction in food allergic responses, as indicated by decreased levels of diarrhea occurrence, OVA-specific IgE, *Il4* mRNA expression, and numbers of mucosal mast cells in proximal colon compared to those in offspring from unsensitized and unchallenged (naïve) dams. Offspring from dams unsensitized and challenged with OVA exhibited a partial but a significant reduction in food allergic responses compared to those from naïve dams. Offspring from OVA-sensitized dams without oral OVA challenge showed comparable food allergic responses to offspring of naïve dams, indicating that there is no increase in food allergy susceptibility of offspring by *in utero* factors from allergen-sensitized dams in this system. OVA-specific IgG was detectable in breast milk and serum of offspring from OVA-sensitized dams regardless of oral OVA challenge. OVA-specific IgA was detectable in breast milk only from OVA-sensitized and challenged dams. These results suggest that allergen-specific IgG and oral allergen exposure during breastfeeding resulting in production of allergen-specific IgA were required to induce an effective protection toward food allergy in offspring. These results suggest that even allergen-sensitized dams could have preventive effects on offspring allergies through breastfeeding. A recent study also demonstrated that allergen-sensitized dams protect offspring against food allergy through breast milk (117). Offspring of naive dams were fostered after birth by OVA-sensitized dams to avoid *in utero* effects then dams were orally challenged with OVA during breastfeeding. Offspring of naïve dams nursed by OVA-sensitized dams showed a significant reduction in levels of diarrhea score, serum mouse mast cell protease 1 (mMCP1), and OVA-specific IgE antibodies compared to naïve offspring nursed by naïve dams. The duration of oral tolerance elicited by breast milk toward food allergy was assessed in offspring of unsensitized dams nursed by dams preconceptionally immunized *i.p.* with OVA/alum then fed OVA during lactation. Breastfeeding by OVA-dams decreased the frequencies of allergic diarrhea in response to multiple oral OVA challenges in 6-week-old, but not in 13-week-old, offspring. TGF-β supplementation after weaning till 12-week prolonged protection toward diarrhea and enhanced gut barrier in 13-week-old mice nursed by OVA-dams. Lactation by allergen-sensitized dams together with offspring TGF-β supplementation may maintain long-lasting prevention of allergic diarrhea (117).

Previous studies reported the involvement of allergen-specific IgG or IgG-IC from allergen-sensitized mothers in the protection of offspring against allergic airway inflammation via breastfeeding and FcRn (18, 102, 114, 126). In line with these reports, our recent study critically elucidated the fundamental mechanisms by which IC in breast milk regulates the induction

of tolerance in offspring toward food allergy (74). Dams were epicutaneously sensitized with OVA or a clinically relevant allergen peanut followed by epicutaneous sensitization and oral challenge of their offspring with the same allergen. Maternal allergen sensitization through skin prevented food anaphylaxis, allergen-specific IgE production, serum IL-4, serum mMCP1, and intestinal mast cell expansion in offspring. The induction of tolerance was mediated by neonatal FcRn-dependent transfer of maternal OVA IgG-IC via milk and induction of allergen-specific Treg cells in offspring.

Neonatal tolerance was induced by breastfeeding by OVA-sensitized dams or maternal supplementation with IgG-IC, suggesting an essential role of breast milk IgG-IC in the induction of tolerance. In addition to FcRn-dependent IgG-IC transfer, our work demonstrated that FcRn-dependent presentation of antigen by $CD11c^+$ DCs was required for the induction of oral tolerance in offspring. Human breast milk from non-atopic mothers contained OVA-IgG-IC and was sufficient to induce tolerance in humanized FcRn mice, providing an important evidence for the potential relevance of our findings in human food allergy. Collectively, these findings demonstrate that interactions of offspring FcRn and maternal IgG-IC are critical in the induction of Treg cell responses that control food-specific tolerance in neonates, indicating the key role played by maternal allergen-specific Igs in milk in establishing tolerance that protects offspring from food allergy. These outcomes extend well beyond the previously defined roles of maternal antibodies and FcRn in stipulating passive immunity. Additionally, such food-specific IgG antibodies are induced during oral immunotherapy in humans and have been shown to act via FcγRIIb to suppress IgE-mediated hypersensitivity (127). Strategies that modulate maternal IgG responses might be beneficial for the prevention of food allergy in offspring.

