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Targeted innovative immunomodulation of allergy and inflammation by natural molecules and probiotics

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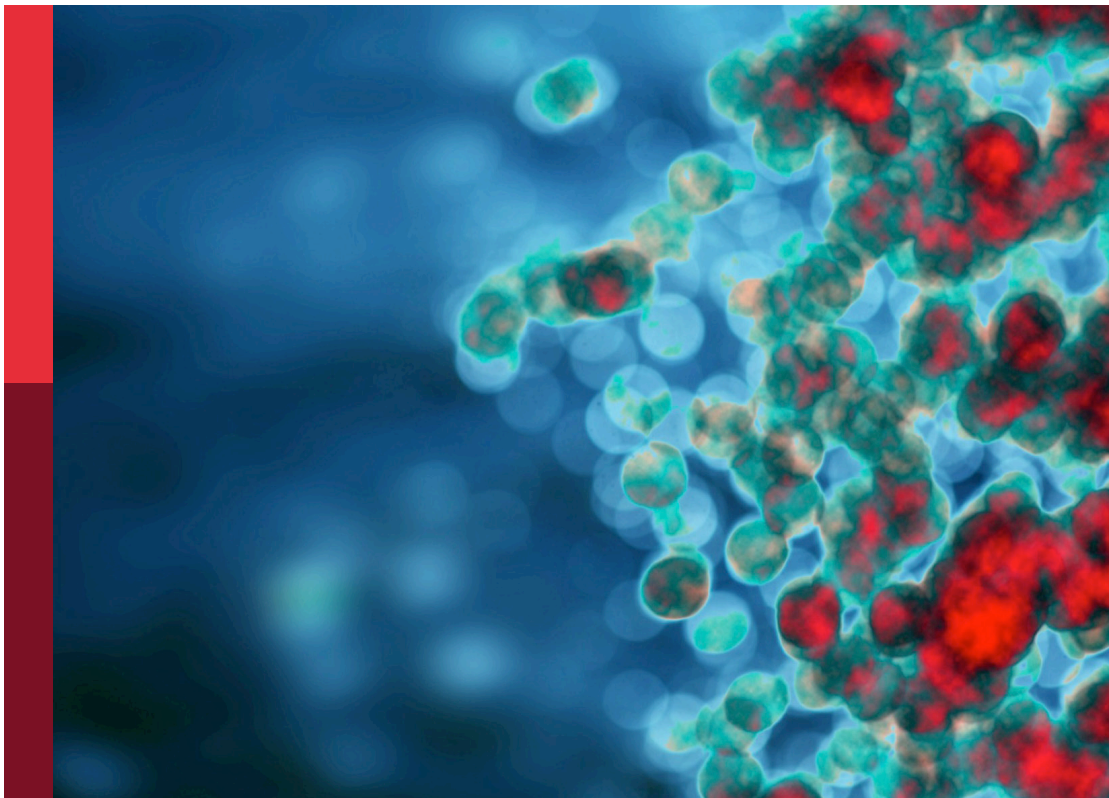

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Targeted innovative immunomodulation of allergy and inflammation by natural molecules and probiotics

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Computational analysis to define efficacy & molecular mechanisms of 7, 4'- Dihydroxyflavone on eosinophilic esophagitis: *Ex-vivo* validation in human esophagus biopsies

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Introduction: Eosinophilic Esophagitis (EoE) is a chronic condition characterized by eosinophilic inflammation of the esophagus which leads to esophageal dysfunction with common symptoms including vomiting, feeding difficulty, dysphagia, abdominal pain. Current main treatment options of EoE include dietary elimination and swallowed steroids. Diet elimination approach could lead to identifying the trigger food(s), but it often requires repeated upper endoscopy with general anesthesia and potentially could negatively affect nutrition intake and growth of the child and individuals' quality of life. Although the swallowed steroid treatment of effective, the EoE will universally recur after discontinuation of the treatment. Digestive Tea formula (DTF) has been used by the Traditional Chinese Medicine (TCM) practice to improve GI symptoms in EoE patients, including abdominal pain, GE reflux, and abnormal bowel movement. Previously, a flavonoid small molecule compound 7, 4 dihydroxy flavone (DHF) from *Glycyrrhiza uralensis* in DTF inhibited eotaxin, Th2 cytokine and IgE production *in vitro* and *in vivo*.

Method: This study comprehensively evaluates the potential therapeutic and immunological mechanisms underlying DHF improvement of symptoms related to EoE using computational modeling, including target mining, gene ontology enrichment, pathway analyses, protein-protein interaction analyses, in silico molecular docking and dynamic simulation followed by ex-vivo target validation by qRT-PCR using cultured human esophagus biopsy specimen with or without DHF from patients with EoE.

Results: Computational analyses defined 29 common targets of DHF on EoE, among which TNF-α, IL-6, IL1β, MAPK1, MAPK3 and AKT1 were most important. Docking analysis and dynamic simulation revealed that DHF directly binds TNF-α with a free binding energy of -7.7 kcal/mol with greater stability and flexibility. Subsequently, in the human esophagus biopsy culture system, significant reduction in levels of TNF-α, IL-6, IL-8 and IL1-β was found in the supernatant of biopsy sample cultured with DHF. Furthermore, the gene expression profile showed significant reduction in levels of TNF-α, IL1-β, IL-6, CCND and MAPK1 in the esophagus biopsy sample cultured with DHF.

Discussion: Taken together, the current study provides us an insight into the molecular mechanisms underlying multi-targeted benefits of DHF in the treatment of EoE and paves the way for facilitating more effective EoE therapies.

KEYWORDS

7, 4 dihydroxy flavone (DHF), eosinophilic esophagitis, anti-inflammation, computational modelling, molecular docking

Introduction

EoE is a chronic, immune/antigen- mediated allergic disease of the esophagus that is characterized clinically by esophageal dysfunction and histologically by eosinophil-predominant inflammation (> 15 eosinophils/HPF). The common symptoms of esophageal dysfunction include vomiting, feeding difficulty, dysphagia, and abdominal pain. The incidence of EoE is increasing with estimated prevalence in the US ranging from 40-90 cases/100000 (1). EoE predominates in Caucasian (81%) (2) and middle-aged (30–40 years) men (3) with a male-to-female ratio approaching 3:1 (4). The potential mechanism of EoE pathogenesis includes over expression of Th2 cytokines, genetic predisposition, and environmental stimulation, which initiate esophageal inflammation and subsequent tissue remodeling and fibrosis. It is possible that other molecular targets exist and yet to be identified and studied to better understand the complexity of EoE pathogenesis.

The current therapy for EoE includes the use of swallowed steroid and dietary elimination (5). The dietary approach is proven to be effective in 90% EoE patients and can lead to identifying the diet trigger(s). Sustained remission can be achieved by permanently eliminating the triggers. But multiple upper endoscopies under general anesthesia are often required and prolonged diet avoidance could negatively affect the child's nutrition intake, physical growth, and quality of life. Systemic steroids although show significant effect in reducing esophageal eosinophils, long-term use is associated with serious side effect. Swallowed steroid treatment is effective and of less side effect, but relapse of EoE is universal after the treatment is stopped (6). Proton-pump inhibitors (PPI) are effective in 15-30% of EoE patients who are PPI-responsive, but long-term use of PPI is associated with a number of side effect (7). Several humanized antibody therapies have been designed to block IL-5 and IL-13 and have showed promising effect in treating EoE (8). These specific therapies are likely to target only particular aspects of the disease but due to complexity and involvement of multiple players in the disease, persistence of inflammation even after the blockage of these single molecules has been observed (8).

Compound 7,4-dihydroxyflavone (DHF)- a flavonoid purified from *Glycyrrhiza uralensis* is one the most commonly used herbs in traditional Chinese medicine (TCM). We have shown that *G. uralensis* significantly inhibits TNF-α production (9). Likewise, we have highlighted key effects of DHF on suppression of Th2 cytokines-IL-4, IL-5 and IL-13 and serum IgE (10) and chemo attractant protein eotaxin/CCL-11 (11). The regulatory effect of DHF was further demonstrated in another study where DHF inhibited MUC5AC mRNA and protein expression (12) Recently, we also showed a case study of an 11 yr. old boy with complete clinical and tissue remission of EoE with TCM therapy, including Digestion teas (13). Based on our previous findings, we believe that DHF improvement of symptoms of EoE may be due to its anti-inflammatory effect, which ultimately reduces GI smooth muscle spasm, reverses the tissue inflammation, facilitates epithelium repair, and restores GI bacterial homeostasis.

Given the complexity of pathological mechanisms of EoE and multiple targets of DHF on immunological responses, we

utilized computational modeling—target mining, gene ontology enrichment, protein-protein interaction analyses, and *in silico* molecular docking to estimate the potential therapeutic mechanisms underlying the efficacy of DHF in EoE. The entire workflow of the study is shown in Figure 1. Guided by computational defined therapeutic targets, we employed human biopsy samples obtained from esophagus of EoE patients and determined DHF effects on those targets by tissue culture, ELISA, and qRT-PCR. The approaches in combination of computational modeling and *ex vivo* biological validation allowed us for the first time to identify potential therapeutic targets of DHF for EoE. This study provides guidance for our future *in vivo* experimental studies and future clinical trials.

Materials and methods

Target mining

Biological targets of DHF were identified from literature reports (14, 15) and published databases including HitPick (16), Swiss Target Prediction (17, 18), Similarity Ensemble Approach (19, 20), PubChem (21, 22), PharmMapper (23), and DrugBank (24, 25). The relevant human genes associated with EoE were selected as drug targets from various databases including Therapeutic Target Database (26, 27), Malacards (28), GeneCards (29, 30), and Open Targets Platform (31, 32). To ensure the predominance of targets, only the top 300 genes in each database were considered. Selected targets were finally mapped to UniProt Database (33, 34) for normalization. Next, the shared targets of DHF with EoE were obtained and these were considered to be potentially regulated targets of DHF for the management of patients with EoE.

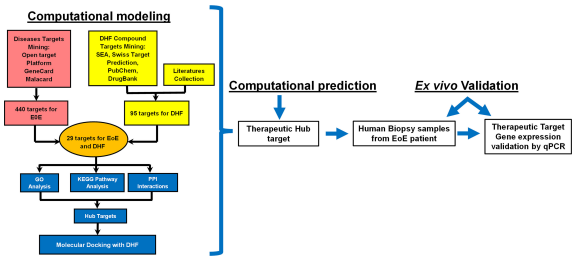

FIGURE 1
The workflow of computational modeling used for analysis of DHF as one of the promising candidates for Eosinophilic Esophagitis. First, total 440 target genes for EoE were selected. Likewise, 95 biological targets of DHF were collected based on literatures and following published databases including Swiss target prediction, SEA, pubchem and drug-bank. Mining DHF targets into obtained disease targets uncovered that DHF might potentially regulate 29 targets for EoE. The GO, KEGG pathway and PPI analysis were further conducted to uncover the regulated details of DHF. Moreover, the hub proteins were determined for further molecular docking analysis. Furthermore, the therapeutic hub targets predicted by the computational modeling were validated *ex-vivo* in human esophageal biopsy samples from patients with EoE by ELISA and qRT-PCR. DHF: 7,4 dihydroxy flavone; SEA, similarity ensemble approach; PPI, protein-protein interactions, GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; EoE, eosinophilic esophagitis.

Gene ontology (GO), pathway and protein-protein interaction (PPI) analysis

Target enrichment gene ontology, pathway, and protein-protein interaction (PPI) analyses provided a molecular-level mechanistic insight into biological function. GO was introduced by mapping potential targets to the DAVID database (35, 36). The GO biological process terms with a false discovery rate of (FDR) <0.01 were selected. Pathways were obtained by mapping targets to KOBAS 3.0 (37, 38) and the significant pathways with FDR <0.01 were selected. Potential targets were mapped to String database, obtaining their interaction. The protein interactions were further used to construct the PPI network using Cytoscape (v3.2.1).

Compound-target-pathway-disease network construction and analysis

With obtained targets and significant pathways, C-T-P-D biological networks were constructed using Cytoscape (v3.2.1). This network, containing DHF, its related targets for EoE, and significant principal pathways were constructed to comprehensively elucidate the complex relationship among compound, targets, and disease related pathways. This analysis provided general information about pharmacological mechanisms of DHF for the treatment and management of EoE at a molecular level. The properties of C-T-P-D networks were validated by NetworkAnalyzer (39), a plugin of Cytoscape.

Molecular docking analysis

The binding modes of DHF with critical targets were predicted through molecular docking by AutoDock Vina (40). Protein crystal structures including IL1β (PDB:5R85), IL6 (PDB: 1ALU) (41), TNF (PDB:2AZ5) (42), and CCND1 (PDB:2W96) with excellent resolution were obtained from RCSB protein data bank (43, 44). The structure of DHF was directly downloaded from PubChemwithout further optimization. The molecular graphics were displayed by PyMOL system (45) (46), and Discovery Studio (47). Generally, all hydrogens and Gasteriger charges were added to each molecule. Docking areas and Autogrid parameters were set based on the binding pockets of proteins.

Molecular dynamic simulation

The molecular dynamic simulations were carried out by Groningen Machine for Chemicals Simulations (GROMACS) with amber99sb-ildn force field and tip3p water model. 50 ns molecular dynamics simulation was performed for protein-ligand complex. The Root Mean Square Deviation (RMSD) analysis, and the toot-mean-square fluctuation (RMSF) were carried out using Xmgrace software.

Esophageal biopsy culture

Pediatric subjects (aged 0-18 years old) with potential or known EoE were recruited under an IRB approved protocol at Mount Sinai Medical Center when undergoing an endoscopy as part of their routine clinical care. All subjects were determined to have active EoE based on clinical criteria including symptoms consistent with EoE and biopsies showing >15 eosinophils per high power field. Subjects 'characteristics are listed in Table 1.

Biopsies were digested with DNases I and Collagenase IV as previously described48 and divided equally into two wells then cultured with or without DHF (0.02mg/ml) in complete RPMI with 10% FBS overnight. Cytokines were measured by ELISA (BD Biosciences, NJ) as per the manufacturer's instructions.

Quantitative real-time PCR

RNA was extracted from precipitation of esophageal biopsy culture using TRIZOL (Invitrogen, Carlsbad, CA) followed by isopropanol precipitation. RNA was then reverse transcribed to complementary DNA (cDNA) using a PrimeScriptTM RT Reagent Kit (TaKaRa, Mountain View, CA). Real-time PCR was performed using SYBRTM Green Master Mix (Thermo Fisher Scientific, Fair lawn, NJ) as previously described (48). The target gene mRNA expression was normalized to the control group and calculated using the ΔΔCT method. The primer sequences are shown in Table S1.

Results

Target mining identifies the shared biological targets between DHF and EoE

The Venn diagram shows that 440 target genes for EoE were selected. Likewise, 95 biological targets of DHF were collected from the literature (14, 15), and published databases. Among them, 29 shared targets between DHF and EoE were discovered, which were finally selected as the potential therapeutic targets of DHF in the treatment and management of EoE (Figure 2A).

Pathway analysis reveals complex signal transduction regulated by DHF

Further, to clarify the potential pharmacological effects of DHF on EoE from the pathway level, the pathway enriched analysis was performed by connecting the predicted targets to DAVID database. The top 15 significant KEGG pathways with False Discovery Rate (FDR) <0.01 and genes associated with each pathway were ranked and picked out by enrichment score –log (FDR) as shown in Figure 2B. Most pathways were related to host immune responses to inflammation and included "IL-17 signaling pathway", "TNF-signaling pathway", "AGE-RAGE signaling pathway in diabetic complications" "TH17 cell differentiation", "Tuberculosis", "Leishmaniasis", "C-type lectin

TABLE 1 Subject demographics.

Patient	Age (yr.)	Date of diagnosis	Sex	t-IgE (IU/L)	Food specific IgE	Treatment	Other allergies	Other conditions
P1	6Y	8/7/2018	F	–	Almond, milk, wheat, treenut, sesame, fish, shellfish, poppy	None	Allergic rhinitis	none
P2	8Y	8/2/2018	M	–	Egg white, egg yolk, pea, walnut, milk, almond, chickpea	budesonide	Asthma, allergic rhinitis, atopic dermatitis	None
P3	17Y	5/17/2018	F	–	Tree nuts (walnut, almond, brazil, cashew, pecan, macadamia), peanut	Omeprazole	Food allergies (peanut and tree nut), asthma	None
P4	5Y	9/8/2018	F	–	All negative	Omeprazole and milk avoidance	Asthma	None
P5	17Y	7/10/2018	M	–	All negative	omeprazole	None	GH Deficiency
P6	10Y	8/28/2018	F	–	All negative	None	Asthma, allergic rhinitis	None
P7	4Y	11/1/18	M	–	Tree nuts, salmon, beef	Protein Pump Inhibitor, TCM	Atopic dermatitis, allergic rhinitis	None
P8	10Y	8/24/2019	M	8	All negative	Fluticasone	None	celiac

TCM, Traditional Chinese Medicine; GH, Growth hormone; t-total.

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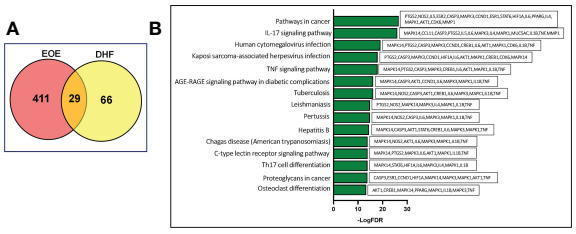

FIGURE 2
Computational identification and KEGG pathway analysis of the therapeutic targets. **(A)** Venn diagram showing the number of targets predicted using computational modeling. Among them 29 targets were shared between DHF and EoE. **(B)** KEGG Pathway analysis. Y-axis: The top 15 pathway relevant to the enriched targets (left) and genes associated with each pathway (right); X-axis: the significance of each term ranked with −log FDR. DHF, 7,4-dihydroxyflavone, EoE, Eosinophilic Esophagitis, FDR, False discovery rate.

receptor signaling pathway" and "Pathway in cancer". One of the main features of EoE is the accumulation of activated eosinophil at the site of inflammation. Eosinophils, generally, have a short life span of about 4 days and prolonged survival of eosinophil at the site of inflammation is mediated by IL-5, a potent type 2 cytokine (16). IL-5 along with IL-3 and GM-CSF mediate prolonged eosinophilic survival and release their cationic granular proteins, oxygen radicals and lipid mediators to cause tissue damage (16). Similarly, other pathways like "Human cytomegalovirus infection", "Hepatitis B", "Kaposi sarcoma-associated herpesvirus infection", "Proteoglycans in cancer", and "osteoclast differentiation" are involved in cell survival, proliferation, and progression. Overall, multiple pathways associated with EoE that were regulated by DHF were identified.

Gene ontology reveals potential regulation of DHF in inflammation, apoptosis, oxidation, and transcription factor activity

The GO biological process terms in DAVID database were obtained with the identified targets as an enriched gene-set. The top 15 biological processes GO terms with False Discovery Rate (FDR) <0.01 were ranked by enrichment score (–log FDR) (Figure S1). The most significant GO biological process terms were closely associated with anti-apoptotic, anti-inflammatory, and anti-oxidative and anti-tumor properties. The most important pathways associated with these properties were "positive regulation of specific DNA binding transcription factor activity", "positive regulation of transcription DNA-template", "lipopolysaccharide-mediated signaling pathway", "positive regulation of nitric oxide biosynthesis", "positive regulation of transcription from RNA polymerase II promoter", "inflammatory response", "positive regulation of fever generation", and "activation of MAPK". Interestingly, most of the genes associated with these pathways (Figure S1) were inflammatory genes and were consistent with the KEGG pathway analysis. This further allowed us to explore the mechanism of DHF in treating EoE with respect to associated processes and function.

Compound-target-pathway-disease network construction to select the crucial proteins

The C-T-P-D network containing DHF, selected targets, top 15 pathways and EoE was developed to interpret the potential pharmacological mechanisms of DHF for management and treatment of EoE at the molecular level (Figures 3A, B). The C-T-P-D network provides general information about the complex interactions of compound, targets, and their related diseases. The frequency of targets appearing in the top 15 pathways implies their influence and importance. Node color from green to red is proportional to degree value, displaying importance from high to low in the network. The most important protein targets based on its degree from the C-T-P-D network were TNF, MAPK1, MAPK3, IL6, IL1B, AKT1 and CASP3 and the most important pathways associated with these protein targets were "Pathways in cancer", "IL-17 signaling pathway", and "Human cytomegalovirus infection". Overall,

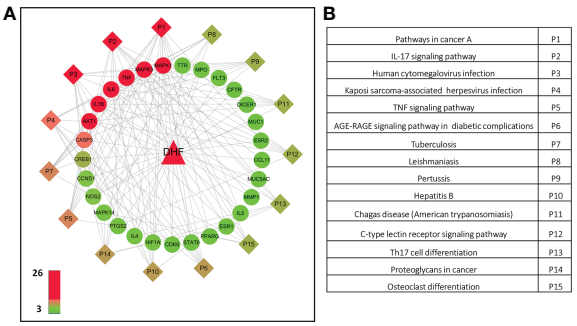

FIGURE 3
Compound-target- pathway-disease network of DHF for EoE. **(A)** By computational modeling, the role of DHF was deciphered through the drug-target network, and the target-disease network from the molecular level to the systems level. Triangle, circle, and diamond represent DHF, key targets and pathways, respectively. Node color from green to red is proportional to its degree. Black lines stand for interaction between the nodes The most important targets were TNF, MAPK1, MAPK3, IL6, IL1B, AKT1 and CASP3 (Red). The most important pathways associated with the protein targets were "Pathways in cancer" (P1), "IL-17 signaling pathway" (P2), and "Human cytomegalovirus infection" (P3). **(B)** Pathways associated in the compound-target-disease network.

the C-T-P-D network helped to simplify the complex interaction between proteins, and pathways associated with EoE and DHF. Furthermore, it helped to select key target proteins which could potentially be targeted by DHF in treating EoE.

Protein-protein interaction network construction to confirm the vital function of proteins

The PPI network was constructed by mapping potential targets to the String database (17). The size of the node from large to small is proportional to its degree value in the network. It is well known that protein-protein interactions are critical to a wide range of biological processes, including cell-to-cell interaction and metabolic and developmental control (18). A deeper understanding of such complex relationships among disease-related proteins provides new opportunities to investigate the molecular mechanisms of diseases (19). Recently, PPI has become a reliable tool to evaluate protein functions in the network and determine hub proteins in the regulation of diseases. In this study, we found TNF occupying the central position in the network along with IL6, MAPK3, MAPK1 and AKT1 (Figure 4). The branching lines from these proteins represent its interaction with other proteins and we found highest interaction of these key proteins with others in the protein-protein interaction analysis. The other important proteins in the network were IL1B, IL4, CASP3, CCND1, and PTGS2 and these proteins showed moderate rank in the PPI network. Some of the less important proteins formed the outer layer of the network and showed lowest interaction with others. Overall, the PPI network led to specifically select key proteins with the highest interaction index to better understand how DHF could potentially regulate these proteins in management of EoE.

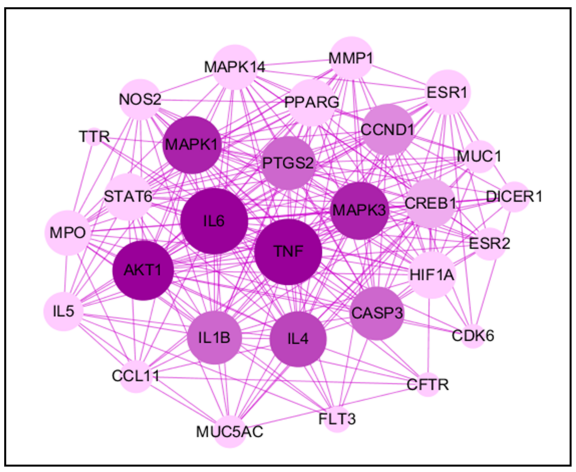

FIGURE 4
Protein-protein interactions. The PPI network was constructed by mapping potential targets to the Strings database. The size of the node from large to small is proportional to its degree value in the network. The circles represent the therapeutic targets, and the purple lines represent the interaction between the nodes.