Perspective

In utero, the timing and dose of allergen exposure, allergen-specific Igs, and ICs along with the combination and balance of cytokine milieu and microbiota profile that reflective of maternal diet likely regulate the potential preventive effects in offspring toward allergies. Increasing evidences represent the importance of breastfeeding in the induction of neonatal tolerance, which is also modulated by maternal allergens, allergen-specific Igs, ICs, as well as cytokine profile (**Table 3**). Allergen alone in breast milk may induce offspring tolerance in synergy with TGF-β. Breastfeeding by allergen-specific IgG-positive dams protect offspring against allergic sensitization and allergic responses, particularly when dams are exposed to allergen during breastfeeding. The induction of tolerance in offspring by IgG or IgG-IC is more efficient than allergen alone and does not require TGF-β, although supplementation of TGF-β may prolong the efficacy of IgG-IC-induced protection in offspring. In humans, increased allergy risk during early childhood indicates inadequate immune regulation in this life period. Both in prenatal and postnatal periods, it seems that maternal factors should interact with offspring immune system within specific time of window to successfully induce neonatal tolerance. More mechanistic studies are needed to elucidate the precise molecular mechanisms by which maternal factors modulate allergen-specific Treg cells and tolerance in offspring. For example, a particular DC subset and Fc receptors that are responsible for the induction of Treg cell responses remain elusive. An inhibitory Fc receptor FcγRIIb and allergen-specific IgG have been shown to promote food tolerance (127, 128), but whether this receptor also plays a role in the context of maternal tolerance transfer needs to be examined. These issues will be the important subjects of future studies.

MATERNAL INFLUENCES OVER OFFSPRING ALLERGY

Human Studies

The early appearance of childhood allergies and the rise in their prevalence suggest that genetics and changes in early-life exposures *in utero* may increase the predisposition. In this section, we focus on the potential maternal risk factors influence on development of offspring allergy, including genetic variance, diet, immunomodulating factors, as well as health status and environmental exposure.

In utero Factors

Genetics

A family history of allergy has been shown to be a risk factor for development of allergies in offspring (129). Common genetic factors driving allergic diseases including atopic dermatitis, asthma, and allergic rhinitis have been suggested (130, 131). Regarding food allergy, the first large-scale genome-wide association study (GWAS) identified 10 loci associating with allergic sensitization as indicated by the presence of allergen-specific IgE against common environmental allergens and self-reported allergic symptoms (132). The report suggests that allergic heritability is associated more with the severity of allergic symptoms and a general tendency for allergic sensitization (sensitization against any environmental allergen) than with sensitization against one specific allergen (132, 133). A recent GWAS in 2017 has identified five susceptibility loci for the disease; the clade B serpin (SERPINB) gene cluster, the cytokine gene cluster, the filaggrin gene, the C11orf30/LRRC32 locus, and the human leukocyte antigen region were identified using a multi-ethnic database (134). All of the identified loci are involved in epithelial barrier function or immunological regulation, highlighting the role of both mechanisms in the development of food allergy.

Twin studies have provided the strong evidences for the genetic determination of allergic diseases. A systematic review of twin and sibling studies suggests that the overlaps in loci between different allergic diseases may partly explain allergic co-morbidities and the concept of the "atopic march" (135). A Swedish cohort study including 25,306 twin aged 9 or 12 years showed that asthma and allergic diseases of childhood are greatly heritable. In this study, the heritability of any childhood asthma was 0.82. For the other allergic diseases including wheezing, hay fever, atopic eczema, and food allergy, the range was ∼0.60–0.80 (136). A twin study of eosinophilic esophagitis indicated the frequencies in both monozygotic and dizygotic twins were significantly higher than in siblings (137), suggesting

that eosinophilic esophagitis also shows the heritability. These heritability estimates are variable in different studies, probably due to differences in study designs and target populations.

Racial disparities are also identified in allergic diseases. It has been shown that African Americans are sensitized to food and aeroallergens more frequently than whites (138). The analysis of genetic markers for the ancestry suggests that the racial disparities and ancestry are associated with elevated levels of food allergen-specific IgE (139). The effects of maternal asthma on racial/ethnic disparities in obstetrical and neonatal complications were tested (91). Analysis of data acquired from white (N = 110,603), black (N = 50,284), and Hispanic (N = 38,831) singleton for joint effects of maternal race/ethnicity and asthma on the odds of obstetrical/neonatal complications indicated that maternal asthma status did not confer additional risks for asthma or complications (preeclampsia, neonatal intensive care unit admissions, maternal hemorrhage, small for gestational age, and apnea) to black women and their infants, while maternal asthma status increased risk for both asthma and many complications to white and Hispanic (91). A meta-analysis study demonstrated that maternal asthma is a greater risk for offspring asthma than paternal asthma (OR, 3.04 vs. 2.44, $p = 0.037$) (94). Maternal, but not paternal, total IgE levels correlated with elevated infant IgE levels and infant eczema (95). The effect of parental allergy on food allergy in children is yet to be discovered.

While these studies have been useful and will continue to identify novel genes for food allergy and other allergies, future approaches to investigate the mechanisms by which such genetic factors play a role in maternal influences over food allergy in offspring will improve our understanding of the disease mechanisms and produce potential opportunities for prevention and treatment of the disease.

Food allergen consumption

In contrast to potential preventive effects of allergen exposure that we discussed earlier in this article, an increased risk by maternal consumption of allergenic foods has been previously proposed. A study enrolled 503 infants 3 to 15 months of age with likely milk or egg allergy without previous diagnosis of peanut allergy and investigated the association of maternal peanut intake with peanut sensitization in offspring. They identified a dose-dependent association of maternal peanut ingestion during the third trimester of pregnancy with peanut sensitization in offspring (12). There was no significant effect of maternal peanut consumption during the first or second trimester of pregnancy or during breastfeeding on offspring peanut sensitization in this study. Among infants never breastfed, frequent ingestion of peanut during pregnancy was strongly associated with peanut sensitization in offspring. These results suggest that maternal consumption of peanut during pregnancy is a potential risk factor of offspring food sensitization, although oral food challenges were not performed due to the age of the group. Earlier study that investigated 13,971 children of non-atopic and atopic mothers reported no association of maternal peanut consumption during pregnancy with challenge-proven peanut allergy in preschool children (13). Effects of maternal and infant food-allergen avoidance on development of early onset atopy were investigated in a prenatally randomized, controlled trial of infants of atopic parents (28). Children of prophylactic-treated group (N = 103) (maternal avoidance of egg, cow's milk, and peanut during the third trimester of pregnancy and breastfeeding period, infant use of casein hydrolysate for supplementation or weaning, and avoidance of solid food for 6 months, avoidance of cow's milk, corn, citrus, and wheat for 12 months, and avoidance of egg, peanut, and fish for 24 months) showed a lower prevalence of atopy at 12 months compared to those in control group (N = 185) with unrestricted diets, occurring from reduced food-associated atopic dermatitis, urticaria, and/or gastrointestinal disease by 12 months ($p = 0.007$) and any positive food skin test by 24 months ($p = 0.019$) in the prophylactic-treated group. However, the suppression of prevalence of food allergy and milk sensitization was significant only before 2 years of age and none of the prevalence in food allergy, atopic dermatitis, allergic rhinitis, asthma, any atopic disease, and serum IgE levels were differed at 7 years of age (29), indicating that maternal and offspring avoidance of allergenic foods is not effective in prevention of allergic diseases in offspring.

Allergen-specific Igs and cytokine

As to Ig transmission, the mechanisms responsible for the acquisition of maternal antibodies other than IgG are not fully understood. Cord blood IgE has been proposed as a predictive marker for allergic diseases (140). However, it is suggested that cord blood allergen-specific IgE does not reflect intrauterine sensitization but rather is the result of maternal IgE transfer to the fetus, which is mediated by FcRn that accelerates the transepithelial transport of IgE in the form of IgE-IgG-IC (141, 142). The significance of this IC in allergy development in offspring has not been investigated. A birth cohort study has shown the association of prenatal cytokine production (cord-blood concentrations of IL-4, IFN-γ, and tumor necrosis factor) with the development of atopy and asthma at 6 years of age. The study suggests that neonatal cytokine production patterns are significant predictors of a following risk for allergy and asthma (90). Another study indicated that maternal allergen sensitization is associated with reduced IFN-γ production from stimulated maternal peripheral blood mononuclear cells and increased production of IL-13 in cord blood mononuclear cells from offspring (92).