Molecular docking analysis and dynamic simulation predicts the binding modes between DHF and its crucial targets

We assumed that DHF regulated the crucial targets by interfering in gene expression or binding with proteins directly. Therefore, a molecular docking was introduced to calculate the binding energy and evaluate binding favorability between DHF, and crucial targets. The binding energy indicated that DHF might be a promising regulator for each of the selected targets with binding affinities between -6.3 to -7.7 Kcal/mol (Table S2). The optimal binding modes of DHF with targets including TNF-α, MAPK1, IL1B, IL-6, and CCND1 is shown in Figure 5. The best results were obtained for complex TNF-DHF with a free binding energy of -7.7 kcal/mol. The Hydrogen bonds between DHF with TYR151 and the π-π stacking between DHF and LEU120 stabilized the left structure of DHF in one chain (Figures 5A, F). Likewise, DHF displayed similar binding affinities with MAPK1 and IL1-β. For MAPK1-DHF complex, the hydrogen bond (MET108), π-alkyl interactions (ILE31, ILE84, ALA52, CYS166), and π-anion interactions (ASP111) with DHF stabilizing the configuration of DHF in the complex (Figures 5B, G). Similarly, for complex IL1B-DHF, the hydrogen bond between DHF and residues (TYR24, LEU134, THR79) significantly contributed to the stability of the complex (Figures 5C, H). For complex IL6-DHF, DHF fitted well in the binding cavity of IL-6 surrounded by hydrogen bonds (ASP34, ARG30), π-sigma (LEU33, LEU178), π-alkyl (ARG179) and π-π (ARG30) interactions (Figures 5D, I). Lastly, for the complex CCND1-DHF, the residues ARG87, LEU91, and LYS149 facilitated the binding interactions by hydrogen, π-π stacking, and π-cation effects (Figures 5E, J). To evaluate the stability of binding, TNF-DHF complex were further optimized by dynamic stimulation using Gromacs. The stability and flexibility of complex were estimated by RMSD (Figure S2A) and RMSF (Figure S2B) analysis respectively. The trend of RMSD figure indicated the stable binding of DHF with TNF. It is worth noting that the average RMSD value for TNF was 4.9 Å. The RMSF analysis implied minor deviation of protein during the simulation period. The most stable binding mode of TNF-DHF were displayed in Figures S2C, D respectively. Residues TYR59, ILE155, and LEU57 contributed to maintain the stability of DHF with TNF. Overall, the molecular docking results indicated that DHF bind with the crucial proteins directly to regulate the resulting biological effects.

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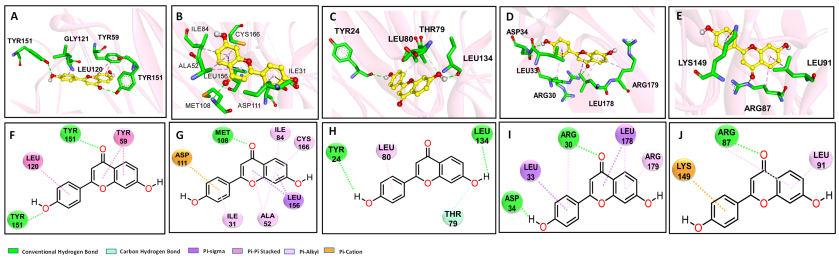

FIGURE 5
Molecular docking analysis to predict the binding modes between DHF and its therapeutic targets. Based on the gene expression results in the esophagus, the binding explorations of complex MAPK1-DHF, IL1B-DHF, IL6-DHF, TNF-DHF, CCND1-DHF were explored. Predicted lowest-energy binding mode of DHF in 3-dimentiaonal figure with the following proteins: **(A)**, **(F)** TNF-α; **(B)**, **(G)** MAPK1; **(C)**, **(H)** IL1B; **(D)**, **(I)** IL6; **(E)**, **(J)** CCND1; the carbon, oxygen and nitrogen are highlighted in yellow, red, and blue, respectively. For residents of proteins, the green, red, and blue stand for C, O and N, respectively.

DHF inhibited pro-inflammatory cytokine levels and its expression *in- vitro* in human esophageal biopsy culture

To provide experimental support and validate the claim that DHF could be an effective alternative for the management of EoE patients, we evaluated the effect of DHF on key therapeutic targets obtained from the computational analysis. We evaluated the levels of different pro-inflammatory cytokines by ELISA and their expression by qRT-PCR in human esophageal biopsy cultures with or without the presence of DHF. In presence of DHF, we found significantly lower levels of pro-inflammatory cytokines- TNF-α, IL-8, IL-6, IL-1B respectively (Figures 6A–D) and slight increase in IL-10 levels (p=0.38) (Figure 6E) in the culture supernatant of esophagus biopsies. Furthermore, in presence of DHF, the gene expression analysis showed marked decrease in the expression levels of TNF-α, IL1-B, IL-6, CCND and MAPK1 respectively (Figures 7A–E). Likewise, there was a slight decrease in expression of CASP3 and PPARγ (p=0.08) respectively (Figures 7F, G). However, there was no statistical differences in the expression levels of MAPK3 and Akt respectively (Figures 7H, I).

Discussion

In this study we, for the first time, used a computational approach that integrated target mining, network technology and pathway analysis to reveal the potential therapeutic pathways and targets to understand pharmacological mechanisms of DHF in treating EoE. The cutting-edge technologies allowed us to uncover key mechanisms involved in the pathogenesis of EoE and further advance our knowledge into new therapeutic applications. It is believed that mechanism of EoE is strictly driven by complex interplay between genetic and early life environmental risk factors (20), interaction and signaling between epithelial, mesenchymal, and immune cells on molecular and intracellular level (21). Based on our results, we selected top 15 pathways of which majority of them were inflammatory. This further adds to the complexity of the disease by deciphering key inflammatory molecular pathways and provide evidence that these pathways may be contributing directly or indirectly to the progression of disease. Some of the important pathways were- pathway in cancer, IL-17 signaling, TNF signaling, AGE-RAGE signaling in diabetic complications, and several other infectious disease related pathways. Furthermore, by mapping these pathways in the C-P-T-D network, we identified the key targets associated with these pathways which could potential be regulated by DHF in EoE. The most prominent targets were MAPK1, MAPK3, TNF, IL6, IL1B and AKT1, also known to as an important cytokine regulating tissue damage. The pathogenesis of EoE is still incompletely understood but it be believed to be a consequence of a complex interplay between genetic, environment and host immune cells (22). Beside these factors, it is plausible that the pathways and genes identified in this study by computational approach could be playing a role in the pathogenesis of EoE. Further studies are necessary to better understand the mechanisms at molecular level. Similarly, the molecular docking and dynamic simulation analysis revealed that DHF can directly bind to the target molecules with similar binding affinities to regulate its action in preventing inflammation. The strongest binding with superior stability and minor deviation was seen between DHF and TNF. The Molecular simulation could accurately predict many important dynamics, but sometimes these simulations are not suitable to systems where metal atoms are involved in binding requiring robust quantum mechanical calculations. Finally, we validated our computational modeling results using esophagus biopsy sample from patient with EoE. We demonstrated that in presence of DHF there was significant reduction in the levels of TNF-α, IL-8, IL-6, IL1B and in the culture supernatant and

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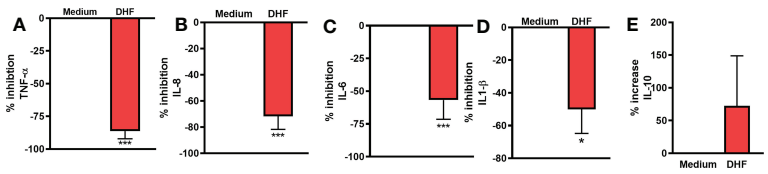

FIGURE 6
Anti-inflammatory effect of DHF on esophageal biopsy sample: Esophageal biopsy samples were cultured with or without DHF for 24 hrs. and cytokine levels in the culture supernatant were measured by ELISA. Percentage inhibition of cytokines TNF-α **(A)**, IL-8 **(B)**, IL-6 **(C)**, IL1-β **(D)** and percentage of increase in cytokine IL-10 **(E)** in presence of DHF were analyzed. Data represents Mean ± SEM. N=6-8 samples. ***p < 0.001; *p < 0.05 compared to Medium. Paired *t* test was used for statistical analysis.

further validated its effect in the molecular level by qRT-PCR. Our gene expression data showed significant decrease in the expression of key pro-inflammatory genes TNF-α, IL-1B, IL-6, CCND and MAPK1. This study provides an evidence DHF could potentially be regulating inflammation in EoE and further studies with large sample size should be conducted to better understand the mechanisms associated with it.

At present, dupilumab is the only the FDA approved treatment for EoE (23), and highlighting the complex nature of the disease, it is crucial to advance our knowledge and strengthen the research into therapeutic agents that can target multiple aspects of the disease. Evidence of successful management of a patient with GI symptoms secondary to EoE using digestion tea containing DHF (13) opens up the area to study the role of flavonoid in treating EoE. With this study, we were able to successfully identify key targets and subsequently validate it using the human esophagus biopsy sample from patient with EoE. It could be possible that DHF mechanism of acting in successfully treating EoE patient was due to its action on the pathways and genes that we identified in our study. This study may provide a potential role of DHF as an active therapeutic candidate in the management and treatment of EoE. The direct effect of DHF in animal models of eosinophilic disease is the next step towards understanding and determining if DHF will be efficacious for the treatment of EoE.

The current study uses computational approach to identify key targets which are potentially regulated by DHF in EoE. However, the key targets obtained using this approach are not primary drivers of eosinophilic esophagitis but based on the evidence on the literature the role of TNF in EoE has been actively investigated. It has been reported that the inflamed epithelial cells prime esophageal fibroblasts to secrete the profibrogenic cytokines IL-1β and TNF-α, which in turn promote epithelial-to-mesenchymal transition and esophageal fibrosis (48). Additionally, it has been reported that cross talk

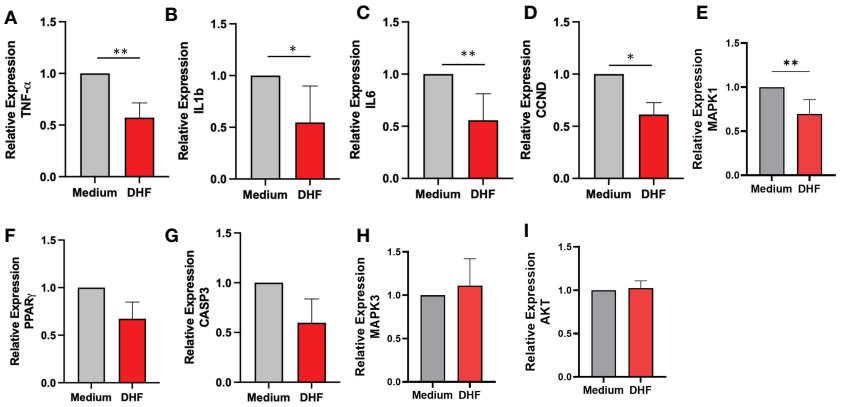

FIGURE 7
Effect of DHF on therapeutic target gene in esophageal biopsy sample: Based on the therapeutic hub targets predicted by the computational modeling, the expression levels of key targets were validated *ex-vivo* using human esophageal biopsy samples cultured in the presence of DHF. The relative expression levels of TNF-α **(A)**, IL1β **(B)**, IL-6 **(C)**, CCND **(D)** and MAPK1 **(E)** were significantly inhibited in the presence of compound DHF. Similarly, the expression levels of PPARγ **(F)**, CASP3 **(G)**, MAPK3 **(H)** and AKT **(I)** showed no significant difference as compared to the medium. Data represents Mean ± SEM. N = 6-8 samples. *p < 0.05; **p < 0.01 compared to Medium. Paired *t* test was used for statistical analysis.

between esophageal epithelial cells and fibroblast leads to robust production of TNF, contributing to fibrostenotic EoE (49). Furthermore, several TNF superfamily of proteins has been shown to be transcribed by several different subsets of T cells in the esophagus including the pathogenic effector Th2 cells (50). This makes us further believe that TNF play an important role in EoE, possibly at the chronic stage and a therapy targeting TNF in EoE would potentially contribute to one endotype of EoE. With current therapy having limited effects on treating fibrostenotic EoE, and ample evidence of production of TNF by esophageal fibroblast to contribute to inflammatory cascade in EoE (51), this study opens up a new area in EoE therapeutics by showing the role of DHF in modulating pro-inflammatory markers in EoE. In addition, most of the targets that we found in this study shared common pathways of inflammation and it plausible that all these inflammatory players could possibly be playing a role in exacerbating eosinophilic inflammation. Even though the effect of DHF on predicted target using computational approach seems promising, further studies are required to test the effect of DHF clinically for proposed activity against inflammatory EoE as well as the detailed mechanism of action on how these genes and targets interact with primary drivers of EoE, including Th2 genes- IL-4, IL-5, and IL-13 needs to be studied. This may be done either alone or in combination with an FDA approved treatment, Dupixent.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by IRB approved protocol at Mount Sinai Medical Center. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

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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Conflict of interest

X-ML received consultancy fees from FARE and Johnson & Johnson Pharmaceutical Research & Development, L.L.C. Bayer Global Health LLC; received royalties from UpToDate; is an Honorary Professor of Chinese Medical University, Taichung, Taiwan; Henan University of Chinese Medicine Zhenzhou, China, and Professorial Lecture at Icahn School of Medicine at Mount Sinai, New York, NY, US; received travel expenses from the NCCAM and FARE; share US patent PCT/US05/008417 (FAHF-2), PCT 14/875,772 (XPP), PCT/US2014/012306 (S. Flavescens), PCT/US14/68396 (WL); PCT/US2017/056822) (nanoBBR, pending), and is a member of Herbs Springs LLC and General Nutraceutical Technology LLC and Health Freedom LLC; take compensation from her practice at Integrative Health and Acupuncture PC. NY shares PCT/US2014/012306 (S. Flavescens), PCT/US14/68396 (WL), is a member of General Nutraceutical Technology LLC and Health Freedom LLC.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1015437/full#supplementary-material

References

1. Dellon ES, Jensen ET, Martin CF, Shaheen NJ, Kappelman MD. Prevalence of eosinophilic esophagitis in the united states. *Clin Gastroenterol Hepatol* (2014) 12 (4):589–96.e1. doi: 10.1016/j.cgh.2013.09.008

2. Hruz P, Straumann A, Bussmann C, Heer P, Simon HU, Zwahlen M, et al. Escalating incidence of eosinophilic esophagitis: a 20-year prospective, population-based study in olten county, Switzerland. *J Allergy Clin Immunol* (2011) 128:1349–50.e5. doi: 10.1016/j.jaci.2011.09.013

3. Sperry SL, Woosley JT, Shaheen NJ, Dellon ES. Influence of race and gender on the presentation of eosinophilic esophagitis. *Am J Gastroenterol* (2012) 107 (2):215–21. doi: 10.1038/ajg.2011.342

4. Mansoor E, Cooper GS. The 2010-2015 prevalence of eosinophilic esophagitis in the USA: A population-based study. *Digestive Dis Sci* (2016) 61 (10):2928–34. doi: 10.1007/s10620-016-4204-4

5. D'Alessandro A, Esposito D, Pesce M, Cuomo R, De Palma GD, Sarnelli G. Eosinophilic esophagitis: From pathophysiology to treatment. *World J Gastrointest Pathophysiol* (2015) 6(4):150–8. doi: 10.4291/wjgp.v6.i4.150

6. Butz BK, Wen T, Gleich GJ, Furuta GT, Spergel J, King E, et al. Efficacy, dose reduction, and resistance to high-dose fluticasone in patients with eosinophilic esophagitis. *Gastroenterology* (2014) 147(2):324–33.e5. doi: 10.1053/j.gastro.2014.04.019

7. Yibirin M, De Oliveira D, Valera R, Plitt AE, Lutgen S. Adverse effects associated with proton pump inhibitor use. *Cureus* (2021) 13(1):e12759. doi: 10.7759/cureus.12759

8. Patel RV, Hirano I, Gonsalves N. Eosinophilic esophagitis: Etiology and therapy. *Annu Rev Med* (2021) 72:183–97. doi: 10.1146/annurev-med-052819-023848

9. Patil SP, Liu C, Yang N, Li X-M. Glycyrrhiza uralensis flavonoids inhibit brain microglial cell TNF-α secretion, p-IκB expression, and increase brain-derived neurotropic factor (BDNF) secretion. *J Traditional Chin Med Sci* (2014) 1(1):28–37. doi: 10.1016/j.jtcms.2014.11.004

10. Yang N, Patil S, Zhuge J, Wen MC, Bolleddula J, Doddaga S, et al. Glycyrrhiza uralensis flavonoids present in anti-asthma formula, ASHMI™, inhibit memory Th2 responses *in vitro* and *in vivo*. *Phytother Res* (2013) 27 (9):1381–91. doi: 10.1002/ptr.4862

11. Jayaprakasam B, Doddaga S, Wang R, Holmes D, Goldfarb J, Li XM. Licorice flavonoids inhibit eotaxin-1 secretion by human fetal lung fibroblasts *in vitro*. *J Agric Food Chem* (2009) 57(3):820–5. doi: 10.1021/jf802601j

12. Liu C, Weir D, Busse P, Yang N, Zhou Z, Emala C, et al. The flavonoid 7,4'-dihydroxyflavone inhibits MUC5AC gene expression, production, and secretion *via* regulation of NF-kappaB, STAT6, and HDAC2. *Phytother Res* (2015) 29 (6):925–32. doi: 10.1002/ptr.5334

13. Soffer G, Kaman K, Li XM. Successful management of eosinophilic esophagitis using traditional Chinese medicine: A case report. *Yale J Biol Med* (2020) 93(5):685–8.

14. Chen XW, Di YM, Zhang J, Zhou ZW, Li CG, Zhou SF. Interaction of herbal compounds with biological targets: a case study with berberine. *ScientificWorldJournal* (2012) 2012:708292. doi: 10.1100/2012/708292

15. Liu H, You L, Wu J, Zhao M, Guo R, Zhang H, et al. Berberine suppresses influenza virus-triggered NLRP3 inflammasome activation in macrophages by inducing mitophagy and decreasing mitochondrial ROS. *J leukocyte Biol* (2020) 108 (1):253–26. doi: 10.1002/JLB.3MA0320-358RR

16. *HitPick*. mips.helmholtz-muenchen.de/hitpick/. Accessed June 2022.

17. *Swiss Target prediction*. swisstargetprediction.ch/. Accessed June 2022.

18. Daina A, Michielin O, Zoete V. SwissTargetPrediction: updated data and new features for efficient prediction of protein targets of small molecules. *Nucleic Acids Res* (2019) 47(W1):W357–w64. doi: 10.1093/nar/gkz382

19. *Similarity ensemble approach*. sea.bkslab.org/. Accessed June 2022.

20. Keiser MJ, Roth BL, Armbruster BN, Ernsberger P, Irwin JJ, Shoichet BK. Relating protein pharmacology by ligand chemistry. *Nat Biotechnol* (2007) 25 (2):197–206. doi: 10.1038/nbt1284

21. *PubChem*. pubchem.ncbi.nlm.nih.gov/. Accessed June 2022.

22. Kim S, Chen J, Cheng T, Gindulyte A, He J, He S, et al. PubChem 2019 update: improved access to chemical data. *Nucleic Acids Res* (2019) 47(D1):D1102–d9. doi: 10.1093/nar/gky1033

23. Wang X, Shen Y, Wang S, Li S, Zhang W, Liu X, et al. PharmMapper 2017 update: a web server for potential drug target identification with a comprehensive target pharmacophore database. *Nucleic Acids Res* (2017) 45(W1):W356–w60. doi: 10.1093/nar/gkx374

24. *DrugBank*. drugbank.ca. Accessed June 2022.

25. Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D, et al. DrugBank: A knowledgebase for drugs, drug actions and drug targets. *Nucleic Acids Res* (2008) 36(Database issue):D901–6. doi: 10.1093/nar/gkm958

26. *Therapeutic target database*. db.idrblab.net/ttd/. Accessed June 2022.

27. Wang Y, Zhang S, Li F, Zhou Y, Zhang Y, Wang Z, et al. Therapeutic target database 2020: enriched resource for facilitating research and early development of targeted therapeutics. *Nucleic Acids Res* (2020) 48(D1):D1031–d41. doi: 10.1093/nar/gkz981

28. Rappaport N, Twik M, Plaschkes I, Nudel R, Iny Stein T, Levitt J, et al. MalaCards: an amalgamated human disease compendium with diverse clinical and genetic annotation and structured search. *Nucleic Acids Res* (2017) 45(D1):D877–d87. doi: 10.1093/nar/gkw1012

29. *GeneCards*. genecards.org/. Accessed June 2022.

30. Safran M, Solomon I, Shmueli O, Lapidot M, Shen-Orr S, Adato A, et al. GeneCards 2002: towards a complete, object-oriented, human gene compendium. *Bioinformatics* (2002) 18(11):1542–3. doi: 10.1093/bioinformatics/18.11.1542

31. . *Open targets platform*. targetvalidation.org/. Accessed June 2022.

32. Carvalho-Silva D, Pierleoni A, Pignatelli M, Ong C, Fumis L, Karamanis N, et al. Open targets platform: New developments and updates two years on. *Nucleic Acids Res* (2019) 47(D1):D1056–d65. doi: 10.1093/nar/gky1133

33. . UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res* (2019) 47(D1):D506–d15. doi: 10.1093/nar/gky1049

34. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res* (2019) 47(D1):D506–d15. doi: 10.1093/nar/gky1049

35. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* (2008) 37(1):1–13. doi: 10.1093/nar/gkn923

36. *DAVID*. david.ncifcrf.gov/. Accessed June 2022.

37. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* (2011) 39(suppl_2):W316–W22. doi: 10.1093/nar/gkr483

38. *KOBAS 3.0*. kobas.cbi.pku.edu.cn/. Accessed June 2022.

39. Assenov Y, Ramírez F, Schelhorn S-E, Lengauer T, Albrecht M. Computing topological parameters of biological networks. *Bioinformatics* (2007) 24(2):282–4. doi: 10.1093/bioinformatics/btm554

40. Trott O, Olson AJ. AutoDock vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* (2010) 31(2):455–61. doi: 10.1002/jcc.21334

41. Somers W, Stahl M, Seehra JS. 1.9 a crystal structure of interleukin 6: implications for a novel mode of receptor dimerization and signaling. *EMBO J* (1997) 16(5):989–97. doi: 10.1093/emboj/16.5.989

42. He MM, Smith AS, Oslob JD, Flanagan WM, Braisted AC, Whitty A, et al. Small-molecule inhibition of TNF-alpha. *Science* (2005) 310(5750):1022–5. doi: 10.1126/science.1116304

43. . rcsb.org 5-30-2021 [.

44. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The protein data bank. *Nucleic Acids Res* (2000) 28(1):235–42. doi: 10.1093/nar/28.1.235

45. DeLano WL. Pymol: An open-source molecular graphics tool. *CCP4 Newslett On Protein Crystallography* (2002) 40:82–92.

46. . pymol.org 5-30-2021 [.