Microbiota

Increasing evidences highlight the role of microbiota in development of allergies [reviewed in (143)]. Children born by caesarian section are suggested to be more susceptible to develop allergies, as appropriate exposure to microbiome of newborns through natural birth may be required for the maturation of their immune system in early life. Consistently, use of antibiotics by both mothers and children were associated with an increased risk of cow's milk allergy (88). In 2018, it is reported that maternal asthma status did not alter the microbial populations in the children. They showed that maternal asthma was, however, a key modifier between the microbiome and asthma risk, which pointed to susceptibility to host-microbial interactions particularly for these children (96). The study suggests that such

susceptibility could occur from an inborn immune deviation determined by maternal asthma status (144).

Dietary supplements

Early-life exposures such as infection, environmental pollutants and nutrients provided via the mother may act upon the developing fetal immune system and lead to allergic diseases. Studies investigating the association of maternal nutrients such as vitamin D, PUFAs, and folate (or the synthetic form, folic acid) have been equivocal (145). Eicosanoids, signaling molecules made from PUFAs play a role in inducing inflammations. High levels of eicosanoids are associated with an increase in the prevalence and severity of allergic diseases (63). The association of high levels of maternal dietary intake of linoleic acid, a common n-6 fatty acid, both during pregnancy and after birth with an increased risk of allergic diseases in children has been demonstrated. A Finnish study showed that higher consumption of n-6 than n-3 during pregnancy was associated with higher risk of rhinoconjunctivitis in offspring at 5 years of age (89). A study on German schoolchildren in Leipzig in 1995-1996 ($N = 2{,}334$) showed that schoolchildren who consumed linoleic acid-rich margarine exhibited a higher risk of hay fever as compared to schoolchildren who did not consume margarine (146). Similarly, a longitudinal study on Australian schoolchildren in 1980 showed higher levels of margarine consumption and lower levels of butter consumption among children with atopic disease as compared to non-atopic children (147). Since n-6 and n-3 compete for metabolism by the same enzyme, too high intake of n-6 would reduce the amount of enzyme available for n-3, decrease the n-6/n-3 ratio, and result in the production of more proinflammatory modulators that may lead to inflammation including asthma (148). Maternal serum concentration of Fe states (ferritin concentration) during first-trimester of pregnancy was inversely correlated with risk of childhood wheeze and impaired lung function (81). Beneficial effects of maternal consumption of specific nutrients on preventing offspring are still largely unknown.

Maternal health and environment

Maternal health condition including control of maternal allergies and maternal exposure to certain chemicals are important factor to influence neonatal health condition. Maternal severe asthma without using inhalant corticosteroid treatment related to reduced birth weights of female children (149). However, prenatal exposure of preterm infants to dexamethasone, a type of corticosteroid medication, increased their susceptibility to allergic diseases, especially asthma and allergic rhinitis (82). In a Danish cohort study that employed 675,379 singletons, children from mothers with mild uncontrolled asthma, moderate-to-severe controlled asthma, and moderate-to-severe uncontrolled asthma showed higher prevalence of early-onset persistent asthma than those of mothers with mild controlled asthma (93), indicating that the severity and control of maternal asthma increase risk for offspring asthma. Although poorly controlled maternal asthma is associated with augmented risk of development of allergy, the use of corticosteroid during pregnancy is only recommended when there are no alternatives and benefit outweighs risk. Maternal exposure to H2 blockers or proton pump inhibitors also increase the risk of developing asthma, especially in children whose mothers purchased these medication more than 3 times within 2 months prior to conception and during pregnancy (83, 84). Preeclampsia, maternal pre-pregnancy overweight, and maternal obesity were associated with an increased risk of wheezing in offspring in a pooled analysis of 14 birth cohort studies (85). High levels of maternal exposure during pregnancy to phthalate reduced the number of Treg cells in cord blood as well as in children aged 2 years (86). Furthermore, high concentrations of phthalate metabolites in maternal urine were significantly associated with a higher risk for atopic dermatitis in children until 3 years of age (OR, 2.21; $p = 0.026$). The study suggests that the reduction in Treg cells in offspring by maternal exposure to phthalate may facilitate the development of atopic dermatitis in early childhood.

Maternal adverse life events such as separation or divorce, marital problems, problems with the children, pregnancy problems, involuntary job loss experience, involuntary job loss of partner, money problems, a residential move, death of a close relative, and death of a close friend during pregnancy are suggested to link an increased risk for atopic disorders in offspring (87).

In addition to genetic predispositions linked to allergies, these studies clearly suggest a critical involvement of maternal health and environmental factors in the development and manifestation of allergy in offspring. Optimal management of maternal health might be essential in determining the allergic predisposition and clinical symptoms in offspring.