47. DassaultSystèmesBIOVIAD. *Discovery studio*. DassaultSystèmesBIOVIAD (2020). Accessed June 2022.

48. Hill DA, Spergel JM. The immunologic mechanisms of eosinophilic esophagitis. *Curr Allergy Asthma Rep* (2016) 16(2):9. doi: 10.1007/s11882-015-0592-3

49. Kasagi Y, Dods K, Wang JX, Chandramouleeswaran PM, Benitez AJ, Gambanga F, et al. Fibrostenotic eosinophilic esophagitis may reflect epithelial lysyl oxidase induction by fibroblasts-derived tumor necrosis factor-alpha. *J Allergy Clin Immunol* (2019) 144(1):171–82. doi: 10.1016/j.jaci.2018.10.067

50. Manresa MC, Miki H, Miller J, Okamoto K, Dobaczewska K, Herro R, et al. A deficiency in the cytokine TNFSF14/LIGHT limits inflammation and remodeling in murine eosinophilic esophagitis. *J Immunol* (2022) 26:ji2200326. doi: 10.4049/jimmunol.2200326

51. Manresa MC, Chiang AWT, Kurten RC, Dohil R, Brickner H, Dohil L, et al. Increased production of LIGHT by T cells in eosinophilic esophagitis promotes differentiation of esophageal fibroblasts toward an inflammatory phenotype. *Gastroenterology* (2020) 159(5):1778–92.e13. doi: 10.1053/j.gastro.2020.07.035

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Effect of immunostimulation with bacterial lysate on the clinical course of allergic rhinitis and the level of γδT, iNKT and cytotoxic T cells in children sensitized to grass pollen allergens: A randomized controlled trial

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Background: There are many drugs for allergic rhinitis (AR), however, these drugs show variable clinical effectiveness and some side effects. Therefore, new methods of AR pharmacotherapy are being sought.

Objectives: The objectives of this study were to evaluate the efficacy of polyvalent mechanical bacterial lysate (PMBL) therapy in improving the clinical course of grass pollen-induced AR (seasonal AR, SAR) in children and its effect on changes in the blood level of the γδT, iNKT and cytotoxic T cell subsets.

Methods: Fifty children with SAR were enrolled in this study and were randomly assigned to either the PMBL group or the placebo group. The severity of SAR symptoms was assessed using the total nasal symptom score (TNSS) and visual analogue scale (VAS). During two visits (V1, V2), peak nasal inspiratory flow (PNIF) was measured and peripheral blood was collected for immunological analyses. The study also included 2 telephone contacts (TC1, TC2).

Results: The severity of the nasal symptoms of SAR on the TNSS scale was revealed to have a significantly lower impact in the PMBL group vs the placebo group at measuring points TC1 and V2 ($p = 0.01$, $p = 0.009$, respectively). A statistically significantly lower mean severity of nasal symptoms of SAR on the VAS scale was recorded for children in the PMBL group compared to the placebo group at measuring points TC1, V2 and TC2 ($p = 0.04$, $p = 0.04$, $p = 0.03$, respectively). The compared groups do not show significant differences in terms of PNIF values at

individual measuring points. There were no statistically significant changes in immune variables. For both groups, there was a statistically significant association between the level of Th1-like γδT cells and the severity of SAR symptoms expressed on the TNSS scale ($p = 0.03$) – the lower the level of Th1-like γδT cells, the higher the TNSS value.

Conclusion: Administration of sublingual PMBL tablets during the grass pollen season proves to have a high efficacy in alleviating SAR symptoms in children sensitized to grass pollen allergens. Th1-like γδT cells may be used as potential markers for SAR severity in children.

Clinical trial registration: ClinicalTrials.gov, identifier (NCT04802616).

KEYWORDS

allergic rhinitis, bacterial lysate, children, γδT cells, iNKT cells, cytotoxic T cells

Introduction

Allergic rhinitis (AR) is one of the most common allergic inflammatory diseases worldwide and it has a significant economic impact through its effects on education, productivity and use of healthcare resources (1, 2). Importantly, it is also a significant risk factor for sinus infections, otitis, and the development or exacerbation of asthma (3).

There are many drugs for AR, including: intranasal corticosteroids, oral and intranasal H1-antihistamines, leukotriene receptor antagonists, intranasal anticholinergic, intranasal and oral decongestants, intranasal saline, allergen immunotherapy and anti-IgE therapy (4, 5). However, these drugs show variable clinical effectiveness, and some side effects or poor efficacy (1, 6, 7). Therefore, new methods of AR pharmacotherapy are sought (8, 9). Recent data indicate possible clinical benefits from the use of probiotics (10), phosphodiesterase 4 inhibitors (11), various methods of immunomodulation (12–15), biologic agents such as mepolizumab or dupilumab (16) and autologous gold-activated patient serum (17). Clinical trials from recent years indicate that bacterial lysates (BLs) may also be one of the options. These preparations are a mixture of bacterial antigens obtained by mechanical (polyvalent mechanical BL, PMBL) or chemical (polyvalent chemical BL, PCBL) lysis of the bacteria most frequently responsible for respiratory infections. It is postulated that the former, due to the lower degree of antigen damage, may have greater immunoregulatory properties (18). BLs have been successfully used in the treatment of recurrent respiratory tract infections for years (19). They have been shown to be highly effective in the treatment of both seasonal (SAR) (20) and perennial (PAR) (21–23) AR. They are widely available, relatively cheap, easy to take by patients, and well tolerated (24, 25).

γδT cells are a small subset of human T cells, comprising approximately 1–10% of T cells. They have a T cell receptor (TCR) built of γ and δ chains instead of α and β. These cells combine characteristics of both innate and acquired immune response and are frequently recognized as the 'bridge' between them (26). γδT cells are able to quickly produce and release significant amounts of pro- and anti-inflammatory cytokines and chemokines after being stimulated by several factors, including autoantigens, lipopeptides, and microbial antigens (27). As such, they play an essential role in human anti-infectious and anti-tumor defense. However, they also may contribute to autoimmunity and allergy (28, 29). It is postulated that γδT lymphocytes are involved in the development and modulation of the course of allergic diseases, however, this ambiguous role has not been yet confirmed (30, 31).

Another lymphocyte population capable of a sudden release of large amounts of cytokines is invariant natural killer T (iNKT) cells, a rare subpopulation (up to 0.5% of T cells) that combines features of typical T and NK cells (32). Activated iNKT cells secrete large amounts of IFN-γ and IL-4 as well as IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF-α, TGF-β, and GM-CSF (33). Rapid synthesis of cytokines makes these cells important regulators of inflammatory processes, including allergic ones. It is postulated that these cells play a double role in the modulation of the course of allergy and its pathogenesis (34).

The purpose of this study was to evaluate the effect of PMBL therapy on the clinical course of grass pollen-induced AR in children, and primarily to characterize the immunomodulatory effects of this treatment.

Methods

Study design

It was a 1:1 randomized, double-blind, placebo-controlled study in parallel groups (PMBL vs placebo). The study received a favorable opinion from the Bioethics Committee of the Medical University of Lublin (Resolution No KE-0254/251/2020, 26 November 2020) and was prospectively registered with ClinicalTrials.gov (Trial Registration No NCT04802616, 17 March 2021). The study was conducted in accordance with Good Clinical Practice standards, and the ethical principles that have their origin in the Declaration

of Helsinki. The project was implemented in 3 centers in eastern Poland from 22 March 2021 to 29 October 2021.

The primary objectives of this study were to evaluate the effectiveness of 3-month PMBL therapy in improving the clinical course of grass pollen-induced AR in children, and above all its effect on changes in the blood level of the γδT cell subsets: Th1-like, Th2-like, Th10-like, Th17-like, Treg-like; iNKT cell subsets: iNKT1, iNKT2, iNKT10, iNKT17, iNKTreg; cytotoxic T (Tc) cell subsets: Tc1, Tc2, Tc10, Tc17, Treg-like and to assess the relationship between the level of these lymphocytes and the severity of SAR symptoms assessed using the total nasal symptom score (TNSS).

The secondary study objectives were to assess the effect of PMBL therapy on the need for oral H1-antihistamines and intranasal corticosteroids during the grass pollen season and to evaluate the safety and tolerability of this therapy.

Patients

Eligible for the study were children aged 5 to 17 years with SAR diagnosed, as per current ARIA recommendations (4), with predominant sensitization to grass pollen allergens determined by skin prick testing (wheal diameter ≥ 3 mm and greater than negative control; Allergopharma-Nexter Ltd, Poland) or based on serum allergen-specific immunoglobulin E (asIgE) levels (≥ class 2; Polycheck, Biocheck GmbH, Germany), with SAR symptoms present in at least two previous grass pollen seasons. Exclusion criteria were as follows: allergy to tree pollens, receipt of PMBL within 12 months or PCBL within 6 months before the randomization visit, oral or subcutaneous allergen-specific immunotherapy within 3 years before the start of the study, treatment with systemic corticosteroids within the last 6 months before the start of the study, history of respiratory infection within 2 weeks before the randomization visit, systemic immunologic disorders, history of transfusion of blood, blood components or blood products.

Children were recruited for the study in the second half of March 2021, before the start of the grass pollen season in Poland. For all patients, written informed consent was obtained from a parent or legal guardian and assent was obtained from the patient.

Interventions

The study used PMBL obtained by sonification, whereby ultrasound is used to inactivate the bacterial cell wall (Ismigen, Lallemand Pharma AG, Switzerland). The product is available in the form of sublingual tablets containing 7 mg of bacterial lysate of the following bacteria: Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus viridans, Staphylococcus aureus, Neisseria catarrhalis, Haemophilus influenzae, Klebsiella pneumoniae and Klebsiella ozaenae. PMBL was taken by patients according to its dosage regimen in recurrent respiratory infections. Thus, children in the PMBL group received 3-cycles of PMBL treatment, each consisting of sublingual administration of one PMBL tablet on an empty stomach for 10 consecutive days followed by a 20-day break (25). In the control group, the patients received placebo tablets indistinguishable from the PMBL tablets in shape, color, smell/taste and dissolution time under the tongue.

Randomization and masking

The randomization list was generated by hospital pharmacy staff using Random Allocation Software version 2.0. Pharmacists also prepared identical packs containing 30 PMBL or placebo tablets each and labelled them with a unique code consistent with the randomization list. The individuals responsible for preparing the randomization list and tablet packs were not involved in the implementation of the study. Children attending the first visit and meeting all inclusion criteria and none of the exclusion criteria drew a card with a unique package number. Patients and investigators, including those responsible for immunoassays, were blind to allocation. Unblinding was performed after receiving patient diaries from all participants, that was in early November 2021.

Measurement of grass pollen concentration in ambient air

The dates of visits to the centers and the periods of assessment of SAR symptoms subjected to statistical analysis were determined based on measurements of grass pollen grain concentrations in ambient air.

A Hirst-type apparatus (Lanzoni VPPS 2000) (35) placed on the flat roof of the building of the University of Life Sciences in Lublin (22032'25" E and 51014'37" N; 197 m a.s.l.) at 18 m above ground level was used to monitor the concentration of grass pollen grains in the air. The apparatus operated in continuous mode, sucking in air along with bioaerosol elements. The pollen grains stuck to a sticky-coated tape placed inside the apparatus on a moving drum. After a week's exposure, the tape was cut into sections corresponding to 24 hours and microscope slides were prepared. The results were expressed as the number of pollen grains in 1 m^3 of air per day. Pollen monitoring was conducted in accordance with the recommendations of the International Association for Aerobiology and Quality Control Working Group (36). The beginning and end of the grass pollen season in eastern Poland were determined using the 95% method (Figure 1) (37).

Study protocols

The study included two site visits: visit 1 (V1) – a screening/randomization visit, before the start of the grass pollen season (22–31 March 2021), visit 2 (V2) – at the peak of the grass pollen season (23 June – 2 July 2021), and two telephone contacts (TC1, TC2; the study protocol registered at ClinicalTrials.gov provided for 4 telephone contacts, but two contacts were dropped due to the late start of the grass pollen season) (Figure 2). For 7 days after each visit, parents rated the severity of four SAR symptoms (nasal congestion, rhinorrhea, itching of the nose and sneezing) in the patient's diary, each scoring from 0 to 3 (0 – no symptom, 3 – severe symptom), and then summed the scores to obtain the TNSS (weekly averages were

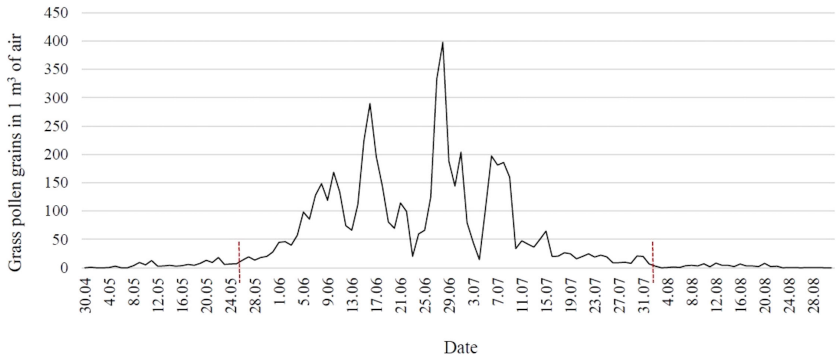

FIGURE 1
Concentration of grass pollen grains in the air in eastern Poland in 2021 (red lines indicate the time frame of the pollen season).

analyzed statistically) (38). Patients started the first cycle of sublingual tablets on 1 April 2021. In addition, the use of an oral antihistamine (desloratadine) or an intranasal corticosteroid (mometasone furoate) was allowed in case of severe allergy symptoms, and the intake of these drugs was recorded daily in the diary (the number of days of the grass pollen season in which patients took the above drugs was analyzed statistically).

During the visits to the center (V1, V2), blood was taken for immunological tests, the severity of four SAR symptoms was assessed cumulatively on a visual analogue scale (VAS), and peak nasal inspiratory flow (PNIF) was measured using the Youlten Peak Flow Meter (Clement Clarke International, UK) (all subjects received appropriate instructions on how to use the Peak Flow Meter correctly; three measurements were recorded for each subject and the highest flow was used for analysis) (39). During telephone contacts, patients were asked to rate the severity of their SAR symptoms cumulatively on a VAS scale using an appropriately prepared tool in a patient diary (instructions, 10 cm line).

Immunological analyses

The peripheral blood taken from patients was collected into 4.9 ml tubes: K2-EDTA S-Monovette (Sarstedt, Germany). Next, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation in Gradisol L (Aqua-Med, Poland) at 700 rcf for 20 minutes at room temperature. PBMC were counted in the Neubauer chamber. PBMC at a density of 1×10^6 per ml were distributed into cell culture plates and stimulated at 37°C in a CO_2 incubator for 4 hours in the presence of monensin (2 μM/ml), ionomycin (1 μg/ml) and phorbol 12-myristate 13-acetate (50 ng/ml). After stimulation, the cells were incubated with the combination of monoclonal antibodies. The following monoclonal antibodies were used in the current study: anti-TCR V alpha 24 J alpha 18 FITC (cat. no 342906, clone: 6B11, BioLegend), anti-TCR V alpha 24 J alpha 18 PE-Cy7 (cat. no 342912, clone: 6B11, BioLegend), anti-CD3 PE (cat. no 555333, BD Biosciences), anti-CD3 Krome Orange (cat. no B00068, clone: UCHT1, Beckman Coulter), anti-CD4V450 (cat. no

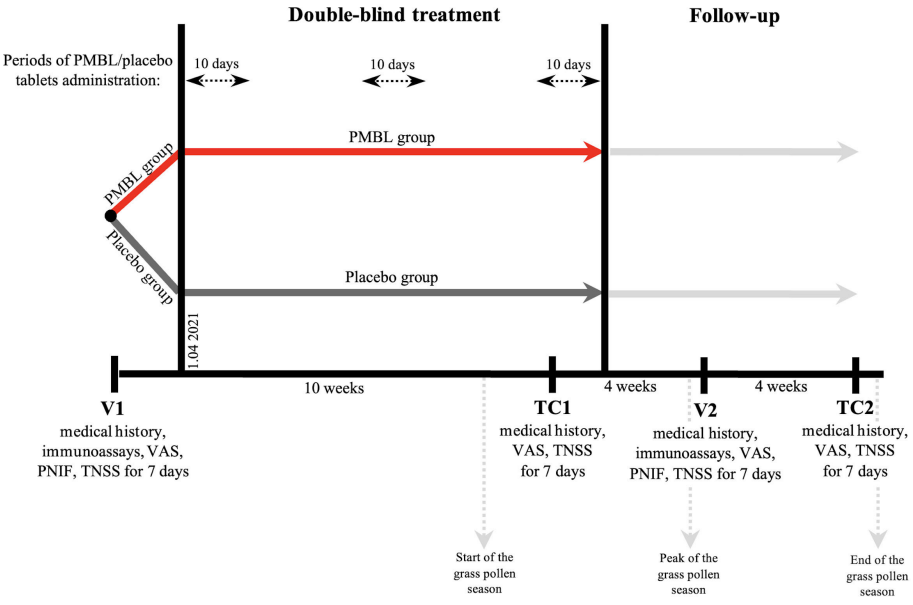

FIGURE 2
Study design. PMBL, polyvalent mechanical bacterial lysate; VAS, visual analogue scale; PNIF, peak nasal inspiratory flow; TNSS, total nasal symptom score.

560345, clone: RPA-T4, BD Biosciences), anti-CD8 Alexa Fluor 700 (cat. no B76279, clone: B9.11, Beckman Coulter), anti-CD8 PE-Cy5 (cat. no 300909, clone: HIT8a, BioLegend), anti-TCR PAN delta/gamma PE-Cy5 (cat. no IM2662U, clone: IMMU510, Beckaman Coulter), anti-TCR PAN delta/gamma FITC (cat. no 347903, clone: 11F2, BD Biosciences), anti-CD45BUV395 (cat. no 563792, clone: HI30, BD Biosciences) and ViaKrome 808 (cat. no C36628, Beckman Coulter). After 20 minutes of incubation, cells were permeabilized with methanol at 4–8°C for 30 minutes in the darkness.

Next, the cells were washed twice with phosphate buffered saline (PBS) for 10 minutes, 700 rcf. Afterwards, intracellular labelling was performed using the following antibodies: anti-FoxP3 Pacific Blue (cat. no B90432, clone: 259D, Beckman Coulter), anti-IFN-gamma PE-CF594 (cat. no 562392, clone: B27, BD Biosciences), anti-IL-10 Brilliant Violet 786 (cat. no 564049, clone: JES3-9D7, BD Biosciences), anti-IL-17A APC-Cy7 (cat. no. 512320, clone: BL168, BioLegend), anti-IL-4 Brilliant Violet 605 (cat. no. 500828, clone: MP4-25D2, BioLegend), anti-E4BP4 PE (cat. no. 12-9812-42, clone: MABA223, Invitrogen), anti-T-bet APC (cat. no. 644814, clone: 4B10, BioLegend), anti-GATA-3 PerCP-eFluor 710 (cat. no. 44-9966-42, clone: TWAJ, Invitrogen) and anti-ROR gamma T Brilliant Violet 650 (cat. no. 563424, clone: RPA-T4, BD Biosciences). Incubation was performed at room temperature for 30 minutes in the darkness. After final wash with PBS, samples were analyzed on CytoFlex LX flow cytometer (Beckman Coulter, USA). Kaluza v2.1.1 (Beckman Coulter, USA) was used to analyze and graphically present the collected data (Figure 3).

Sample size

Sample size was determined using sample size test in Statistica 13.3 software (StatSoft, Poland) based on previous studies on the effect of immunostimulant products on the number of CD3+ lymphocytes in children with allergic asthma, recurrent respiratory tract infections and adults with chronic obstructive pulmonary disease (40–42). It was calculated that 21 patients should be included in the PMBL and placebo groups, respectively (power 85%, alpha value 0.05). The total sample size calculation required to enroll 50 patients in anticipation of a dropout rate of 15%.

Statistical analysis

Statistical analysis was conducted by an International Statistical Institute Elected Member not involved in the study using IBM SPSS Statistics 25 package. Using a two-way mixed model analysis of variance, checks were made to assess whether there were statistically significant differences between the study periods analyzed in the two groups, as well as whether the groups differed

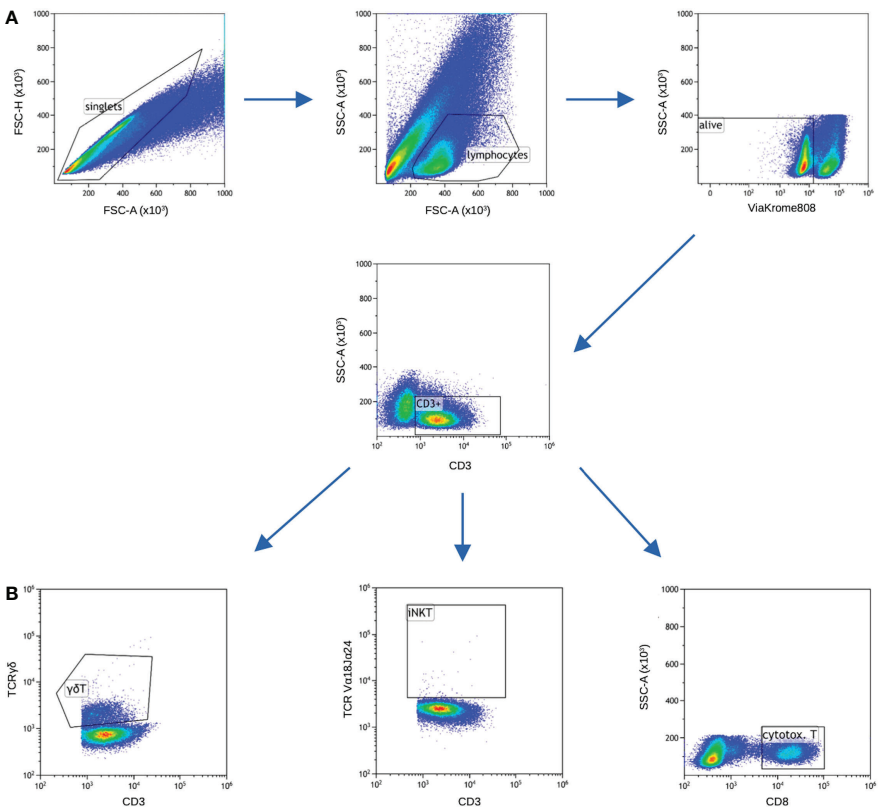

FIGURE 3
Gating strategy. Samples for cytokines and transcription factors were stained in separate tubes, nevertheless majority of gating is common for both (panels A and B), only gating of cytotoxic T cells differed. **(A)** FSC-A vs FSC-H was used to gate only single cells, next lymphocytic gate was set on FSC-A vs SSC-A. ViaKrome 808 was used to gate out dead cells. Next, CD3+ cells were gated. **(B)** Two subsets of unconventional T cells (iNKT and γδT) were gated based on specific TCR chains, cytotoxic T cells were gated as CD8+ cells among total T cells.

in terms of the variable under analysis in the same study period. Spearman's rank correlation made it possible to check whether there was a statistically significant relationship between the analyzed variables. The Mann-Whitney U test was used to assess the presence of statistically significant differences between the two independent groups (in terms of age). A two-tailed P value lower than 0.05 was considered as statistically significant. Analyses were based on the intent-to-treat (ITT) population (patients who took ≥ 1 tablet and had ≥ 1 post-randomization assessment).

Results

Participant flow

62 children were enrolled in the study. After excluding 8 patients who did not meet the inclusion criteria or met at least one exclusion criterion and 4 patients who did not agree to participate in the study, 25 children were randomized to the PMBL group and 25 to the placebo group. A total of 41 children completed the trial, 21 in the PMBL group and 20 in the placebo group (Figure 4). Of the 41 children, 29 (71%) were male and 12 (29%) were female. Mean age of participants was 9.1 ± 2.55 years. Demographic characteristics were similar between the groups as shown in Table 1.

Primary outcome

In both groups, during the grass pollen season, a statistically significant increase and then a decrease in the severity of SAR symptoms expressed on the TNSS and VAS was observed (Table 2). In the placebo group there was a statistically significant decrease in PNIF ($p = 0.007$), while in the PMBL group there was a statistically insignificant ($p = 0.07$) but clinically significant increase of 11.19 L/min (minimally clinically important difference [MCID] is 5 L/min) (Table 3).

Children taking PMBL during the grass pollen season displayed much less intensity of nasal symptoms of SAR compared to children receiving placebo. The severity of the nasal symptoms of SAR on the TNSS scale was revealed to have a significantly lower impact in the PMBL group vs the placebo group at measuring points TC1 and V2 ($p = 0.01$, $p = 0.009$, respectively) (Figure 5A). A statistically significantly lower mean severity of nasal symptoms of SAR on the VAS scale was recorded for children in the PMBL group compared to the placebo group at measuring points TC1, V2 and TC2 ($p = 0.04$, $p = 0.04$, $p = 0.03$, respectively) (Figure 5B). The compared groups do not show significant differences in terms of PNIF values at individual measuring points, although a p-value on the verge of statistical significance ($p = 0.053$) was obtained at measuring point V2, indicating less nasal obstruction in the PMBL group versus the placebo group (Figure 5C).

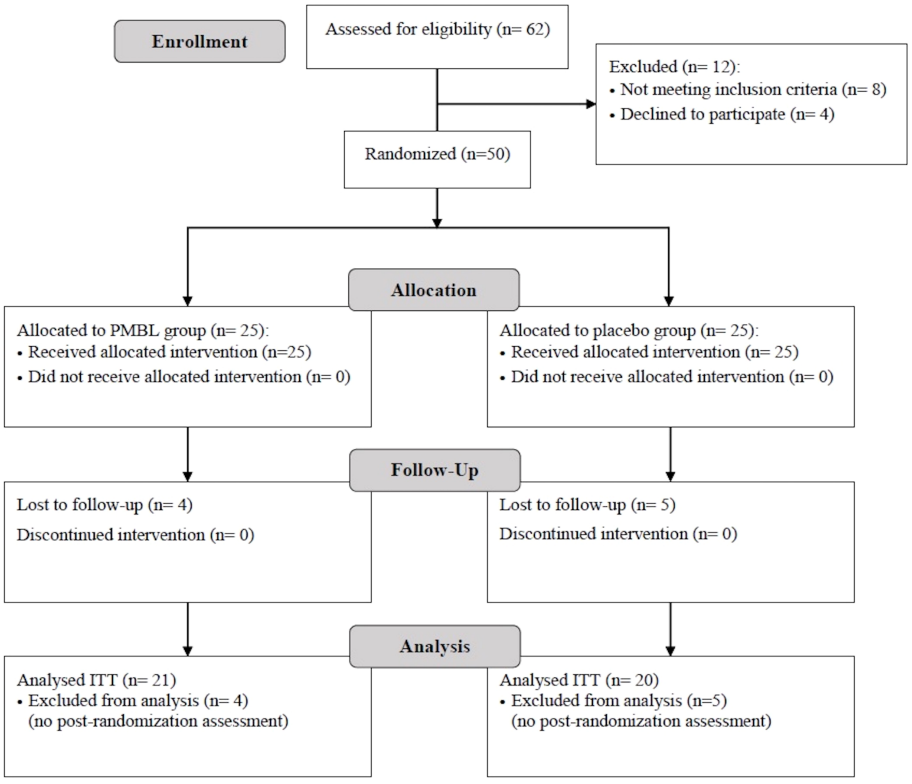

FIGURE 4
Patient flowchart (presented in accordance with the Consolidated Standards of Reporting Trials guidelines). PMBL, polyvalent mechanical bacterial lysate; ITT, intent-to-treat.

TABLE 1 Baseline overall characteristics of patients.

	PMBL group (n = 21)	Placebo group (n = 20)	p-value
Sex, n (%)			
Male	14 (66.7)	15 (75)	0.73
Female	7 (33.3)	5 (25)	
Age, mean (SD) [years]	9.43 (2.68)	8.7 (2.49)	0.32
Place of residence, n (%)			
Village	7 (33.3)	6 (30)	1.00
City	14 (66.7)	14 (70)	
Sensitizing allergen, n (%)			
Grasses	21 (100)	20 (100)	–
Weeds	2 (9.5)	2 (10)	1.00
House dust mite	8 (38.1)	10 (50)	0.54
Pet dander	6 (28.6)	4 (20)	0.72
Moulds	3 (14.3)	1 (5)	0.61

PMBL, polyvalent mechanical bacterial lysate.

TABLE 2 The severity of nasal symptoms assessed using the total nasal symptom score and visual analogue scale.

	PMBL group (n = 21)				
	V1	TC1	V2	TC2	p-value
TNSS[a], mean (SD)	0.69 (0.86)	2.22 (1.84)	1.92 (1.37)	1.12 (1.11)	V1 vs TC1 0.01 V1 vs V2 0.03 TC1 vs TC2 0.02
VAS, mean (SD)	9.52 (6.46)	23.24 (14.77)	22.81 (13.48)	14.27 (9.71)	V1 vs V2 0.02 V2 vs TC2 0.03
	Placebo group (n=20)				
	V1	**TC1**	**V2**	**TC2**	**p-value**
TNSS[a], mean (SD)	0.82 (0.77)	3.82 (2.15)	3.36 (1.94)	1.96 (2.19)	V1 vs TC1 < 0.001 V1 vs V2 < 0.001 TC1 vs TC2 < 0.001 V2 vs TC2 < 0.001
VAS, mean (SD)	7.65 (6.18)	38.25 (28.7)	34.45 (21.37)	25.35 (19.61)	V1 vs others < 0.001

PMBL, polyvalent mechanical bacterial lysate; TNSS, total nasal symptom score; VAS, visual analogue scale; V1, V2, visit 1 and 2; TC1, TC2, telephone contact 1 and 2; [a] TNSS symptom severity was assessed daily for 7 days after each visit, statistical analysis was performed on weekly mean values.

TABLE 3 Peak nasal inspiratory flow.

	PMBL group (n=21)		
	V1	V2	p-value
PNIF (l/min), mean (SD)	105.71 (40.6)	116.9 (61.57)	0.07
	Placebo group (n=20)		
	V1	**V2**	**p-value**
PNIF (l/min), mean (SD)	104.5 (31.11)	86.75 (28.57)	0.007

PMBL, polyvalent mechanical bacterial lysate; PNIF, peak nasal inspiratory flow; V1, V2, visit 1 and 2.

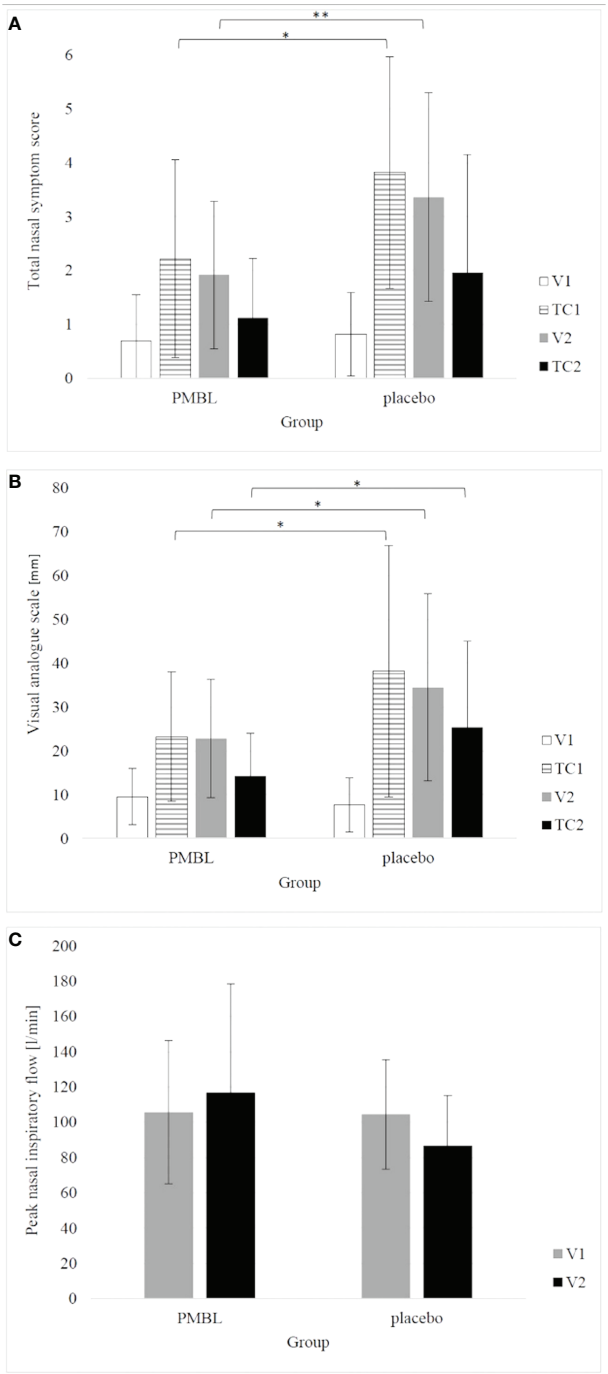

FIGURE 5
(A) total nasal symptom score; **(B)** visual analogue scale; **(C)** peak nasal inspiratory flow. The error bars represent standard deviation. (*p < 0.05; **p < 0.01). PMBL, polyvalent mechanical bacterial lysate; V1, V2, visit 1 and 2; TC1, TC2, telephone contact 1 and 2.

There were no statistically significant changes in immune variables between V1 and V2 in both groups, and between the groups at both measurement points (Table 4). For both groups, there was a statistically significant association between the level of Th1-like γδT cells and the severity of SAR symptoms expressed on the TNSS scale (p = 0.03) – the lower the level of Th1-like γδT cells, the higher the TNSS value (Figure 6).

Secondary outcome

The mean number of days of use of desloratadine and mometasone furoate per patient was respectively 26% (18.62 ± 4.6 vs 25.2 ± 7.4 days, p = 0.005) and 31% (28.57 ± 7.41 vs 41.6 ± 11.17 days, p < 0.001) lower in the PMBL group vs the placebo group. The therapy was associated with a high safety and tolerability profile. In the PMBL group only one adverse event in forms of mild abdominal pain was reported, which resolved spontaneously without any medication. No adverse event was reported in the placebo group.

Discussion

As mentioned earlier, the present study was designed to evaluate the efficacy of PMBL therapy in children with SAR, and primarily to further understand the immune mechanisms of bacterial immunostimulants responsible for reducing the severity of allergic disease symptoms. To the best of our knowledge, this is the first study to evaluate the effect of PMBL therapy on changes of γδT, iNKT and Tc cell subsets in the serum levels in children with AR.

The study presented here is a continuation of a series of studies on the efficacy of BLs in the treatment of grass pollen-induced AR in children, but it focuses primarily on the immunological mechanisms of these preparations. The results of the first project were published in 2019. It was a pilot, randomized, open-label study that included 38 children with SAR. The addition of sublingual BL to standard AR treatment (oral antihistamine, intranasal corticosteroid) has been shown to reduce the severity of nasal symptoms as assessed by a standard scoring scale and to improve nasal patency expressed as an increase in PNIF values. Nevertheless, the drug has not been confirmed to alleviate symptoms of allergic conjunctivitis (43). As a clinically significant reduction in nasal symptom severity on the TNSS scale was found (a reduction of 1.54, with a MCID of 0.55), decision was made to conduct a randomized, double-blind, placebo-controlled trial in the next grass pollen season. The project involved 70 children who were randomly assigned to a group receiving PMBL or placebo. During the grass pollen season, an improvement in the clinical course of SAR was observed, expressed as a statistically significant decrease in TNSS (p = 0.001) and VAS values (p < 0.001) and a statistically significant increase in PNIF values (p = 0.04) in the PMBL treatment group. Patients taking the immunostimulant had significantly lower nasal symptom severity than children in the placebo group. The compared groups did not differ in the severity of ocular allergy symptoms. In addition, the use of PMBL was associated with a reduction in the need for desloratadine and mometasone furoate. In order to assess the effect of PMBL therapy on Th1/Th2 balance, nasal swabs were taken from the patients for eosinophils and nasal lavage fluids for asIgE against timothy grass. Relation between the number of eosinophils in nasal swabs and the concentration of grass pollen grains in ambient air was recorded for both groups, although the number of these cells was significantly lower in the PMBL group (p = 0.01). Furthermore, no changes in asIgE concentrations during the grass pollen season was recorded in the PMBL group, while in the control group, these concentrations increased significantly (p = 0.03). The above observations indirectly indicate the influence of PMBL on the suppression of the dominant Th2 lymphocyte response in SAR

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TABLE 4 Variation in γδT, iNKT and cytotoxic T lymphocytes subsets and cytokines throughout the study period.

Lymphocytes	Subsets/cytokines	PMBL group (n = 21)		Placebo group (n = 20)		p-value[a]
		V1	V2	V1	V2	
γδT cells mean (SD) [MFI]	T-bet (Th1-like)	5726.85 (3484.87)	4476.69 (2493.26)	4933.24 (4687.07)	3603.62 (1787.91)	0.63
	GATA3 (Th2-like)	4274.66 (1995.52)	4125.33 (1876.21)	3866.94 (3873.73)	3986.63 (1271.24)	0.77
	E4BP4 (Th10-like)	3392.55 (3905.4)	929.97 (220.23)	3036.41 (3256.12)	911.39 (209.6)	0.84
	RORγT (Th17-like)	340.16 (483.1)	85.75 (53.15)	285.75 (501.88)	100.15 (40.33)	0.88
	FoxP3 (Treg-like)	1786.23 (766.63)	1826.17 (816.32)	2005.91 (801.78)	5666.8 (4510.67)	0.35
	IL-4	2371.86 (835.47)	2573.19 (890.13)	2384.62 (917.63)	44336.75 (178971.66)	0.32
	IL-10	793.17 (664.88)	814.03 (706.85)	962.53 (742.4)	5211.2 (19631.84)	0.34
	IL-17A	12968.59 (11180.63)	16201.53 (13949.6)	15163.8 (13732.43)	11270.24 (10250.44)	0.28
	IFN-γ	758.8 (958.35)	623.73 (461.38)	968.38 (1122.13)	695.03 (984.39)	0.95
iNKT cells mean (SD) [MFI]	T-bet (iNKT1)	27441.93 (19084.15)	2737.11 (2197.81)	28015.4 (34328.33)	2610.76 (1310.58)	0.98
	GATA3 (iNKT2)	6530.14 (8944.69)	2736.17 (1391.58)	6065.95 (7690.32)	6012.15 (8977.67)	0.17
	E4BP4 (iNKT10)	3927.27 (4554.89)	1028.29 (468.21)	3776.73 (4576.69)	1124.01 (1194.18)	0.88
	RORγT (iNKT17)	3591.45 (8045.12)	119.1 (98.62)	3814.15 (8198.44)	533.74 (1512.65)	0.95
	FoxP3 (iNKTreg)	4264.5 (3056.07)	3925.12 (3436.62)	3818.86 (2835.85)	2979.88 (1335.06)	0.71
	IL-4	4994.25 (2183.97)	4663.55 (1983.26)	4720.92 (2467.46)	5407.15 (4709.77)	0.43
	IL-10	1144.93 (388.95)	1653.82 (2633.29)	2123.1 (2269.84)	1286.43 (996.84)	0.11
	IL-17A	19069.63 (16766.35)	21533.56 (20930.87)	24564.27 (20688.77)	15287.22 (11747.6)	0.16
	IFN-γ	348.34 (405.38)	732.35 (756.52)	704.08 (784.79)	568.14 (634.45)	0.08
Cytotoxic T cells mean (SD) [MFI]	T-bet (Tc1)	4325.1 (1261.6)	4658.24 (1594.22)	3977.56 (1026.32)	4217.21 (1170.27)	0.43
	GATA3 (Tc2)	3798.56 (2132.26)	2012.49 (2183.65)	3515.95 (3526.99)	5743.84 (1321.61)	0.89
	E4BP4 (Tc10)	3899.59 (4869.09)	666.39 (508.88)	3648.8 (4409.43)	610.52 (438.2)	0.69
	RORγT (Tc17)	288.66 (236.78)	37.34 (31.81)	221.4 (359.53)	40.05 (33.07)	0.9
	FoxP3 (Treg-like)	1726.32 (733.62)	1522.49 (463.29)	1848.78 (704.86)	1630.25 (609.61)	0.88
	IL-4	2298.71 (835.18)	2223.63 (679.1)	2162.73 (873.79)	2068.96 (609.24)	0.9
	IL-10	931.94 (752.56)	703.49 (557.84)	975.45 (817.07)	789.32 (773.14)	0.53
	IL-17A	12602.21 (10871.44)	12626.69 (9680.75)	13749 (11957.41)	13456.06 (10917.35)	0.87
	IFN-γ	832.33 (743.81)	747.11 (619.32)	947.52 (1093.35)	530.92 (392.53)	0.63

PMBL, polyvalent mechanical bacterial lysate; V1, V2, visit 1 and 2; MFI, mean fluorescent intensity.
[a]The analysis of variance showed no statistically significant interaction of the group with the level of measurement for individual variables, meaning that the two groups do not differ in V1 and V2 for individual variables, and that there are no differences between V1 and V2 in individual groups.
Data is presented as mean fluorescent intensity (MFI).

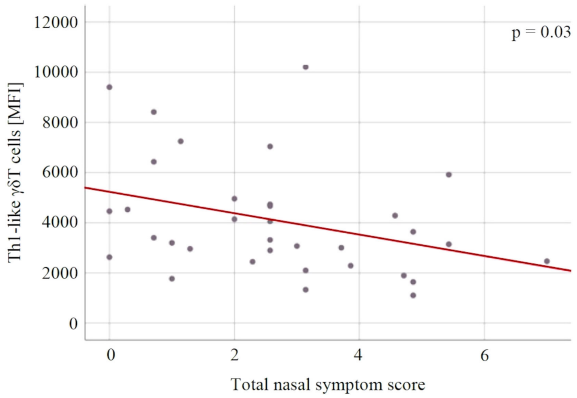

FIGURE 6
The relationship between the Th1-like γδT cells and the severity of allergic rhinitis symptoms expressed in the total nasal symptom score. MFI, mean fluorescent intensity.

patients (20). Similar conclusions can be drawn based on the results of the present study, which showed statistically significant lower severity of SAR symptoms expressed on the TNSS and VAS scales, as well as lower allergy medication use in the PMBL treatment group vs the placebo group. A clinically significant increase in PNIF values during the grass pollen season was observed in the PMBL group, while a significant decrease was observed in the placebo group, and the differences found between groups on this variable were close to the level of statistical significance. The beneficial effect of BL therapy on the clinical course of AR in adult patients was also confirmed by Banche et al. (21) and Meng et al. (23). Italian researchers have shown that PMBL therapy reduces the severity of AR symptoms (in 62% of patients) and improves asthma control (in 40% of patients), with the achieved effects lasting at least 3 months. In contrast, in the placebo group, more than half of the patients experienced a worsening of AR symptoms. The authors signal that the achieved effects of PMBL therapy may be related to a significant reduction in IL-4 levels in the blood (21). On the other hand, Meng et al. evaluated the efficacy of PCBL in adult patients with PAR, demonstrating, like us, the effect of immunostimulation with the bacterial product on reducing nasal symptoms and reducing the need for oral antihistamines by almost 40%. Moreover, the researchers demonstrated the effect of these preparations on reducing Th2-type cytokines (IL-4 and IL-13) and increasing Th1-type cytokine (IFN- γ) (23). The role of BLs in restoring and maintaining Th1/Th2 balance, as well as other mechanisms of action of BLs in allergic diseases known so far, have been thoroughly discussed by us in other articles (44, 45).

Recent data indicate that Tc cells play a role in the chronic inflammatory diseases, including allergic ones (46). Exposure to specific antigens can trigger these cells to release inflammatory molecules. In AR, researchers have noted that Tc cells release IL-4, which is involved in the pathogenesis of the disease, and contributes to the production of asIgE by B lymphocytes (47). γδT and iNKT cells are also able to produce and release large amounts of pro- and anti-inflammatory cytokines and chemokines after being stimulated by several factors, such as microbial antigens (27, 32). Rapid synthesis of cytokines makes these cells important regulators of inflammatory processes, including allergic processes. It is postulated that γδT and iNKT cells are involved in the development and modulation of the course of allergic diseases (30, 31, 34).

It has been shown that some molecules can modulate the activation of the above-mentioned cells. Johansson et al. investigated if lactobacilli-derived factors could beneficially change immune responsiveness of γδT and NK cells *in vitro*. They showed that molecules present in the lactobacilli cell-free supernatants act directly on these cells, reducing their activation, which provided a novel insight on the immunomodulatory nature of probiotic lactobacilli (48). Wang et al. showed that probiotic treatment (mixture of lactobacillus, bifidobacterium, and Streptococcus thermophilus) can restore adipose iNKT cell frequency and enhance the function of the iNKT cell anti-inflammatory phenotype in high-fat diet-induced obese mice (49). Furthermore, Olszak et al. concluded that early-life colonization of germ-free mice with a conventional microbiota protected them from mucosal iNKT accumulation and related pathologies such as asthma and inflammatory bowel disease (50). Mentioned studies suggest that some bacteria molecules can influence the activation of γδT and iNKT cells. It can be presumed that BLs, as preparations consisting of inactivated antigens derived from respiratory pathogens, are also able to modulate the immune response by restoring these cells balance. However, our study did not confirm this hypothesis. To the best of our knowledge, our study is the first to evaluate the effect of BL therapy on changes of γδT, iNKT and Tc cell subsets in the serum levels in children with AR. Our lack of confirmation of the results of previous studies may be due to the fact that they involved probiotics, were *in vitro* or animal studies, and were not conducted on an allergic disease model. It is worth mentioning that we focused mostly on unconventional T cells that undergo significant changes during childhood and early adolescence. This is especially true for γδT cells. At birth the Vδ1 subset prevails, it comprises usually more than 95% of total circulating γδT cells. In adults Vδ2 dominates, while Vδ1 rarely exceeds 25% of total γδT in peripheral blood. Both subsets recognize different stimuli and may respond in slightly different ways (28). Thus, data obtained in children and adults is usually not fully comparable.

γδT can be divided similarly to the conventional T helper cells into populations based on their cytokine profile and transcription factors. At least the major subsets (Th1-, Th2-, Th10-, Th17- and Treg-like) can be distinguished (51). γδT percentage in peripheral blood tends to be significantly lowered in asthmatic patients, which suggests a possible role in asthma and allergy pathogenesis (28). Moreover, γδT may be also involved in regulation of IgE production, this observation has been so far noted only in murine model of asthma, not in patients' samples (52). In the current study, we have observed a decrease in Th1-like (T-bet+) γδT cells with the increase in allergic symptoms expressed on the TNSS scale. This is generally in line with the general theory of immunopathogenesis, namely the dysregulation of Th1/Th2 balance.

Our study has some limitations. First, a longer follow up, more numerous population and more detailed studies are needed to elucidate the mechanism of action of PMBL in patients with AR. Second, since we have only studied systemic effects, it is possible that different or stronger immune effects can be found locally in the respiratory tract.