Breastfeeding

Allergen, allergen-specific Igs, and cytokine

Maternal and environmental factors modify the milk composition and may facilitate development of allergic diseases in neonate. Dietary allergens such as peanut, egg, and cow's milk proteins have been detected in human milk (72, 150–152) and may induce symptoms in already sensitized infants (152). However, their role in early sensitization vs. tolerance induction in offspring is still unclear. Airborne allergens from house dust mite are contained in human breast milk that may facilitate sensitization to food allergens in mice (153). Infants with cow's milk allergy have specific IgE that cross-reacts with endogenous human protein in breast milk (154), potentially suggesting that cow's milk allergy may trigger allergic march because cow's milk proteins could be the first foreign proteins recognized by infant. Breastfeeding may increase a risk for atopic eczema and food allergy in breast-fed infant (155). As we discussed earlier, food avoidance by allergic mothers was associated with offspring allergy (75). Lower levels of milk-specific IgG4 and IgA in allergic mothers who have eliminated milk from their diet during breastfeeding were associated with the development of cow's milk allergy, implying the potential protective roles of maternal food allergen consumption against offspring allergies via food-specific IgG4 and IgA in breast milk. Concentrations of chemoattractant factors were higher in breast milk of allergic mothers than in those of non-allergic mothers. The breast milk of allergic mothers contained higher levels of IL-4, regulated on

activation, normal T cell expressed and secreted (RANTES) and IL-8 as compared to those of non-allergic mothers (97). Another study reported that the levels of IL-1β ($p = 0.001$), IL-10 ($p = 0.04$), and β-lactoglobulin-specific IgA ($p = 0.005$) were lower in breast milk from mothers of infants with cow's milk allergy than in breast milk from mothers of infants without cow's milk allergy (98). The component of breast milk of allergic mothers may be different from that of non-allergic mothers, although the effects of these differences on offspring allergies are poorly understood.

Perspective

Evidences indicate that genetics are one of the most critical factors that determine the susceptibility of allergic sensitization and severity of allergic symptoms in the offspring. The impacts of genetics are likely amplified by maternal nutrition and environmental factors including drug usage and exposure to harmful chemicals during pregnancy. Breastfeeding by allergic mothers may be another modifier of allergic reactions in offspring. Allergen, Ig, and IC transferred *in utero* and via breast milk likely play important roles in promoting the susceptibility of offspring by regulating the balance of Th2 vs. Treg cell responses. It is still unclear which factor is crucial to determine susceptibility to allergic sensitization and clinical manifestation of allergic inflammation in offspring (**Table 2**). Further prospective birth cohort studies and meta-analysis are needed to elucidate crucial risk factors for allergic sensitization in offspring transferred from allergic mothers.

Mouse Studies

In this section, we focus on literatures demonstrated the increased susceptibility of offspring from allergic dams to allergic inflammation and discuss the role of maternal transmission of allergies to offspring.

Preconceptional and *in utero* Factors

Cytokine, T cell, and genetic factors

Mouse models of experimental allergies have been utilized to dissect how maternal factors affect the susceptibility of offspring to allergic inflammation. The susceptibility of offspring from asthmatic dams to development of allergic airway inflammation has been intensely studied. Hamada et al. preconceptionally immunized BALB/c WT dams with OVA/alum and exposed to OVA aerosol during pregnancy. Offspring of OVA-dams, but not of unsensitized dams, exhibited allergic airway inflammation in response to suboptimal sensitization with OVA/alum and OVA aerosol, suggesting maternal transfer of asthma risk to offspring (107). This model of maternal transmission of asthma risk to offspring was not allergen-specific (107), as offspring of asthmatic dams sensitized and challenged with OVA also showed higher responsiveness to casein-induced allergic airway inflammation. Blocking of IL-4 signaling in asthmatic dams by administration of anti-IL-4 antibodies before mating abolished the increased susceptibility of offspring from asthmatic dams to suboptimal sensitization protocol, suggesting that the maternal transfer of asthma risk to offspring is IL-4 dependent (107). Th2 responses in asthmatic dams skewed fatal and neonatal immunity toward Th2 and were associated with the increased asthma susceptibility in offspring that persisted through the young adulthood (∼3 or 6 weeks) in offspring (156, 157). Adoptive transfer of DO11.10-derived T cells to naive dams prior to mating resulted in higher responsiveness of offspring to suboptimal challenge of OVA (108). Donor T cells were identified in spleen and placenta of the recipient female dams but were not detectable in fetal tissues, suggesting that the factors produced by maternal allergen-specific T cells mediated the response. Naïve offspring of asthmatic dams showed significantly high levels of spleen cellularity due to an increase in $CD4^+CD25^+Foxp3^-$ effector T cell population (109). Interestingly, depletion of $CD25^{high}$ T cell population by anti-CD25 antibody (clone PC61) in 4-day-old newborn mice before induction of airway inflammation by suboptimal sensitization protocol resulted in the increased susceptibility to allergic airway inflammation in normal offspring. In contrast, depletion of $CD25^{high}$ T cell resulted in a substantial reduction in asthma symptoms in offspring of allergic dams. Anti-glucocorticoid-induced tumor necrosis factor receptor-family related receptor (GITR) antibody (clone DTA-1) treatment before induction of airway inflammation with suboptimal sensitization protocol significantly attenuated the susceptibilities to allergic airway inflammation in offspring of both naïve and asthmatic dams. Contrary, anti-GITR antibody treatment after induction of airway inflammation enhanced the development of asthma-like allergic inflammation in normal offspring, but induced only a slight deterioration of allergic airway inflammation in the offspring of asthmatic dams. The authors suggest that the increased asthma susceptibility in offspring of asthmatic dams can be prevented by modulating maternal Th2 responses as well as neonatal regulatory responses with anti-CD25 (pre-sensitization in asthma-prone offspring) or -GITR antibodies (pre-sensitization in both normal and asthma-prone offspring). As OVA-dams were *not* exposed to OVA during lactation, these data may indicate that lack of allergen transfer through milk together with maternal sensitization is critical for maternal transmission of asthma risk to offspring (51, 118, 156, 158–160).

Skin barrier impairment also affects the susceptibility to food allergy in offspring born from allergen-sensitized dams. WT dams were *i.p.* sensitized with OVA/alum and exposed to OVA aerosols before mating with unsensitized flaky tail (ft) male mice that exhibit an impaired skin barrier due to homozygous mutations of filaggrin (*Flg*) and mattrin (*Tmem79*^{ma}) genes (*Flg*$^{ft/ft}$/*Tmem79*$^{ma/ma}$). Resulting *Flg*$^{ft/+}$/*Tmem79*$^{ma/+}$ offspring showed an increase in food allergen sensitization, serum mMCP1, and anaphylaxis to food allergen sensitization through tape stripped skin with peanut extract and *Alternaria alternate* extract followed by food allergen challenge compared to those born from unsensitized WT dams (110). These food allergic responses were associated with higher levels of transcripts for CCL11, TSLP and IL-33 in the skin, and TSLP in the jejunum. This study suggests the possibility that responses to food allergens of neonatal mice are dependent on genetic defects in cutaneous barrier function and on exposure to environmental allergens.

Breastfeeding

Both maternal allergic sensitization and allergen consumption seem to alter the immunological components of breast milk that affect offspring allergy. Using the mouse model of maternal asthma transfer as discussed above, Leme et al. demonstrated the role of breast milk from allergen-sensitized dams in the development of allergy in offspring (161). Both offspring from asthmatic dams nursed by unsensitized dams and offspring from unsensitized dams nursed by asthmatic dams showed enhanced airway hyperresponsiveness and allergic airway inflammation after allergen sensitization compared to offspring from and nursed by unsensitized dams. The authors suggest an equal transmission of asthma to the offspring either *in utero* or through breast milk.

With regards to food allergy, Song et al. demonstrate that maternal peanut allergy increases susceptibility to offspring allergy in link with Th2-biased epigenetic alterations in a mouse model of peanut allergy (118). C3H/HeJ dams were sensitized with oral peanut/CT and challenged before mating. Offspring of dams sensitized and challenged with peanut exhibited increased food allergic responses following suboptimal dose of oral peanut/CT sensitization and peanut challenge, associated with hypomethylation at CpG sites of *Il4* gene promoter. Interestingly, unlike their previous study demonstrating that maternal exposure to peanut during pregnancy and lactation reduces peanut allergy risk in offspring (17), dams did not receive sensitization during pregnancy and breastfeeding in this study. These results suggest that maternal sensitization may increase food allergy susceptibility in offspring in the absence of maternal allergen exposure during pregnancy and breastfeeding. Together, these studies suggest that maternal immune responses during lactation are critical for the reduction of food allergy susceptibility in offspring, consistent with the increasing numbers of studies demonstrating that breastfeeding by allergen-sensitized dams results in the induction of tolerance rather than the maternal risk transfer (as discussed in section Breastfeeding in Mouse studies for maternal protective influences).