Conclusion

Administration of sublingual PMBL tablets during the grass pollen season proves to have a high efficacy in alleviating SAR symptoms in children sensitized to grass pollen allergens. PMBL treatment has not been shown to alter the levels of individual subsets of γδT, iNKT and Tc cells. Th1-like γδT cells may be used as potential markers for SAR severity in children.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Bioethics Committee of the Medical University of Lublin. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Conceptualization: KJ and MZ; methodology: KJ, WK, MZ, AE, and JR; investigation: KJ, WK, MZ, DS, MM, EM-M, IM-M, AB, AT, and KP-W; data interpretation: KJ, WK, and MZ; supervision: KJ, AE, and JR; writing original draft: KJ, WK, MZ, DS, IM-M, AB, AT, AK, AE, and KP-W. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Wise SK, Lin SY, Toskala E. International consensus statement on allergy and rhinology: Allergic rhinitis - executive summary. *Int Forum Allergy Rhinol* (2018) 8(2):85–107. doi: 10.1002/alr.22070

2. Strozek J, Samolinski BK, Klak A, Gawinska-Druzba E, Izdebski R, Krzych-Falta E, et al. The indirect costs of allergic diseases. *Int J Occup Med Environ Health* (2019) 32 (3):281–90. doi: 10.13075/ijomeh.1896.01275

3. Sih T, Mion O. Allergic rhinitis in the child and associated comorbidities. *Pediatr Allergy Immunol* (2010) 21(1 Pt 2):e107–13. doi: 10.1111/j.1399-3038.2009.00933.x

4. Bousquet J, Schunemann HJ, Togias A, Bachert C, Erhola M, Hellings PW, et al. Next-generation allergic rhinitis and its impact on asthma (ARIA) guidelines for allergic rhinitis based on grading of recommendations assessment, development and evaluation (GRADE) and real-world evidence. *J Allergy Clin Immunol* (2020) 145(1):70–80 e3. doi: 10.1016/j.jaci.2019.06.049

5. Bayar Muluk N, Bafaqeeh SA, Cingi C. Anti-IgE treatment in allergic rhinitis. *Int J Pediatr Otorhinolaryngol* (2019) 127:109674. doi: 10.1016/j.ijporl.2019.109674

6. Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. *Lancet* (2011) 378(9809):2112–22. doi: 10.1016/S0140-6736(11)60130-X

7. Meltzer EO, Wallace D, Friedman HS, Navaratnam P, Scott EP, Nolte H. Meta-analyses of the efficacy of pharmacotherapies and sublingual allergy immunotherapy tablets for allergic rhinitis in adults and children. *Rhinology* (2021) 59(5):422–32. doi: 10.4193/Rhin21.054

8. Heffler E, Brussino L, Del Giacco S, Paoletti G, Minciullo PL, Varricchi G, et al. New drugs in early-stage clinical trials for allergic rhinitis. *Expert Opin Investig Drugs* (2019) 28 (3):267–73. doi: 10.1080/13543784.2019.1571581

9. Linton S, Burrows AG, Hossenbaccus L, Ellis AK. Future of allergic rhinitis management. *Ann Allergy Asthma Immunol* (2021) 127(2):183–90. doi: 10.1016/j.anai.2021.04.029

10. Steiner NC, Lorentz A. Probiotic potential of lactobacillus species in allergic rhinitis. *Int Arch Allergy Immunol* (2021) 182(9):807–18. doi: 10.1159/000515352

11. Janosova V, Calkovsky V, Pedan H, Behanova E, Hajtman A, Calkovska A. Phosphodiesterase 4 inhibitors in allergic Rhinitis/Rhinosinusitis. *Front Pharmacol* (2020) 11:1135. doi: 10.3389/fphar.2020.01135

12. Ramchandani R, Hossenbaccus L, Ellis AK. Immunoregulatory T cell epitope peptides for the treatment of allergic disease. *Immunotherapy* (2021) 13(15):1283–91. doi: 10.2217/imt-2021-0133

13. Gevaert P, De Craemer J, De Ruyck N, Rottey S, de Hoon J, Hellings PW, et al. Novel antibody cocktail targeting bet V 1 rapidly and sustainably treats birch allergy symptoms in a phase 1 study. *J Allergy Clin Immunol* (2022) 149(1):189–99. doi: 10.1016/j.jaci.2021.05.039

14. Shamji MH, Singh I, Layhadi JA, Ito C, Karamani A, Kouser L, et al. Passive prophylactic administration with a single dose of anti-fel d 1 monoclonal antibodies REGN1908-1909 in cat allergen-induced allergic rhinitis: A randomized, double-blind, placebo-controlled clinical trial. *Am J Respir Crit Care Med* (2021) 204(1):23–33. doi: 10.1164/rccm.202011-4107OC

15. Skaarup SH, Schmid JM, Skjold T, Graumann O, Hoffmann HJ. Intralymphatic immunotherapy improves grass pollen allergic rhinoconjunctivitis: A 3-year randomized placebo-controlled trial. *J Allergy Clin Immunol* (2021) 147(3):1011–9. doi: 10.1016/j.jaci.2020.07.002

16. Eschenbacher W, Straesser M, Knoeddler A, Li RC, Borish L. Biologics for the treatment of allergic rhinitis, chronic rhinosinusitis, and nasal polyposis. *Immunol Allergy Clin North Am* (2020) 40(4):539–47. doi: 10.1016/j.iac.2020.06.001

17. Schneider U, Hollands P. Intravenous gold-induced autologous serum injection therapy (Go ACT) as a new treatment for seasonal pollen-based allergies. *Eur Rev Med Pharmacol Sci* (2021) 25(11):4121–7. doi: 10.26355/eurrev_202106_26055

18. Li C, Zhou H, Zhang W, Che D. Bacterial lysate treatment in allergic disease: A systematic review and meta-analysis. *Pediatr Allergy Immunol* (2021) 32(8):1813–23. doi: 10.1111/pai.13572

19. Cazzola M, Anapurapu S, Page CP. Polyvalent mechanical bacterial lysate for the prevention of recurrent respiratory infections: a meta-analysis. *Pulm Pharmacol Ther* (2012) 25(1):62–8. doi: 10.1016/j.pupt.2011.11.002

20. Janeczek K, Emeryk A, Rachel M, Duma D, Zimmer L, Poleszak E. Polyvalent mechanical bacterial lysate administration improves the clinical course of grass pollen-induced allergic rhinitis in children: A randomized controlled trial. *J Allergy Clin Immunol Pract* (2021) 9(1):453–62. doi: 10.1016/j.jaip.2020.08.025

21. Banche G, Allizond V, Mandras N, Garzaro M, Cavallo GP, Baldi C, et al. Improvement of clinical response in allergic rhinitis patients treated with an oral immunostimulating bacterial lysate: *In vivo* immunological effects. *Int J Immunopathol Pharmacol* (2007) 20(1):129–38. doi: 10.1177/039463200702000115

22. Koatz AM, Coe NA, Ciceran A, Alter AJ. Clinical and immunological benefits of OM-85 bacterial lysate in patients with allergic rhinitis, asthma, and COPD and recurrent respiratory infections. *Lung* (2016) 194(4):687–97. doi: 10.1007/s00408-016-9880-5

23. Meng Q, Li P, Li Y, Chen J, Wang L, He L, et al. Broncho-vaxom alleviates persistent allergic rhinitis in patients by improving Th1/Th2 cytokine balance of nasal mucosa. *Rhinology* (2019) 57(6):451–9. doi: 10.4193/Rhin19.161

24. Jurkiewicz D, Zielnik-Jurkiewicz B. Bacterial lysates in the prevention of respiratory tract infections. *Otolaryngol Pol* (2018) 72(5):1–8. doi: 10.5604/01.3001.0012.7216

25. Emeryk A, Vallet T, Wawryk-Gawda E, Jedrzejewski A, Durmont F, Ruiz F. Acceptability of a sublingual drug formulation for respiratory tract infections in children aged 3 to 5 years. *Pharmaceutics* (2021) 13(2):294. doi: 10.3390/pharmaceutics13020294

26. Dar AA, Patil RS, Chiplunkar SV. Insights into the relationship between toll like receptors and gamma delta T cell responses. *Front Immunol* (2014) 5:366. doi: 10.3389/fimmu.2014.00366

27. Hayes SM, Love PE. Distinct structure and signaling potential of the gamma delta TCR complex. *Immunity* (2002) 16(6):827–38. doi: 10.1016/s1074-7613(02)00320-5

28. Zarobkiewicz MK, Wawryk-Gawda E, Kowalska W, Janiszewska M, Bojarska-Junak A. γδT lymphocytes in asthma: a complicated picture. *Arch Immunol Ther Exp (Warsz)* (2021) 69(1):4. doi: 10.1007/s00005-021-00608-7

29. de Oliveira Henriques MD, Penido C. Gammadelta T lymphocytes coordinate eosinophil influx during allergic responses. *Front Pharmacol* (2012) 3:200. doi: 10.3389/fphar.2012.00200

30. Hahn YS, Taube C, Jin N, Sharp L, Wands JM, Aydintug MK, et al. Different potentials of gamma delta T cell subsets in regulating airway responsiveness: V gamma 1+ cells, but not V gamma 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation. *J Immunol* (2004) 172(5):2894–902. doi: 10.4049/jimmunol.172.5.2894

31. Cui ZH, Joetham A, Aydintug MK, Hahn YS, Born WK, Gelfand EW. Reversal of allergic airway hyperreactivity after long-term allergen challenge depends on gammadelta T cells. *Am J Respir Crit Care Med* (2003) 168(11):1324–32. doi: 10.1164/rccm.200305-634OC

32. Krovi SH, Gapin L. Invariant natural killer T cell subsets-more than just developmental intermediates. *Front Immunol* (2018) 9:1393. doi: 10.3389/fimmu.2018.01393

33. Bojarska-Junak A, Tabarkiewicz J, Rolinski J. NKT cells: Their development, mechanisms and effects of action. *Postepy Hig Med Dosw (Online)* (2013) 67:65–78. doi: 10.5604/17322693.1034001

34. Ose R, Weigmann B, Schuppan D, Waisman A, Saloga J, Bellinghausen I. Depletion of CD56(+)CD3(+) invariant natural killer T cells prevents allergen-induced inflammation in humanized mice. *J Allergy Clin Immunol* (2021) 148(4):1081–7 e2. doi: 10.1016/j.jaci.2021.05.005

35. Hirst JM. An automatic volumetric spore trap. *Ann Appl Biol* (1952) 39:257–65. doi: 10.1111/j.1744-7348.1952.tb00904.x

36. Galan C, Smith M, Thibaudon M, Frenguelli G, Oteros J, Gehrig R, et al. Pollen monitoring: Minimum requirements and reproducibility of analysis. *Aerobiologia (Bologna)* (2014) 30:385–95. doi: 10.1007/s10453-014-9335-5

37. Myszkowska D, Jenner B, Stepalska D, Czarnobilska E. The pollen season dynamics and the relationship among some season parameters (Start, end, annual total, season phases) in Krakow, Poland, 1991-2008. *Aerobiologia (Bologna)* (2011) 27(3):229–38. doi: 10.1007/s10453-010-9192-9

38. Ellis AK, Soliman M, Steacy L, Boulay ME, Boulet LP, Keith PK, et al. The allergic rhinitis - clinical investigator collaborative (AR-CIC): Nasal allergen challenge protocol optimization for studying AR pathophysiology and evaluating novel therapies. *Allergy Asthma Clin Immunol* (2015) 11(1):16. doi: 10.1186/s13223-015-0082-0

39. Starling-Schwanz R, Peake HL, Salome CM, Toelle BG, Ng KW, Marks GB, et al. Repeatability of peak nasal inspiratory flow measurements and utility for assessing the severity of rhinitis. *Allergy* (2005) 60(6):795–800. doi: 10.1111/j.1398-9995.2005.00779.x

40. Lanzilli G, Traggiai E, Braido F, Garelli V, Folli C, Chiappori A, et al. Administration of a polyvalent mechanical bacterial lysate to elderly patients with COPD: Effects on circulating T, b and NK cells. *Immunol Lett* (2013) 149(1-2):62–7. doi: 10.1016/j.imlet.2012.11.009

41. Niu H, Wang R, Jia YT, Cai Y. Pidotimod, an immunostimulant in pediatric recurrent respiratory tract infections: A meta-analysis of randomized controlled trials. *Int Immunopharmacol* (2019) 67:35–45. doi: 10.1016/j.intimp.2018.11.043

42. Bartkowiak-Emeryk M, Emeryk A, Rolinski J, Wawryk-Gawda E, Markut-Miotla E. Impact of polyvalent mechanical bacterial lysate on lymphocyte number and activity in asthmatic children: A randomized controlled trial. *Allergy Asthma Clin Immunol* (2021) 17(1):10. doi: 10.1186/s13223-020-00503-4

43. Janeczek KP, Emeryk A, Rapiejko P. Effect of polyvalent bacterial lysate on the clinical course of pollen allergic rhinitis in children. *Postepy Dermatol Alergol* (2019) 36(4):504–5. doi: 10.5114/ada.2019.87457

44. Kaczynska A, Klosinska M, Janeczek K, Zarobkiewicz M, Emeryk A. Promising immunomodulatory effects of bacterial lysates in allergic diseases. *Front Immunol* (2022) 13:907149. doi: 10.3389/fimmu.2022.907149

45. Janeczek K, Kaczynska A, Emeryk A, Cingi C. Perspectives for the use of bacterial lysates for the treatment of allergic rhinitis: A systematic review. *J Asthma Allergy* (2022) 15:839–50. doi: 10.2147/JAA.S360828

46. Hennino A, Jean-Decoster C, Giordano-Labadie F, Debeer S, Vanbervliet B, Rozieres A, et al. CD8+ T cells are recruited early to allergen exposure sites in atopy patch test reactions in human atopic dermatitis. *J Allergy Clin Immunol* (2011) 127(4):1064–7. doi: 10.1016/j.jaci.2010.11.022

47. Qiu S, Du Y, Duan X, Geng X, Xie J, Gao H, et al. Cytotoxic T lymphocytes mediate chronic inflammation of the nasal mucosa of patients with atypical allergic rhinitis. *N Am J Med Sci* (2011) 3(8):378–83. doi: 10.4297/najms.2011.3378

48. Johansson MA, Bjorkander S, Mata Forsberg M, Qazi KR, Salvany Celades M, Bittmann J, et al. Probiotic lactobacilli modulate staphylococcus aureus-induced activation of conventional and unconventional T cells and NK cells. *Front Immunol* (2016) 7:273. doi: 10.3389/fimmu.2016.00273

49. Wang X, Ba T, Cheng Y, Zhang P, Chang X. Probiotics alleviate adipose inflammation in high-fat diet-induced obesity by restoring adipose invariant natural killer T cells. *Nutrition* (2021) 89:111285. doi: 10.1016/j.nut.2021.111285

50. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* (2012) 336(6080):489–93. doi: 10.1126/science.1219328

51. Pang DJ, Neves JF, Sumaria N, Pennington DJ. Understanding the complexity of gammadelta T-cell subsets in mouse and human. *Immunology* (2012) 136(3):283–90. doi: 10.1111/j.1365-2567.2012.03582.x

52. Huang Y, Jin N, Roark CL, Aydintug MK, Wands JM, Huang H, et al. The influence of IgE-enhancing and IgE-suppressive gammadelta T cells changes with exposure to inhaled ovalbumin. *J Immunol* (2009) 183(2):849–55. doi: 10.4049/jimmunol.0804104

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Inhibition of pathologic immunoglobulin E in food allergy by EBF-2 and active compound berberine associated with immunometabolism regulation

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Introduction: Food allergy is a significant public health problem with limited treatment options. As Food Allergy Herbal Formula 2 (FAHF-2) showed potential as a food allergy treatment, we further developed a purified version named EBF-2 and identified active compounds. We investigated the mechanisms of EBF-2 on IgE-mediated peanut (PN) allergy and its active compound, berberine, on IgE production.

Methods: IgE plasma cell line U266 cells were cultured with EBF-2 and FAHF-2, and their effects on IgE production were compared. EBF-2 was evaluated in a murine PN allergy model for its effect on PN-specific IgE production, number of IgE^+ plasma cells, and PN anaphylaxis. Effects of berberine on IgE production, the expression of transcription factors, and mitochondrial glucose metabolism in U266 cells were evaluated.

Results: EBF-2 dose-dependently suppressed IgE production and was over 16 times more potent than FAHF-2 in IgE suppression in U266 cells. EBF-2 significantly suppressed PN-specific IgE production (70%, $p<0.001$) and the number of IgE-producing plasma cells in PN allergic mice, accompanied by 100% inhibition of PN-induced anaphylaxis and plasma histamine release ($p<0.001$) without affecting IgG1 or IgG2a production. Berberine markedly suppressed IgE production, which was associated with suppression of XBP1, BLIMP1, and STAT6 transcription factors and a reduced rate of mitochondrial oxidation in an IgE-producing plasma cell line.

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Conclusions: EBF-2 and its active compound berberine are potent IgE suppressors, associated with cellular regulation of immunometabolism on IgE plasma cells, and may be a potential therapy for IgE-mediated food allergy and other allergic disorders.

KEYWORDS

berberine, IgE, food allergy, metabolism, anaphylactic allergic reaction

Introduction

Food allergy (FA) has rapidly increased over the past 2 decades affecting 32 million Americans, with annual costs of $25 billion (1–9). FA anaphylaxis, a potentially life-threatening condition, increased 200-400% in toddlers to teens (10) accounting for up to 81% of pediatric anaphylaxis (1). Peanut allergies are lifelong and cause severe reactions and there is currently no cure (1, 10–15). Common treatments, such as prophylactic-food avoidance, or therapeutic-food allergen oral immunotherapy (OIT) are limited and impractical (16–22). Therefore, there is a significant need for safe, effective, and non-food restricted therapeutics. FA is primarily mediated by food protein specific immunoglobulin E (sIgE) (23). IgE-producing long-lived plasma cells (IgE^+LPC) cause "lifelong allergy" (24–26). Persistent IgE is a significant barrier to FA mitigation. Omalizumab, an anti-IgE antibody, "traps" IgE but does not target its production. OIT, including Palforzia®, does not decrease IgE production, but in fact may paradoxically increase IgE levels, carrying a significant immune reaction risk (27–32). Therefore, a safe and effective therapy that targets excess IgE production represents an important strategy for food allergy treatment.

In recent years, a substantial number of findings have been made in the area of immunometabolism, the changes in intracellular metabolic pathways in immune cells that alter their function (33). Glycolysis is one of the major metabolic pathways involved in immune cell regulation. Immunoglobulins are glycoproteins that are produced, glycosylated, and secreted in the endoplasmic reticulum (ER), requiring energy and metabolites. Mitochondria are a highly efficient organelle for fueling the ER through ATP consumption *via* oxidation (respiration) (34). Alternative energy (ATP) production is *via* glycolysis though pyruvate. However, this pathway generates less ATP than mitochondrial oxidation (3 vs. 31 ATP molecules). IgE is the most heavily glycosylated isotype, with sugar moieties accounting for 12-14% of its molecular weight compared to approximately 3% for IgG (35–38). As more than 90% of glucose uptake in plasma cells (PCs) is utilized for antibody glycosylation, IgE^+PCs face greater metabolic stress compared to other isotype PCs. It was reported that inhibition of glycosylation essentially shuts down IgE release while IgG is largely unaffected (39, 40). Plasma cells require high energy and are regulated by specific transcription factors. XBP1 expression, which is high in plasma cells, is important for plasma cell differentiation (41) and secretory function (42, 43). XBP1 promotes gene expression involved in mitochondrial and ER biogenesis (44) and is required for antibody generation of heavy and light chain (IgH/IgL) transcripts (45). Loss of XBP-1 is associated with complete absence of plasma cells and circulating immunoglobulins (46, 47). This is due to inefficient processing and exportation of immunoglobulins and accumulation of unfolded proteins further contributing to ER stress (48).Thus, modulating XBP1 and mitochondrial glucose metabolism may affect IgE^+ PC antibody production and secretion (44), but direct evidence is limited.

Like FA, immunologic responses in parasite infections are associated with excessive production of IgE. FAHF-2 derived from *Fructus mume formula*, which has been used to treat parasite infection traditionally, showed a reduction of peanut specific IgE and protected against anaphylaxis in murine models of peanut allergy (49–53), suggesting a possible FA treatment. A new purification method was developed using ethyl acetate and butanol to concentrate active ingredients. The objective of this study is to investigate the potency of EBF-2 on IgE production *in vitro* and *in vivo*, and to understand underlying mechanisms on immunometabolism regulation of IgE producing cells. We first compared the effects of EBF-2 and FAHF-2 on IgE production across-multiple batches using IgE producing plasma cells and chromatographic approaches. We also assessed EBF-2's effects on PN-specific IgE production, IgE^+PC count, and anaphylactic symptoms in a murine peanut allergy model. Furthermore, we investigated the effect of the bioactive compound berberine (BBR) on XBP1 expression and mitochondrial metabolism in a human IgE^+PC line.

Material and methods

EBF-2 constituents, and production

EBF-2 was generated by purification of FAHF-2 with a safe solvents consisting of butanol and ethyl acetate (52). 8 herb constituents (*Prunus mume*, *Zanthoxylum schinifolium*, *Angelica sinensis*, *Zingiber officinalis*, *Cinnamomum cassia*, *Phellodendron chinense*, *Coptis chinensis*, and *Panax ginseng*) were extracted using butanol, while the *Ganoderma lucidum* was extracted using ethyl

Abbreviations: FAHF-2, Food Allergy Herbal Formula 2; PN, Peanut; DMSO, Dimethyl sulfoxide; HPLC, High performance liquid chromatography; EBF-2, Ethyl acetate and butanol purified FAHF-2; BBR, berberine; FA, food allergy; IgE^+PC, IgE plasma cells; PNA: peanut allergy; TCM, Traditional Chinese Medicine; wk (s), week or weeks; i.g., intragastrically; SPC (s), splenocyte (s); CPE, crude peanut extract; Ab, antibody; CT, cholera toxin; ER, Endoplasmic reticulum; XBP1, X-box binding protein; BLIMP, B lymphocyte-induced maturation protein; STAT6, Signal Transducer and Activator of Transcription.

acetate and the dried extracts were combined to generate the EBF-2 powder substance. Three batches of FAHF-2 and EBF-2 were tested in this study-manufacturing date and shelf life are listed in Supplemental Table 1. Botanical information for individual herbs, including geographical location, harvest season, pre-processing, heavy metal and pesticide residues, and quality control methods, have been published previously (54).

Cell culture and IgE measurement

The IgE-producing human plasma cell line U266 (ATCC, MD) was grown in complete media containing RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 1×10^{-5} M β-ME and 0.5% penicillin-streptomycin (55, 56) at 2×10^{4} cells/mL in 48 well plates. Three batches of FAHF-2 and 3 batches of EBF-2 at serial dilution concentrations starting at 500 µg/mL and 120 µg/mL, respectively, were used in culture for 6 days. Berberine (purity>98%, Sigma Aldrich, St Louis, MO) at serial concentrations starting at 5 µg/mL were also tested on U266 cells. Supernatants were harvested, IgE levels were determined by ELISA (Mabtech Inc, OH) and cell viability was evaluated by trypan blue exclusion (55).