Perspective

In some mouse studies, offspring of asthmatic dams have been shown to be more susceptible to asthma than offspring of non-allergic dams. However, the efficacy of breastfeeding by asthmatic dams in ameliorating allergic symptoms have not been thoroughly investigated. It is currently unclear whether offspring of allergic dams who did not receive breast milk may exhibit higher susceptibilities to allergies and move severe allergies than breastfed offspring. Multitudes of factors likely contribute to the maternal life during pregnancy and breastfeeding period, including allergen exposure and allergic responses. Depending on doses, timing, and number of antigen challenges, multiple factors in breast milk could transfer mediators that either increase or decrease offspring responses to allergen (**Table 3**). In the recent dramatic shift in recommendations from avoidance of common food allergens to early ingestion to prevent the development of allergy in offspring support the concept that early allergen introduction, potentially through breastfeeding, may decrease the risk of offspring allergies. Investigations of exact timing and dose of allergen exposure as well as early critical factors are necessary for development of safe and effective strategies for high-risk infant.

CONCLUSIONS

Evidences from human and mouse studies suggest that maternal allergen exposure and resulting maternal immune responses that are transferred through placenta and breast milk likely influence offspring immune responses toward those allergens. Such interactions are regulated by genetic factors and environmental factors (maternal microbiota, nutrients, and health) to determine the induction of tolerance vs. allergy outcome in offspring through Th1, Th2, and Treg cell responses.

Current human data are not sufficient to draw firm conclusions regarding the role of breastfeeding in preventing or delaying the development of food allergies in offspring (69), however, there is now evidence that early introduction of peanuts may prevent peanut allergy (**Table 1**). Mouse studies suggesting maternal transfer of asthma risk to offspring *lack* maternal allergen exposure during pregnancy and breastfeeding. Other studies suggest maternal transmission of tolerance to offspring against asthma through breast milk allergen and IC. Although the investigations using experimental food allergy within this area are still developing, those studies consistently suggest that maternal sensitization and exposure with allergen induce tolerance in offspring, especially via breast milk. These results from experimental asthma and food allergy models may seem inconsistent at first glance, yet they are consistent in that maternal allergen sensitization plus allergen exposure *during pregnancy and breastfeeding*, which are likely tolerogenic periods, are critical in the induction of tolerance vs. allergies in offspring (**Table 3**). Those studies, including our own, are in line with several recent findings in humans as (1) maternal exposure to food allergens reduces allergy in offspring in humans, (2) recent decisions to cancel recommendations of allergen avoidance during pregnancy and breastfeeding, and (3) early food introduction potentially decreasing the risk of food allergy development. The last finding is particularly relevant to the results from mouse studies, as food allergen transfer via milk may be the first food exposure for the infant.

Development of a feasible and optimal instruction for maternal and offspring consumption of potentially allergenic food and nutrient during pregnancy and breastfeeding are urgently needed. Thorough analysis of human amniotic fluid and breast milk component based on challenge-proven food allergies in offspring in combination with genetic analysis may help with development of such instruction. Consideration of dose, frequencies, and period of food consumption during pregnancy and breastfeeding together with nutrient or immunological supplementation of breast milk may help with the novel strategies for prevention and treatment of food allergy in offspring.

The precise mechanisms by which maternal factors, such as allergen exposure during breastfeeding, exert a lasting effect on determining the susceptibility of offspring toward allergy need further investigations. Findings in mouse studies need to be

carefully interpreted due to the differences between mice and humans. However, the powers of animal models are being able to investigate specific questions that are impossible or unethical to address in human subjects. To date, epidemiological studies have been performed at population levels and not at personal levels. Combined future research of translational and clinical science will help us with understandings of the role of maternal factors on offspring food allergy and will lead to effective preventive care of the disease.

AUTHOR CONTRIBUTIONS

TF, SL, YN, and MO wrote the paper. TF and MO arranged and revised the manuscript. SK and MO conceived and supervised the paper. TF, SL, and MO have agreed to be accountable for all aspects of the manuscript and in securing that questions related to the accuracy of any part of the manuscript. MO has given a final approval of the version of the manuscript to be published.

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