Mice, peanut sensitization, and EFB-2 treatment

Six-week-old female C3H/HeJ mice purchased from the Jackson Laboratory (Bar Harbor, ME) were maintained in pathogen-free facilities at the Mount Sinai vivarium according to standard guidelines (57). Mice were intragastrically (i.g.) sensitized with 10 mg of homogenized peanut (PN) in 0.5 mL PBS containing 75 mg sodium bicarbonate, 10 µg of the mucosal adjuvant cholera toxin (CT) (List Laboratories, Campbell, CA), and 16.5 µL (1.1 µL/g body weight) of 80 proof Stolichnaya Vodka® (a source of food grade ethanol) to neutralize stomach pH and increase gastrointestinal permeability, three times during week 0 (50). Thereafter, sensitization was done weekly as above except that the CT dose given was 20 µg. The boosting dose of 50 mg PN was given at weeks 6 and 8 using the same gavage solution as in weeks 1 through 5. These mice were defined as peanut allergic (PNA) mice. One day following the last boost, at which hypersensitivity was developed (58), PNA mice received EBF-2 treatment at 3.84 mg in 0.5 mL drinking water, twice a day for 4 weeks. One week after completing the treatment, mice were challenged with ground peanut (200 mg) i.g. and again 4 weeks later. Anaphylactic reactions were accessed (Figure 1A). Sham treated PNA mice and naïve mice (unsensitized/untreated) were used as disease and normal controls, respectively. In a separate experiment, to determine the persistent impact of EBF-2 on IgE producing cells by flowcytometry analysis, EBF-2 treated, and sham treated PNA mice received periodic oral exposure of either boiled (10 mg/mouse) or roasted peanut (200 mg/mouse) approximately every 10-15 weeks. Mice were terminated using ketamine/xylazine euthanasia protocol. Briefly, mice were given over-dose (15µL/g body weight) of ketamine-xylazine mixture (100mg/mL and 10 mg/mL respectively) intraperitoneally. After the mice were in deep anesthetics, blood samples were collected, and mice were sacrificed by cervical dislocation after which tissue samples were collected. All animal experiments were approved and performed according to the instruction and guidelines of the Institutional Animal Care and Use Committee (IACUC) of Icahn School of Medicine at Mount Sinai."

TABLE 1 *In vivo* sub-chronic safety assessment of EBF-2.

	Treatment (5x daily dose)		Reference range
	Water (n=5)	EBF-2 (n=5)	
Morbidity	0/10	0/10	NA
Mortality	0/10	0/10	NA
ALT (U/L)	32.6±12.4	55.8±27.7	28-129
BUN (mg/dL)	18.8±3.5	23.2±1.9	7.0-28
WBC (10^3/µL)	5.1±1.1	6.2±1.8	3.9-13.9
RBC (10^3/µL)	10.4±0.7	9.9±0.9	7.14-12.2
Hb (g/dL)	13.3±1.1	12.4±1.2	10.8-19.2
PLT (10^3/µL)	645.6±243.2	923.6±483.2	565-2159
Neu (10^3/µL)	1.3±0.3	1.7±0.6	0.42-3.09
L (10^3/µL)	3.3±0.9	3.4±0.9	2.88-11.15
Eos (10^3/µL)	0.1±0.0	0.1±0.0	0.01-0.50
Bas (10^3/µL)	0.0±0.0	0.0±0.0	0-0.14

Naive mice were fed a therapeutic dose 5 times daily for 14 days. Sham fed mice served as controls (sham). Blood samples were collected after termination of experiments. Blood urea nitrogen (BUN) and alanine aminotransferase (ALT) measurements for evaluation of kidney and liver functions, respectively, and complete blood count (CBC) testing were performed. ALT, Alanine Aminotransferase; BUN, Blood Urea Nitrogen; WBC, White Blood Cells; RBC, Red Blood Cells; Hb, Hemoglobin; PLT, Platelets; Neu, Neutrophils; L, Lymphocytes; Eos, Eosinophils; Bas, Basophils; NA, Not available.

Assessment of hypersensitivity reactions

Symptoms were evaluated 30-40 minutes following oral PN challenge as described previously (51, 59), and symptoms were scored utilizing the scoring system ranging from 0 (no reaction) to 5 (fatal reactions), described previously (59). Rectal temperatures were also measured immediately after scoring using a rectal probe (Harvard Apparatus, Holliston, MA).

Measurement of plasma histamine levels

Blood samples were collected *via* sub-mandibular bleeding 30 minutes after scoring and measurement of body temperature following oral peanut challenge. Plasma was harvested within 20 minutes after blood collection and stored at -80°C until analyzed. Histamine was measured using a commercial enzyme immunoassay kit (Fisher Scientific, NJ) as described by the manufacturer (58).

Measurement of serum peanut specific-IgE, IgG1 and IgG2a

PN-specific-IgE, IgG1 and IgG2a in serum was measured as reported previously (50, 51, 59). Briefly, microtiter plates were coated with peanut extract (sample wells), anti-mouse IgE (BD Biosciences, San Jose, CA, for IgE reference wells), or DNP-HSA (Sigma-Aldrich for IgG2a and IgG1 reference wells) and incubated overnight at 4° C. Subsequently, the plates were blocked with 2% BSA-PBS after washing. Washed plates were incubated with diluted serum samples, mouse IgE (BD Biosciences), anti-DNP-IgG2a, or anti-DNP-IgG1 (Accurate Antibodies, Westbury, NY) overnight at 4°C and later developed by using biotinylated anti-IgE, IgG2a or IgG1 detection antibodies (BD Biosciences), avidin-peroxide, and ABTS substrate (KPL, St Paul, MN).

Flow cytometry analysis of IgE producing plasma cells

Mice were sacrificed at week 78 of the protocol and single-cell suspensions of splenocytes were prepared in ice cold staining buffer (PBS including 0.5 mM EDTA, 0.05 mM sodium azide, 0.5% BSA). First, surface staining with unlabeled anti-IgE (to block membrane IgE), APC anti-CD138, BV711-anti-CD3, and anti-CD16/32 (Fc-block) (all from BD Biosciences), CA was performed. Live-dead discriminating dye (Live-Dead Aqua, Invitrogen, CA) was included. Cells were washed and incubated with fixation/permeabilization buffer (BD Biosciences, CA) for 15 mins, washed with permeabilization buffer (BD Biosciences, CA), and then incubated with FITC-anti-IgE, in permeabilization buffer. After washing, cells were treated with Cytofix buffer (BD Biosciences, CA) for 15 mins for post-fixation, washed, and then data were acquired on an LSRII flow cytometer (Becton Dickinson, CA). Flow cytometry analysis was performed using Flow Jo (Tree Star, CA) as follows. Live singlet cells were then analyzed for IgE^+ plasma cells (FITC-IgE +; APC-CD138+ cells).

Safety testing of EBF-2 in a mouse model

To evaluate the safety of EBF-2, the sub-chronic toxicity assay was performed on C3H/HeJ mice as in our previous studies (52). Naïve C3H/HeJ mice were fed 40 mg/mL of EBF-2, which is 5 times the daily therapeutic dose, for 14 days. Sham (water) fed mice served as controls (sham). Blood samples were collected at the end of the experiment. Liver and kidney function and complete blood count (CBC) were performed by ALX laboratories, NY.

High performance liquid chromatography fingerprint analysis of EBF-2 and ex vivo detecting EBF-2 active compound by liquid chromatography–mass spectrometry

HPLC analysis was performed on a Waters 2690 HPLC system coupled with a 2996 PDA detector (Waters, Milford, MA) for each batch of FAHF-2 and EBF-2 using the method described previously (52). Each sample was first dissolved in 2 mL of the mixture of mobile phases at a 1:1 ratio and centrifuged at 10,000 rpm for 10 mins. The sample amount injected for HPLC fingerprint analysis was based on the human daily dose used (1:200 of human daily dose). FAHF-2's human daily dosage is 19.8g, therefore the concentration of FAHF-2 injected was 99mg/mL. For EBF-2, the human daily dose is 4.58g, therefore the concentration of EBF-2 injected to HPLC was 22.9mg/mL. 10 µL of the supernatant was injected into the HPLC system and separated on a ZORBAX SB-C18 (5 µm, 150 mm x 4.6 mm, column (Agilent, Santa Clara, CA). Aqueous formic acid (0.1%) was used as mobile phase A, while acetonitrile (Fisher Scientific, NJ) served as mobile phase B. The separation was performed using a linear gradient elution of 2% to 25% mobile phase B in 45 min, 25% to 35% in the following 25 mins, 35% to 55% in the next 15 mins, and 55% to 75% in the final 10 mins. The flowrate was maintained at 1 mL/min. Data was collected and processed using Waters Empower software.

EBF-2 active compound in tissue samples were analyzed using LC-MS system. Briefly, tissue samples were cut into small pieces and soaked in methanol. The extracts were analyzed on an Agilent 1200 HPLC instrument with an Agilent Eclipse Plus-C18 column (5 µm, 250 mm × 4.6 mm), coupled with an Agilent 6130 Single Quad Mass Spectrometry. The samples were eluted with acetonitrile-water (5-95%) containing 0.1% formic acid (v/v) over 100 mins.

Real time polymerase chain reaction

U266 cells (1.0×10^6 cells/mL) were incubated with or without berberine for 3 days. Cells were harvested and total RNA was isolated using Trizol (Gibco BRL, Rockville, MD). The RNA concentrations were quantified by triplicate optical density (OD) readings (Bio-Rad SmartSpect 3000; Bio-Rad, Hercules, CA). Reverse transcription was performed to yield cDNA using ImProm-II™ Reverse Transcriptase (Promega Corporation, Madison, WI) as per the manufacturer's instructions. The RT-PCR amplification was performed using Maxima™ SYBR Green qPCR Master Mix (2X) kit (Fisher Scientific, Pittsburgh, PA). Primer sequences of XBP1, BLIMP1, STAT-6, BCL-6 and GAPDH were from previously published literature and listed in Supplemental Table 2 (55, 60–62).

Seahorse mitochondrial stress assay

To determine the effect of EBF-2's active compound berberine on mitochondrial metabolism of an IgE producing plasma cell line, we used the Seahorse mitochondrial stress assay. XF cell Mito stress test kits were obtained from Agilent (Santa Clara, CA). Each well of the XF24 cell culture plates was coated with 50 µL of Corning Cell-Tak cell and tissue adhesive at a density of 3.5 µg/cm^2 for 20 mins followed by washing with 200 µL water and 20 min of drying. Fresh assay medium was prepared by supplementing 2 mM glutamine into XF cell base medium, DMEM with an adjusted pH of 7.4. Next, 1 × 10^5 of U266 cells resuspended in 100 µL of assay medium were seeded into each well of the coated plate by centrifugation in a swing-bucket rotor at 450 rpm for 1 min without braking. After reversing the orientation of the plates, they were centrifuged again at 650 rpm for 1 min without braking. Plates were transferred to a 37°C incubator not supplemented with CO_2 and incubated for 25-30 mins. Then, 500 µL of warm assay medium, containing DMSO or various concentrations of BBR was slowly and gently added into the wells. After a 15 mins CO_2-free incubation, the cells were ready for the assay on a Seahorse XFe24 Analyzer. Oligomycin (3 µM final concentration), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, 3 µM final concentration) and rotenone and antimycin (3 µM and 1 µM final concentration, respectively) were diluted in the assay medium and loaded into ports A, B, and C of the XF24 assay plate. The machine was calibrated, and the assay was performed using the Mito stress test assay protocol per the manufacturer's recommendations. The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured under basal conditions after sequential addition of the above-mentioned drugs.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 (San Diego, CA). One-way ANOVA (analysis of variance) was performed followed by Bonferroni correction for all pairwise comparisons. For skewed data, differences between groups were analyzed by one-way ANOVA on ranks followed by Dunn's method for all pairwise comparisons. Data for symptom score correlation with IgE were analyzed using Spearman correlation. Pearson correlation was used for all other correlation analyses. *p*-value calculations were two-tailed and a p value < 0.05 was considered as statistically significant.

Results

EBF-2 dose-dependently inhibited IgE production in an IgE plasma cell line

As compared to the untreated cells, EBF-2 significantly decreased IgE production beginning at 1.9 µg/mL (p<0.05) with complete inhibition of IgE production at 60 µg/mL (p<0.001) (Figure 1A). The IC_{50} value was 4.70 ± 1.16 µg/mL (Figure 1B). There was no observed cytotoxicity at any tested concentrations (Figure 1C). The parent formula FAHF-2 also significantly decreased IgE production at 62.5 µg/mL (p<0.01) with complete inhibition of IgE production at 500 µg/mL(p<0.001) (Figure S1A). The IC_{50} value of the parent formula FAHF-2 was 79.7 ± 17.39 µg/mL (Figure S1B), with no cytotoxic effect across all concentration (Figure S1C). Furthermore, we analyzed the effect of three different batches of FAHF-2 (F2-1106, F2-0202, F2-0909) (Figures S2A–C) and EBF-2 (EBF-2-0303, EBF-2-0808, EBF-2-0130) (Figures S2D–F) on IgE production by U266 cells respectively and found consistent results between different batches. Taken together, EBF-2 is markedly more inhibitory on IgE production than its parent formula while retaining high cellular safety and batch to batch consistency

EBF-2 treatment suppressed peanut anaphylaxis associated with suppression of peanut specific-IgE without affecting IgG1 or IgG2a production

We next determined EBF-2's inhibitory effect on IgE levels and its protective effect against peanut anaphylaxis in a murine model (Figure 2A). Four weeks after discontinuation of EBF-2 treatment (at week 14 following initial PN sensitization), peanut (PN)-specific IgE levels were significantly reduced by approximately 70% in EBF-2 treated mice compared to sham treated mice (Figure 2B, p<0.05 vs. Sham). Following intragastric challenge, all sham-treated mice developed anaphylactic symptoms, with symptom severity scores ranging from 2-3. In sharp contrast, EBF-2 treated mice were completely protected

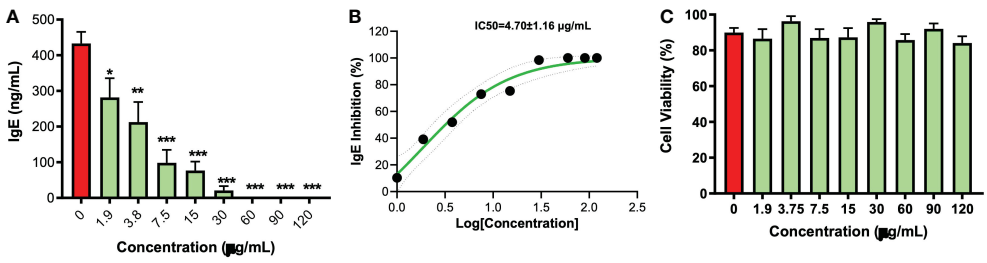

FIGURE 1
Inhibitory effect of EBF-2 on IgE production by plasma cell line U266 cells. **(A)** U266 cells were treated with EBF-2 at different concentrations and cultured for 6 days. Supernatants were collected, and IgE levels were determined by ELISA. **(B)** IC_{50} values for EBF-2 were calculated to be IC50=4.70±1.16 µg/mL. IC_{50} = 4.70 ± 1.16 mg/mL **(C)** Cell viability was measured by trypan blue excursion showed no cell cytotoxicity. IgE levels are expressed as Mean± SEM, and significance is indicated by *p≤0.05, **p≤0.01, and ***p≤0.001 as compared to the untreated control. N=9 independent cultures over 3 batches.

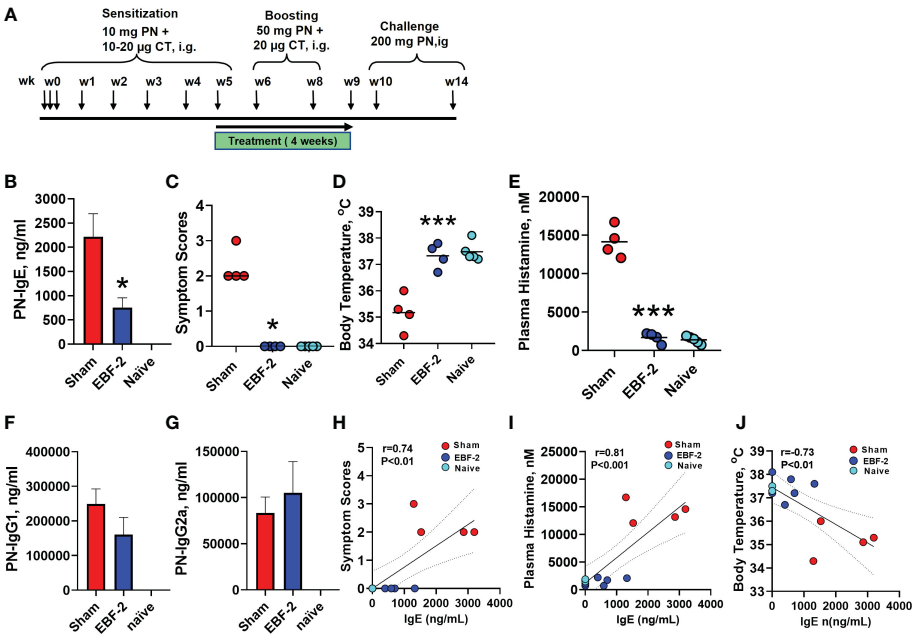

FIGURE 2
Effect of EBF-2 in a peanut allergic mouse model. **(A)** Experimental design for sensitization, treatment, and challenge: 6 weeks old C3H/HeJ mice were orally sensitized with 10 mg PN and 10-20 µg cholera toxin at weeks 0 through 5. Mice were boosted with 50 mg PN and 20 µg cholera toxin at weeks 6 and 8. Daily oral EBF-2 treatment (7.68mg/mouse/day) started at week 5 and continued for four weeks. Mice underwent oral PN challenges (200mg) at week 10 and week 14. **(B)** PN specific IgE measured by ELISA at week 14. **(C)** Symptom scores; **(D)** Body temperatures and **(E)** Plasma histamine levels 30 mints following oral PN challenge at week 14. **(F)** PN-specific IgG1; and **(G)** PN-specific IgG2a were measured by ELISA. **(H)** Spearman correlation between PN-IgE and symptom scores, **(J)** Pearson correlation between PN-IgE and Body temperature. **(I)** Pearson correlation between PN-IgE and plasma histamine Bars indicate group means. *P < 0.05; ***P < 0.001 vs. Sham. N=4-5 mice/group.

from anaphylaxis (Figure 2C, EBF-2 vs. Sham: median score 0 vs. 2, $p<0.05$). Hypothermia, a decrease in core body temperature, is a symptom of anaphylaxis in mice. We measured rectal temperatures every 30 minutes after the intragastric challenge, EBF-2 prevented hypothermia, the mean post-challenge body temperature in EBF-2 group was significantly higher than the Sham group and not different from naïve mice (Figure 2D, sham vs. EBF-2 vs. naïve: 35.18±0.3°C vs. 37.33±0.2°C vs. 37.33±0.2°C, $p<0.001$ vs. sham). Anaphylaxis is associated with an increase in plasma histamine levels and plasma histamine levels in EBF-2 mice were markedly and significantly lower than in sham mice (Figure 2E Sham vs. EBF-2 mean±SEM: 14,134±1004 nM vs. 1689±340 nM). The EBF-2 treated group's plasma histamine level was not significantly different from the normal range of histamine levels in the naïve group (1392±213 nM). PN-specific IgG1 and PN-specific IgG2a production were not affected (Figures 2F, G). In this model, symptom severity and plasma histamine levels strongly correlated with IgE levels (Figures 2H, I, r=0.74, p <0.01; r=0.81, p <0.001, respectively), whereas body temperatures at challenge were inversely correlated with IgE (Figure 2J, r=-0.73, p <0.01).

EBF-2 treatment produced long term protection from peanut anaphylaxis and reduced IgE$^+$ plasma cell numbers

We investigated the long-term protection by EBF-2 and its effect on long-lived IgE producing plasma cells in a peanut anaphylaxis murine model (Figure 3A). EBF-2 significantly reduced PN-specific IgE during therapy (weeks 8-26) ($p<0.001$) and maintained consistent levels even after completion of the treatment, up to week 78 ($p<0.01$, $p<0.001$) (Figure 3B). Post-treatment challenges conducted at week 30, week 40 and week 70, respectively, showed that EBF-2 treated mice were completely protected from anaphylaxis (Figure 3C, EBF-2 vs Sham, $p<0.05$), prevented hypothermia (Figure 3D, $p<0.001$), and reduced plasma histamine levels (Figure 3E, $p<0.001$). Furthermore, EBF-2 treated mice showed significantly lower numbers of the IgE$^+$/CD138+ plasma cells from the spleen compared to Sham treated mice (Figures 3F, G, $p<0.001$). There was a significant correlation between IgE+/CD138+ plasma cells and PN-specific IgE levels (Figure 3H, r=0.84, $p<$ 0.0001). These data highlight the persistent protection of EBF-2 from anaphylaxis following peanut exposure.

EBF-2 formula had a high safety profile

EBF-2's safety was evaluated with a sub-chronic toxicity protocol. C3H/HeJ mice were fed 5 times the normal daily dose of EBF-2 for 14 consecutive days and observed for 2 weeks. No morbidity or mortality was observed. Serum ALT and BUN levels of EBF2 and sham-treated mice were all within normal range (Table 1). CBC results in the EBF-2 treated group were similar to those of sham treated mice and were all within the normal range. Thus, the EBF-2 formula has a high safety profile.

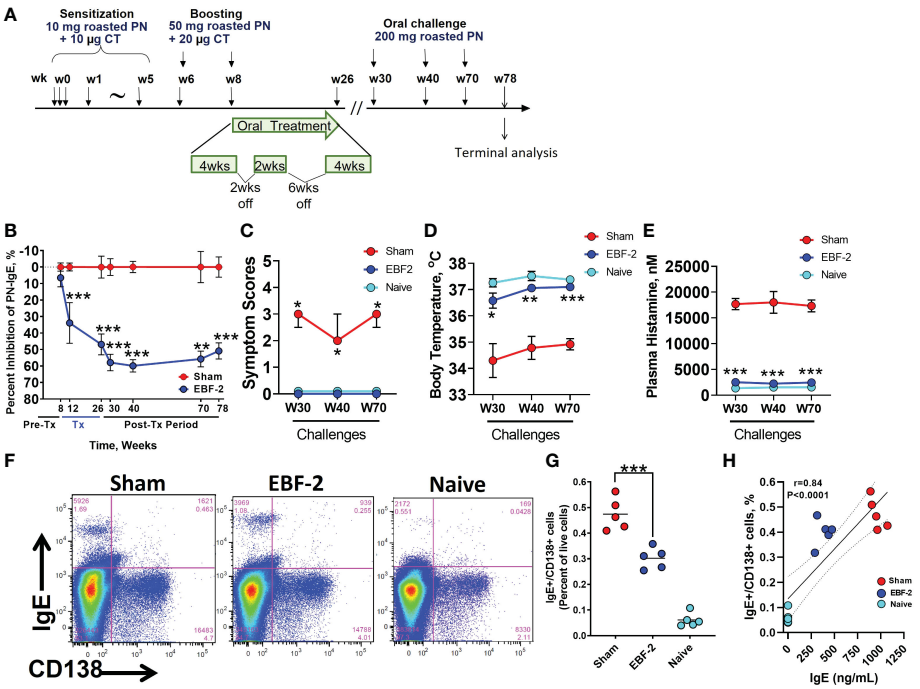

FIGURE 3
Long term effect of EBF-2 on peanut-specific IgE, anaphylaxis and IgE$^+$PC. **(A)** Experimental protocol. **(B)** Effect of EBF-2 on percent inhibition of PN-specific IgE over duration of the experiment. **(C–E)** symptoms scores, body temperature and plasma histamine levels 30 minutes following challenges at weeks 30, 40 and 70. **(F)** Representative flow cytometry panels showing the percentage of IgE+/CD138+ PCs (upper right quadrant) in spleens of mice in sham, EBF-2, and naïve groups. **(G)** Scatter graph showing data for individual mice across experimental groups shown in **(F)** Bars are group means. **(H)** Correlation between IgE+/CD138+ plasma cells and PN-specific IgE levels in mice across all experimental groups. r value is the Pearson coefficient of correlation. *p<0.05, **p<0.01, ***p<0.001 vs sham vs. sham. N=5 mice/group. PC, plasma cells.

HPLC fingerprints reveal a higher berberine peak in EBF-2 than FAHF-2 and detecting BBR ex vivo after feeding a single dose of EBF-2 by LC-MS

We previously showed that berberine (BBR) isolated from FAHF-2 and B-FAHF-2 reduced IgE by plasma cell lines and human PBMCs from food allergic patients (55), and demonstrated that the concentration of BBR can be a pharmacological marker of FAHF-2 and EBF-2. We therefore determined the concentration of BBR in EBF-2 and compared with parent formula FAHF-2 (Figure 4A). A total of 29 peaks (P) were detected in FAHF-2 and EBF-2 batches. The major peak 13 (P13) was identified as BBR (Figure 4B). The peak area of BBR (Mean ± SED) in EBF-2 was significantly higher than that in FAHF-2 (62.83 ± 3.53% vs 33.26 ± 6.40% overall total peaks $p<0.05$ (Supplemental Table 3). We have identified other peaks such as Magnoflorine (P6), Phellodendrine (P8), Jatrorrhizine (P12), Ganolucidic acid D (P18), and Ganoderic acid H (P27), but the differences between these peaks were not statistically significant. After the purification process, the constituents (less polar small molecules) in EBF-2 were more concentrated. We also calculated the BBR concentration by using the equation collected from the peak area of BBR standard versus the concentration (BBR concentration (μg/mL) = BBR pear area/1000/72.23). The BBR in FAHF-2 was calculated as 0.36%, while the EBF-2 contains 4.4% of BBR. The BBR concentration was approximately 12 times higher in EBF2 than in FAHF-2 (data not shown). We detected BBR in liver and fat tissue 5 days after oral administration of EBF-2 by LC-MS and demonstrated that BBR is a major bioavailable compound within EBF-2 when compared to naïve mice (Figure 4C).

EBF-2 bioactive compound BBR inhibited IgE production and transcription factor XBP1, BLIMP1, and STAT6, and increased BCL-6 by IgE producing plasma cell

We evaluated the active compound BBR identified from EBF-2 and determined its effect on the regulation of IgE plasma cells at the transcriptional level, *in vitro* using U266 cells. BBR dose-dependently inhibited IgE production approaching 100% inhibition at 5 μg/mL (Figure 5A), without any cytotoxicity across the doses (0.625 – 5 μg/mL) (Figure 5B) with an IC_{50} value 1.946 μg/mL (Figure 5C). We evaluated BBR's effects on the gene expression of XBP1, BLIMP1 and STAT-6, which are genes that have been shown to be upregulated during plasma cell activation. BBR significantly inhibits the gene expression of XBP1, BLIMP1 and STAT6 compared to untreated cells ($p<0.01$, Figure 5D). BCL-6 reportedly inhibits long-lived plasma cell survival, and it has been shown to be upregulated in plasma cell activation. Therefore, we measured the effect of BBR on the gene expression of BCL-6, however our results showed that the increase expression of BCL-6 gene was not statistically significant. Taken together, we showed that the suppression of IgE in IgE$^+$ plasma B cells by BBR is mediated by down-regulation of XBP1 and BLIMP1.

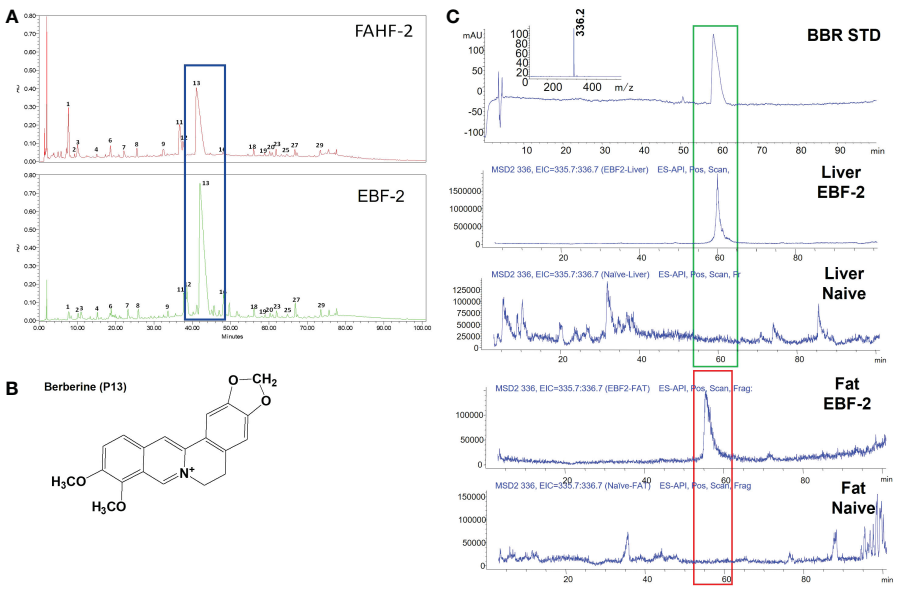

FIGURE 4
Characterization of FAHF-2 and EBF-2 products. **(A)** HPLC fingerprint of FAHF-2 and EBF-2. The x-axis indicates retention time in minutes, while the y-axis indicates an absorbance unit (AU). **(B)** Chemical structure of Berberine. **(C)** The presence of berberine in the liver and fat tissue samples of mice treated with E-B-FAHF-2. Mice were fed with the EBF-2 formula, and tissue samples were collected 5 days after oral administration. Mass spectra of the berberine standard; m/z: 336.2 was detected. Berberine presented in the liver of EBF-2 treated mice. No berberine was detected in liver samples of naïve mice. Berberine presented in fat tissue samples of EBF-2 treated mice. No berberine was detected in fat tissue samples of naïve mice. The illustrations are representative of 3-5 samples.

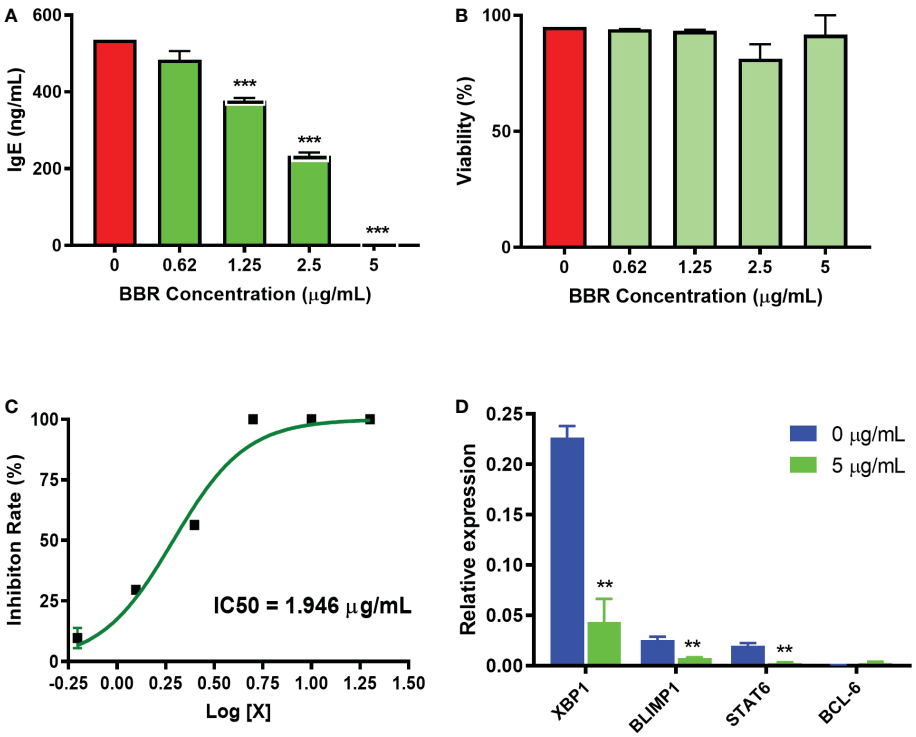

FIGURE 5
Effect of BBR on IgE production and transcription factor gene expression in U266 cells. **(A)** BBR dose-dependently inhibited the IgE production by U266 cells. **(B)** Cell viability of BBR on U266 cells. **(C)**. C_{50} value of BBR on IgE production was 1.946 μg/mL. **(D)** The relative expression level of XBP1, STAT6, BLIMP1, and BCL-6 genes vs. GAPDH. **p<0.001; ***p<0.001 vs. untreated. N=3 independent culture.

Berberine, a major active compound in EBF-2, inhibited IgE producing plasma cell mitochondrial metabolism

Plasma cells have a nutrient uptake and energy demand for the production and secretion of antibodies compared to their counterparts of B cells and plasmablasts (44). Emerging evidence suggests that glucose availability and energy metabolism are important for regulating plasma cell antibody production, secretion and survival at post-transcriptional levels (44, 63). Traditionally, BBR has been used as a glucose lowering agent in Type II diabetes through inhibition of mitochondrial respiratory complex I (64–67). We next asked whether BBR disrupts energy metabolism and changes glucose utilization in IgE plasma cell line U266 by a seahorse Mito stress assay. The assay measures mitochondrial respiration and function glycolysis by directly measuring OCR (Oxygen Consumption Rate) and ECAR (Extracellular Acidification Rate). Vehicle treated U266 cells were used to establish some key parameters of mitochondrial function (Figure 6A) including basal respiration, proton leak (after oligomycin injection, which inhibits ATP production through complex V), maximal respiration capacity (after injecting FCCP, which uncouples ATP production from electron transport), and non-mitochondrial respiration (after injecting complex I and III inhibitors rotenone and antimycin A). Interestingly, BBR pretreatment significantly suppressed basal OCR ($p<0.05$) and FCCP-induced maximal OCR ($p<0.001$) in a dose-dependent manners, with almost complete inhibition of mitochondrial respiration at 3 μg/mL and 10 μg/mL, respectively. To compensate for energy crisis resulted from mitochondrial respiratory inhibition, BBR-treated U266 cells increased the rate of glycolysis to increase ATP production from glycolysis pathway, as indicated by significantly elevated ECAR (Figure 6B, BBR at 10 μg/mL vs. untreated cells, $p<0.05$). These results suggest BBR inhibits mitochondrial respiration in IgE$^+$PCs, which lead to cellular energy crisis and decreased the availability of glucose molecules for other pathways such as IgE Ab glycosylation.

Discussion

IgE plays a key pathogenic role in FA-related hypersensitivity. Enthusiasm about IgE regulatory interventions for FA therapy remains high yet, interventions to modulate IgE$^+$PCs remain undeveloped. We tested direct effects of EBF-2, a refinement of parent formula FAHF-2, on IgE production using U266 cells, a well-established IgE$^+$ plasma cell line. We demonstrated the consistent inhibition of IgE production with EBF-2 across 3 separate batches, without cytotoxicity, and more potently than parent FAHF-2. EBF-2 was 16-fold more potent than parent formula FAHF-2, suggesting a superior effect compared to the parent formula. Using a PN allergic murine model, we demonstrated that EBF-2 decreased PN-specific IgE levels by ~70% following 4 weeks of oral treatment compared to sham-treated mice in an early EBF-2 treatment protocol, with no significant changes in PN-specific IgG1 or IgG2a levels and demonstrated a high safety profile. EBF-2 significantly protected PN allergic mice from anaphylaxis with an 8.5-fold lower daily dose than parent FAHF-2 (68), highlighting its efficacy and potency. The mechanism underlying this persistent therapeutic effect is unknown but may be due to suppression of long-lived IgE$^+$PCs. Using a PN allergy model, we showed that the percentages of IgE$^+$PCs, largely LLPCs at this 8-week post antigen exposure timepoint, were significantly reduced in EBF-2 treated mice and correlated with the peanut-IgE levels. This is important because these IgE$^+$PCs, which are LLPCs known to resist immunosuppressive or ablative therapies (24, 25, 69, 70), showed significant reduction following EBF-2 treatment. This indicates that EBF-2 may have a potential to alter the process of persistent peanut allergy, but this requires further investigation.

As a first attempt to understand EBF-2 suppression of IgE$^+$PCs and given that BBR is found at higher levels in EBF-2 than in FAHF-2 by HPLC, we identified BBR as a bioavailable compound within EBF-2 by LC-MS analysis following oral feeding. This provides a rationale to study BBR as a bioavailable active compound to regulate IgE production. We showed that BBR suppressed IgE production by a

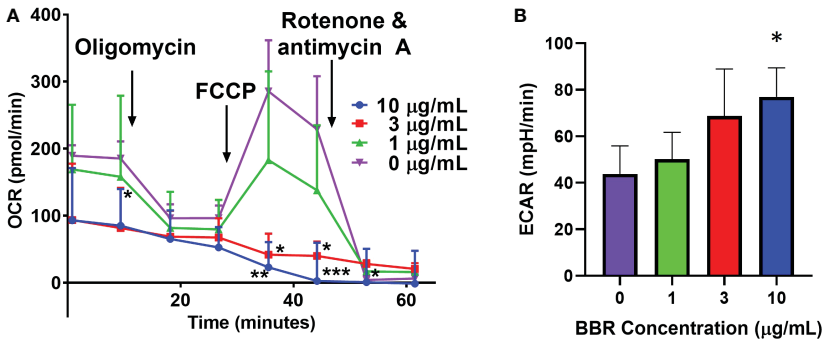

FIGURE 6
Effect of BBR on mitochondrial respiration rate in U266 cells. **(A)** U266 cells were pre-treated with DMSO or the indicated concentration of BBR for 15 min. Oligomycin (3 μM, inhibitor of mitochondrial complex V), FCCP (4 μM, stimulator of mitochondrial complex IV), and combination of rotenone (3 μM) and antimycin A (1 μM) (inhibitors of mitochondrial complex I and III, respectively) were injected at the indicated time per manufacturer instructions. The oxygen consumption rate (OCR) was recorded over time. **(B)** BBR dose-dependently increased basal glycolytic rate 15 min after treatment. Data represents triplicate cultures and is expressed as mean ± SEM. *p <0.05; **P<0.01; ***p<0.001 vs. untreated.

human IgE producing plasma cell line in a dose-dependent manner. Since no cytotoxicity was observed even at the dose (5 μg/mL) which BBR eliminated IgE production, we hypothesized it may act on mechanisms controlling IgE production and secretion. IgE^+PCs are under constant stress from antibody production and secretion. XBP1, a transcription factor, promotes and maintains plasma cells antibody production and secretion under ER stress as a compensating response (44, 63). We found a significant reduction of XBP1 gene expression in BBR-treated cells (~ 5-fold reduction) at a non-toxic dose compared to untreated cells. We also found that BBR inhibited BLIMP1, which promotes plasma cell survival (45). In addition, we found that STAT-6 was significantly reduced in a BBR treated IgE^+ plasma cell. STAT-6 is reported to be mainly involved in cooperating on IL-4-induced up-regulation of an IgE germline promoter (71). However, the role of STAT-6 on terminally differentiated IgE^+PCs has not been reported, requiring further investigation. In contrast, BCL-6, a transcriptional repressor of IgE production (72), tended to be increased. These findings suggest that BBR modulation of XBP1 and other transcription factors may together down-regulate IgE^+PCs.

In addition to transcriptional regulation, energy metabolism has emerged as an important regulator of plasma cell survival and function (44). Previous studies have shown that long-lived plasma cells (LLPCs) used 90% of glucose to glycosylate antibodies; however, when these cells were under energy stress such as challenged with a mitochondrial inhibitor, they diverted glucose to glycolysis to form pyruvate to support energy production, which was accompanied by a marked decrease in antibody secretion (73). Classically, BBR has been used to treat type II diabetes (74). Suppressing mitochondrial metabolism and promoting glycolysis have been suggested as mechanisms underlying BBR's anti-diabetic effects (66). Here we demonstrate, for the first time, that BBR reduces IgE plasma cell mitochondrial metabolism. We believe that BBR inhibits IgE^+PC mitochondrial respiration likely forcing IgE^+PCs to produce ATP through upregulated glycolysis. The metabolic decision point for glucose between glycolysis and hexosamine biosynthesis for glycosylation occurs at fructose-6-phosphate, catalyzed by phosphofructokinase (PFK). It is established that a low cellular ATP level allosterically activates the enzyme PFK, one of the rate-limiting enzymes in glycolysis, thus leading to the increase in glycolysis and the subsequent decrease in hexosamine biosynthesis for glycosylation (Figure 7A). Therefore, we feel that for the scope of this paper, showing the >50% increased glycolytic rate after BBR treatment highly likely led to the diversion of glucose away from IgE glycosylation (Figure 7B), potentially causing accumulation of immature (unglycosylated) IgE in the ER and in turn triggering ER stress. Under normal circumstances this triggers up-regulation of XBP1 to compensate for energy depletion. However, this will not happen in the presence of BBR that suppresses XBP1, leading to a cellular energy crisis (Figure 7B). Thus, BBR switches off IgE production from IgE^+PCs by regulating both transcription factors and mitochondrial metabolism.

In this study, our goal is to test the potency of a refined botanical medicine in murine model of peanut allergy, we therefore orally sensitized using Th2 adjuvant and orally challenged female C3H/HeJ mice. The advantage is that this murine model showed persistent peanut allergy, which allow us to test the durability of EBF-2 on food allergy and study long-lived IgE producing plasma cells. The reason to use female mice is to consider that females are more susceptible to food allergies (75) and have been widely used in food anaphylaxis studies (75–83). Therefore, we intended to use established model. Additional limitation of this study is lack of positive treatment control. At present, there is limited or no peanut therapy showing sustainable protection. Our previous publication showed that the effect of protection again anaphylaxis by peanut oral immunotherapy (OIT) is transient. At 5 weeks post therapy, the reactions returned by 90% of OIT treated mice following peanut challenge (51). Therefore, we included peanut allergic mice treated with water as sham treatment control (equivalent to placebo control in human trials. Since BBR has poor bioavailability,

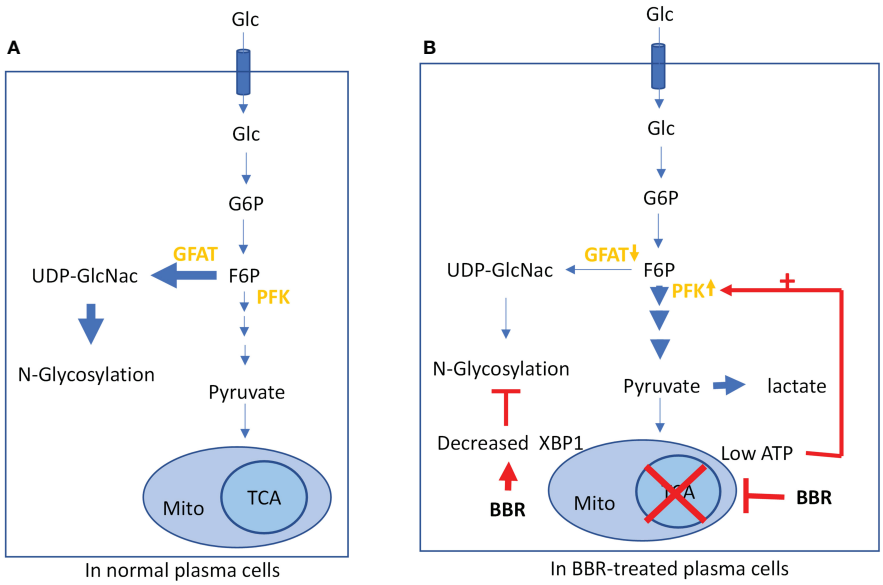

FIGURE 7
Role of BBR in regulating energy consumption: **(A)** Glucose metabolism in normal plasma cell to produce ATP *via* TCA cycle. **(B)** BBR treatment decreases mitochondrial respiration, suppresses XBP1, and inhibits glycosylation.

we further are working to develop a technology to encapsulate BBR with nano particle to prevent degradation and enhance absorption in the gastric tract. In future, we intend to investigate nano-BBR intervention on transcription factors and mitochondrial metabolism in murine PN allergic models. Furthermore, in order to conform the direct evidence of BBR effect on IgE glycosylation, glycoproteomic analyses should be considered in future.

In conclusion, this study demonstrated that a novel botanical medicine, EBF-2, significantly and consistently suppressed IgE production and is markedly more potent than its parent formula FAHF-2. EBF-2 significantly suppressed PN specific IgE production with complete protection against anaphylaxis and long-lasting effects associated with suppression of IgE^{+}PCs in a murine model. The mechanism of BBR, the active EBF-2 compound in suppressing IgE may be partially associated with its inhibitory effect on XBP1 and mitochondrial metabolism leading to insufficient energy and transcriptional activation for IgE IgH/IgL synthesis and antibody glycosylation. Further understanding of how EBF-2 and BBR regulate established IgE production by IgE^{+} PCs may lead to new interventions to target key mechanisms of IgE-mediated food allergy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Icahn School of Medicine at Mount Sinai.

Author contributions

NY, ARM, KS, MK, ZW, IM, YS, OF, and YG were significantly involved in conducting experiments, data analysis, and manuscript preparation. JW, DC, DD, and HS were significantly involved in manuscript revision. JZ, MM, and X-ML were significantly involved in study design, data interpretation, and manuscript revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

X-ML received research support to her institution from the National Institutes of Health (NIH)/National Center for Complementary and Alternative Medicine (NCCAM) # 1P01AT002644725-01"Center for Chinese Herbal Therapy (CHT) for Asthma", and grant #1R01AT001495-01A1 and 2R01 AT001495-05A2, NIH/NIAID R43AI148039, Food Allergy Research and Education (FARE), Winston Wolkoff Integrative Medicine Fund for Allergies and Wellness, the Parker Foundation and Henan University of Chinese Medicine; received consultancy fees from FARE and Johnson & Johnson Pharmaceutical Research & Development, L.L.C. Bayer Global Health LLC; received royalties from UpToDate; is an Honorary Professor of Chinese Medical University, Taichung, Taiwan; Henan University of Chinese Medicine Zhengzhou, China, and Professorial Lecture at Icahn School of Medicine at Mount Sinai, New York, NY, US; received travel expenses from the NCCAM and FARE; share US patent US7820175B2 (FAHF-2), US10500169B2 (XPP), US10406191B2 (S. Flavescens), US10028985B2 (WL); US11351157B2 (nanoBBR): take compensation from her practice at Center for Integrative Health and Acupuncture PC; US Times Technology Inc is managed by her related party; is a member of General Nutraceutical Technology LLC and Health Freedom LLC. NY received research support from the National Institutes of Health (NIH)/National Center for Complementary and Alternative Medicine (NCCAM), NIH/NIAID R43AI148039, shares US patent: US10500169B2 (XPP), US10406191B2 (S. Flavescens), US10028985B2 (WL); and is a member of General Nutraceutical Technology, LLC, and Health Freedom LLC; receives a salary from General Nutraceutical Technology, LLC. HS shares US patents: US7820175B2 (FAHF-2) and US10406191B2 (S. Flavescens), US10028985B2 (WL); is a medical consultant for General Nutraceutical Technology, LLC. KS shares US patent: US11351157B2 (nanoBBR) and received a salary from General Nutraceutical Technology LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1081121/full#supplementary-material

References

1. Cianferoni A, Muraro A. Food-induced anaphylaxis. *Immunol Allergy Clinics North America.* (2012) 32(1):165–95. doi: 10.1016/j.iac.2011.10.002

2. Gupta RS, Warren CM, Smith BM, Jiang J, Blumenstock JA, Davis MM, et al. Prevalence and severity of food allergies among US adults. *JAMA Netw Open* (2019) 2(1): e185630. doi: 10.1001/jamanetworkopen.2018.5630

3. Sasaki M, Koplin JJ, Dharmage SC, Field MJ, Sawyer SM, McWilliam V, et al. Prevalence of clinic-defined food allergy in early adolescence: The SchoolNuts study. *J Allergy Clin Immunol* (2018) 141(1):391–8.e4. doi: 10.1016/j.jaci.2017.05.041

4. Dunlop JH, Keet CA. Epidemiology of food allergy. *Immunol Allergy Clinics North America.* (2018) 38(1):13–25. doi: 10.1016/j.iac.2017.09.002

5. Gupta RS, Springston EE, Warrier MR, Smith B, Kumar R, Pongracic J, et al. The prevalence, severity, and distribution of childhood food allergy in the united states. *Pediatrics.jt/>* (2011) 128(1):e9–17. doi: 10.1542/peds.2011-0204

6. Bunyavanich S, Rifas-Shiman SL, Platts-Mills TA, Workman L, Sordillo JE, Gillman MW, et al. Peanut allergy prevalence among school-age children in a US cohort not selected for any disease. *J Allergy Clin Immunol* (2014) 134(3):753–5. doi: 10.1016/j.jaci.2014.05.050

7. Gupta R, Holdford D, Bilaver L, Dyer A, Holl JL, Meltzer D. The economic impact of childhood food allergy in the united states. *JAMA pediatrics.* (2013) 167(11):1026–31. doi: 10.1001/jamapediatrics.2013.2376

8. Gupta R, Holdford D, Bilaver L, Dyer A, Meltzer D. The high economic burden of childhood food allergy in the united states. *J Allergy Clin Immunol* (2013) 131(2):AB223. doi: 10.1016/j.jaci.2012.12.1464

9. FARE. Food allergy research and education. In: *Facts and statistics.* Available at: https://www.foodallergy.org/resources/facts-and-statistics.

10. Motosue MS, Bellolio MF, Van Houten HK, Shah ND, Campbell RL. National trends in emergency department visits and hospitalizations for food-induced anaphylaxis in US children. *Pediatr Allergy Immunol Off Publ Eur Soc Pediatr Allergy Immunol* (2018) 29(5):538–44. doi: 10.1111/pai.12908

11. Savage J, Sicherer S, Wood R. The natural history of food allergy. *J Allergy Clin Immunol In practice.* (2016) 4(2):196–203. doi: 10.1016/j.jaip.2015.11.024

12. Brough HA, Caubet JC, Mazon A, Haddad D, Bergmann MM, Wassenberg J, et al. Defining challenge-proven coexistent nut and sesame seed allergy: A prospective multicenter European study. *J Allergy Clin Immunol* (2020) 145(4):1231–9. doi: 10.1016/j.jaci.2019.09.036

13. Warren CM, Jiang J, Gupta RS. Epidemiology and burden of food allergy. *Curr Allergy Asthma Rep* (2020) 20(2):6. doi: 10.1007/s11882-020-0898-7

14. Wang J, Sampson HA. Food anaphylaxis. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* (2007) 37(5):651–60. doi: 10.1111/j.1365-2222.2007.02682.x

15. *FDA Encourages manufacturers to clearly declare all uses of sesame in ingredient list on food labels*. U.S. Food and Drug Administration (2020). Available at: https://www.fda.gov/news-events/press-announcements/fda-encourages-manufacturers-clearly-declare-all-uses-sesame-ingredient-list-food-labels.

16. Chooniedass R, Temple B, Martin D, Becker A. A qualitative study exploring parents' experiences with epinephrine use for their child's anaphylactic reaction. *Clin Trans Allergy* (2018) 8:43. doi: 10.1186/s13601-018-0230-y

17. Turner PJ, DunnGalvin A, Hourihane JO. The emperor has no symptoms: The risks of a blanket approach to using epinephrine autoinjectors for all allergic reactions. *J Allergy Clin Immunol In practice.* (2016) 4(6):1143–6. doi: 10.1016/j.jaip.2016.05.005

18. Yamamoto-Hanada K, Futamura M, Takahashi O, Narita M, Kobayashi F, Ohya Y. Caregivers of children with no food allergy–their experiences and perception of food allergy. *Pediatr Allergy Immunol Off Publ Eur Soc Pediatr Allergy Immunol* (2015) 26 (7):614–7. doi: 10.1111/pai.12442

19. Leftwich J, Barnett J, Muncer K, Shepherd R, Raats MM, Hazel Gowland M, et al. The challenges for nut-allergic consumers of eating out. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* (2011) 41(2):243–9. doi: 10.1111/j.1365-2222.2010.03649.x

20. Patel N, Herbert L, Green TD. The emotional, social, and financial burden of food allergies on children and their families. *Allergy Asthma Proc* (2017) 38(2):88–91. doi: 10.2500/aap.2017.38.4028

21. Polloni L, DunnGalvin A, Ferruzza E, Bonaguro R, Lazzarotto F, Toniolo A, et al. Coping strategies, alexithymia and anxiety in young patients with food allergy. *Allergy.jt/>* (2017) 72(7):1054–60. doi: 10.1111/all.13097

22. King RM, Knibb RC, Hourihane JO. Impact of peanut allergy on quality of life, stress and anxiety in the family. *Allergy.jt/>* (2009) 64(3):461–8. doi: 10.1111/j.1398-9995.2008.01843.x

23. Iweala OI, Burks AW. Food allergy: Our evolving understanding of its pathogenesis, prevention, and treatment. *Curr Allergy Asthma Rep* (2016) 16(5):37. doi: 10.1007/s11882-016-0616-7

24. Luger EO, Fokuhl V, Wegmann M, Abram M, Tillack K, Achatz G, et al. Induction of long-lived allergen-specific plasma cells by mucosal allergen challenge. *J Allergy Clin Immunol* (2009) 124(4):819–26.e4. doi: 10.1016/j.jaci.2009.06.047

25. Luger EO, Wegmann M, Achatz G, Worm M, Renz H, Radbruch A. Allergy for a lifetime? *Allergology Int Off J Japanese Soc Allergology* (2010) 59(1):1–8. doi: 10.2332/allergolint.10-RAI-0175

26. He JS, Narayanan S, Subramaniam S, Ho WQ, Lafaille JJ, Curotto de Lafaille MA. Biology of IgE production: IgE cell differentiation and the memory of IgE responses. *Curr topics Microbiol Immunol* (2015) 388:1–19. doi: 10.1007/978-3-319-13725-4_1

27. Wood RA. Food allergen immunotherapy: Current status and prospects for the future. *J Allergy Clin Immunol* (2016) 137(4):973–82. doi: 10.1016/j.jaci.2016.01.001

28. Varshney P, Steele PH, Vickery BP, Bird JA, Thyagarajan A, Scurlock AM, et al. Adverse reactions during peanut oral immunotherapy home dosing. *J Allergy Clin Immunol* (2009) 124(6):1351–2. doi: 10.1016/j.jaci.2009.09.042

29. Vazquez-Ortiz M, Turner PJ. Improving the safety of oral immunotherapy for food allergy. *Pediatr Allergy Immunol Off Publ Eur Soc Pediatr Allergy Immunol* (2015) 27 (2):117–25. doi: 10.1111/pai.12510

30. Virkud YV, Burks AW, Steele PH, Edwards LJ, Berglund JP, Jones SM, et al. Novel baseline predictors of adverse events during oral immunotherapy in children with peanut allergy. *J Allergy Clin Immunol* (2017) 139(3):882–8.e5. doi: 10.1016/j.jaci.2016.07.030

31. Cildag S, Senturk T. The effect of omalizumab treatment on IgE and other immunoglobulin levels in patients with chronic spontaneous urticaria and its association with treatment response. *Postepy dermatologii i alergologii.* (2018) 35 (5):516–9. doi: 10.5114/ada.2017.71422

32. Gonul M, Ozenergun Bittaci A, Ergin C. Omalizumab-induced triphasic anaphylaxis in a patient with chronic spontaneous urticaria. *J Eur Acad Dermatol Venereology JEADV.* (2016) 30(11):e135–e6. doi: 10.1111/jdv.13439

33. O'Neill LA, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol* (2016) 16(9):553–65. doi: 10.1038/nri.2016.70

34. Simmen T, Lynes EM, Gesson K, Thomas G. Oxidative protein folding in the endoplasmic reticulum: tight links to the mitochondria-associated membrane (MAM). *Biochim Biophys Acta* (2010) 1798(8):1465–73. doi: 10.1016/j.bbamem.2010.04.009

35. Arnold JN, Radcliffe CM, Wormald MR, Royle L, Harvey DJ, Crispin M, et al. The glycosylation of human serum IgD and IgE and the accessibility of identified oligomannose structures for interaction with mannan-binding lectin. *J Immunol* (2004) 173(11):6831–40. doi: 10.4049/jimmunol.173.11.6831

36. Wu G, Hitchen PG, Panico M, North SJ, Barbouche MR, Binet D, et al. Glycoproteomic studies of IgE from a novel hyper IgE syndrome linked to PGM3 mutation. *Glycoconjugate J* (2016) 33(3):447–56. doi: 10.1007/s10719-015-9638-y

37. Zauner G, Selman MH, Bondt A, Rombouts Y, Blank D, Deelder AM, et al. Glycoproteomic analysis of antibodies. *Mol Cell Proteomics MCP.* (2013) 12(4):856–65. doi: 10.1074/mcp.R112.026005

38. Schroeder HWJr., Cavacini L. Structure and function of immunoglobulins. *J Allergy Clin Immunol* (2010) 125(2 Suppl 2):S41–52. doi: 10.1016/j.jaci.2009.09.046

39. Hickman S, Kulczycki AJr., Lynch RG, Kornfeld S. Studies of the mechanism of tunicamycin in hibition of IgA and IgE secretion by plasma cells. *J Biol Chem* (1977) 252 (12):4402–8. doi: 10.1016/S0021-9258(17)40279-1

40. Sidman C. Differing requirements for glycosylation in the secretion of related glycoproteins is determined neither by the producing cell nor by the relative number of oligosaccharide units. *J Biol Chem* (1981) 256(18):9374–6. doi: 10.1016/S0021-9258(19) 68766-1

41. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell.jt/>* (2001) 107(7):881–91. doi: 10.1016/S0092-8674(01)00611-0

42. Taubenheim N, Tarlinton DM, Crawford S, Corcoran LM, Hodgkin PD, Nutt SL. High rate of antibody secretion is not integral to plasma cell differentiation as revealed by XBP-1 deficiency. *J Immunol* (2012) 189(7):3328–38. doi: 10.4049/jimmunol.1201042

43. Todd DJ, McHeyzer-Williams LJ, Kowal C, Lee AH, Volpe BT, Diamond B, et al. XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory b cell development. *J Exp Med* (2009) 206(10):2151–9. doi: 10.1084/jem.20090738

44. Lam WY, Bhattacharya D. Metabolic links between plasma cell survival, secretion, and stress. *Trends Immunol* (2018) 39(1):19–27. doi: 10.1016/j.it.2017.08.007

45. Tellier J, Shi W, Minnich M, Liao Y, Crawford S, Smyth GK, et al. Blimp-1 controls plasma cell function through the regulation of immunoglobulin secretion and the unfolded protein response. *Nat Immunol* (2016) 17(3):323–30. doi: 10.1038/ni.3348

46. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, et al. Plasma cell differentiation requires the transcription factor XBP-1. *Nature.jt/>* (2001) 412(6844):300–7. doi: 10.1038/35085509

47. Hu CC, Dougan SK, McGehee AM, Love JC, Ploegh HL. XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in b cells. *EMBO J* (2009) 28(11):1624–36. doi: 10.1038/emboj.2009.117

48. Iwakoshi NN, Lee AH, Glimcher LH. The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response. *Immunol Rev* (2003) 194:29–38. doi: 10.1034/j.1600-065X.2003.00057.x

49. Li XM, Zhang TF, Huang CK, Srivastava K, Teper AA, Zhang L, et al. Food allergy herbal formula-1 (FAHF-1) blocks peanut-induced anaphylaxis in a murine model. *J Allergy Clin Immunol* (2001) 108(4):639–46. doi: 10.1067/mai.2001.118787

50. Srivastava KD, Bardina L, Sampson HA, Li XM. Efficacy and immunological actions of FAHF-2 in a murine model of multiple food allergies. *Ann allergy Asthma Immunol Off Publ Am Coll Allergy Asthma Immunol* (2012) 108(5):351–8.e1. doi: 10.1016/j.anai.2012.03.008

51. Srivastava KD, Song Y, Yang N, Liu C, Goldberg IE, Nowak-Wegrzyn A, et al. B-FAHF-2 plus oral immunotherapy (OIT) is safer and more effective than OIT alone in a murine model of concurrent peanut/tree nut allergy. *Clin Exp Allergy* (2017) 47(8):1038–49. doi: 10.1111/cea.12936

52. Srivastava K, Yang N, Chen Y, Lopez-Exposito I, Song Y, Goldfarb J, et al. Efficacy, safety and immunological actions of butanol-extracted food allergy herbal formula-2 on peanut anaphylaxis. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* (2011) 41(4):582–91. doi: 10.1111/j.1365-2222.2010.03643.x

53. Kattan JD, Srivastava KD, Zou ZM, Goldfarb J, Sampson HA, Li XM. Pharmacological and immunological effects of individual herbs in the food allergy herbal formula-2 (FAHF-2) on peanut allergy. *Phytother Res* (2008) 22(5):651–9. doi: 10.1002/ptr.2357

54. Wang J, Patil SP, Yang N, Ko J, Lee J, Noone S, et al. Safety, tolerability, and immunologic effects of a food allergy herbal formula in food allergic individuals: a randomized, double-blinded, placebo-controlled, dose escalation, phase 1 study. *Ann Allergy Asthma Immunol* (2010) 105(1):75–84. doi: 10.1016/j.anai.2010.05.005

55. Yang N, Wang J, Liu C, Song Y, Zhang S, Zi J, et al. Berberine and limonin suppress IgE production by human b cells and peripheral blood mononuclear cells from food-allergic patients. *Ann allergy Asthma Immunol Off Publ Am Coll Allergy Asthma Immunol* (2014) 113(5):556–64.e4. doi: 10.1016/j.anai.2014.07.021

56. Lopez-Exposito I, Castillo A, Yang N, Liang B, Li XM. Chinese Herbal extracts of rubia cordifolia and dianthus superbus suppress IgE production and prevent peanut-induced anaphylaxis. *Chin Med* (2011) 6:35. doi: 10.1186/1749-8546-6-35

57. National Research Council (US) Institute for Laboratory Animal Research. *Guide for the Care and Use of Laboratory Animals*. Washington (DC): National Academies Press (US) (1996). doi: 10.17226/5140

58. Qu C, Srivastava K, Ko J, Zhang TF, Sampson HA, Li XM. Induction of tolerance after establishment of peanut allergy by the food allergy herbal formula-2 is associated with up-regulation of interferon-gamma. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* (2007) 37(6):846–55. doi: 10.1111/j.1365-2222.2007.02718.x

59. Srivastava KD, Siefert A, Fahmy TM, Caplan MJ, Li XM, Sampson HA. Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. *J Allergy Clin Immunol* (2016) 138(2):536–543. doi: 10.1016/j.jaci.2014.12.1701

60. Barnes NA, Stephenson SJ, Tooze RM, Doody GM. Amino acid deprivation links BLIMP-1 to the immunomodulatory enzyme indoleamine 2,3-dioxygenase. *J Immunol* (2009) 183(9):5768–77. doi: 10.4049/jimmunol.0803480

61. Scheeren FA, Naspetti M, Diehl S, Schotte R, Nagasawa M, Wijnands E, et al. STAT5 regulates the self-renewal capacity and differentiation of human memory b cells and controls bcl-6 expression. *Nat Immunol* (2005) 6(3):303–13. doi: 10.1038/ni1172

62. Diehl SA, Schmidlin H, Nagasawa M, van Haren SD, Kwakkenbos MJ, Yasuda E, et al. STAT3-mediated up-regulation of BLIMP1 is coordinated with BCL6 down-regulation to control human plasma cell differentiation. *J Immunol* (2008) 180(7):4805–15. doi: 10.4049/jimmunol.180.7.4805

63. Tellier J, Nutt SL. Plasma cells: The programming of an antibody-secreting machine. *Eur J Immunol* (2019) 49(1):30–7. doi: 10.1002/eji.201847517

64. Chang W, Chen L, Hatch GM. Berberine as a therapy for type 2 diabetes and its complications: From mechanism of action to clinical studies. *Biochem Cell Biol* (2015) 93 (5):479–86. doi: 10.1139/bcb-2014-0107

65. Lan J, Zhao Y, Dong F, Yan Z, Zheng W, Fan J, et al. Meta-analysis of the effect and safety of berberine in the treatment of type 2 diabetes mellitus, hyperlipemia and hypertension. *J ethnopharmacology.* (2015) 161:69–81. doi: 10.1016/j.jep.2014.09.049

66. Yin J, Gao Z, Liu D, Liu Z, Ye J. Berberine improves glucose metabolism through induction of glycolysis. *Am J Physiol Endocrinol Metab* (2008) 294(1):E148–56. doi: 10.1152/ajpendo.00211.2007

67. Turner N, Li JY, Gosby A, To SW, Cheng Z, Miyoshi H, et al. Berberine and its more biologically available derivative, dihydroberberine, inhibit mitochondrial respiratory complex I: a mechanism for the action of berberine to activate AMP-activated protein kinase and improve insulin action. *Diabetes.jt/>* (2008) 57(5):1414–8. doi: 10.2337/db07-1552

68. Song Y, Qu C, Srivastava K, Yang N, Busse P, Zhao W, et al. Food allergy herbal formula 2 protection against peanut anaphylactic reaction is *via* inhibition of mast cells and basophils. *J Allergy Clin Immunol* (2010) 126(6):1208–17.e3. doi: 10.1016/j.jaci.2010.09.013

69. Lam WY, Jash A, Yao CH, D'Souza L, Wong R, Nunley RM, et al. Metabolic and transcriptional modules independently diversify plasma cell lifespan and function. *Cell Rep* (2018) 24(9):2479–92.e6. doi: 10.1016/j.celrep.2018.07.084

70. Hammarlund E, Thomas A, Amanna IJ, Holden LA, Slayden OD, Park B, et al. Plasma cell survival in the absence of b cell memory. *Nat Commun* (2017) 8(1):1781. doi: 10.1038/s41467-017-01901-w

71. Messner B, Stütz AM, Albrecht B, Peiritsch S, Woisetschläger M. Cooperation of binding sites for STAT6 and NF kappa b/rel in the IL-4-induced up-regulation of the human IgE germline promoter. *J Immunol* (1997) 159(7):3330–7. doi: 10.4049/jimmunol.159.7.3330

72. Harris MB, Chang C-C, Berton MT, Danial NN, Zhang J, Kuehner D, et al. Transcriptional repression of Stat6-dependent interleukin-4-Induced genes by BCL-6: Specific regulation of iε transcription and immunoglobulin e switching. *Mol Cell Biol* (1999) 19(10):7264–75. doi: 10.1128/MCB.19.10.7264

73. Lam WY, Becker AM, Kennerly KM, Wong R, Curtis JD, Llufrio EM, et al. Mitochondrial pyruvate import promotes long-term survival of antibody-secreting plasma cells. *Immunity.jt/>* (2016) 45(1):60–73. doi: 10.1016/j.immuni.2016.06.011

74. Yin J, Xing H, Ye J. Efficacy of berberine in patients with type 2 diabetes mellitus. *Metabolism: Clin experimental.* (2008) 57(5):712–7. doi: 10.1016/j.metabol.2008.01.013

75. Wang J, Guo X, Chen C, Sun S, Liu G, Liu M, et al. Gender differences in food allergy depend on the PPAR γ/NF-κB in the intestines of mice. *Life Sci* (2021) 278:119606. doi: 10.1016/j.lfs.2021.119606

76. Snider DP, Marshall JS, Perdue MH, Liang H. Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin. *J Immunol* (1994) 153(2):647–57. doi: 10.4049/jimmunol.153.2.647

77. Schülke S, Albrecht M. Mouse models for food allergies: Where do we stand? *Cells.jt/>* (2019) 8(6):546. doi: 10.3390/cells8060546

78. Kanagaratham C, Sallis BF, Fiebiger E. Experimental models for studying food allergy. *Cell Mol Gastroenterol hepatology.* (2018) 6(3):356–69.e1. doi: 10.1016/j.jcmgh.2018.05.010

79. Ptaschinski C, Rasky AJ, Fonseca W, Lukacs NW. Stem cell factor neutralization protects from severe anaphylaxis in a murine model of food allergy. *Front Immunol* (2021) 12:604192. doi: 10.3389/fimmu.2021.604192

80. Samadi N, Heiden D, Klems M, Salzmann M, Rohrhofer J, Weidmann E, et al. Gastric enzyme supplementation inhibits food allergy in a BALB/c mouse model. *Nutrients* (2021) 13(3):738. doi: 10.3390/nu13030738

81. Gu Y, Guo X, Sun S, Che H. High-fat diet-induced obesity aggravates food allergy by intestinal barrier destruction and inflammation. *Int Arch Allergy Immunol* (2022) 183 (1):80–92. doi: 10.1159/000517866

82. Kinney SR, Carlson L, Ser-Dolansky J, Thompson C, Shah S, Gambrah A, et al. Curcumin ingestion inhibits mastocytosis and suppresses intestinal anaphylaxis in a murine model of food allergy. *PloS One* (2015) 10(7):e0132467. doi: 10.1371/journal.pone.0132467

83. Jensen-Jarolim E, Pali-Scholl I, Roth-Walter F. Outstanding animal studies in allergy II. from atopic barrier and microbiome to allergen-specific immunotherapy. *Curr Opin Allergy Clin Immunol* (2017) 17(3):180–7. doi: 10.1097/ACI.0000000000000364

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Staphylococcus aureus δ-toxin present on skin promotes the development of food allergy in a murine model

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Background: Patients with food allergy often suffer from atopic dermatitis, in which *Staphylococcus aureus* colonization is frequently observed. *Staphylococcus aureus* δ-toxin activates mast cells and promotes T helper 2 type skin inflammation in the tape-stripped murine skin. However, the physiological effects of δ-toxin present on the steady-state skin remain unknown. We aimed to investigate whether δ-toxin present on the steady-state skin impacts the development of food allergy.

Material and methods: The non-tape-stripped skins of wild-type, *Kit*$^{W\text{-}sh/W\text{-}sh}$, or ST2-deficient mice were treated with ovalbumin (OVA) with or without δ-toxin before intragastric administration of OVA. The frequency of diarrhea, numbers of jejunum or skin mast cells, and serum levels of OVA-specific IgE were measured. Conventional dendritic cell 2 (cDC2) in skin and lymph nodes (LN) were analyzed. The cytokine levels in the skin tissues or culture supernatants of δ-toxin-stimulated murine keratinocytes were measured. Anti-IL-1α antibody-pretreated mice were analyzed.

Results: Stimulation with δ-toxin induced the release of IL-1α, but not IL-33, in murine keratinocytes. Epicutaneous treatment with OVA and δ-toxin induced the local production of IL-1α. This treatment induced the translocation of OVA-loaded cDC2 from skin to draining LN and OVA-specific IgE production, independently of mast cells and ST2. This resulted in OVA-administered food allergic responses. In these models, pretreatment with anti-IL-1α antibody inhibited the cDC2 activation and OVA-specific IgE production, thereby dampening food allergic responses.

Conclusion: Even without tape stripping, δ-toxin present on skin enhances epicutaneous sensitization to food allergen in an IL-1α-dependent manner, thereby promoting the development of food allergy.

KEYWORDS

food allergy, epicutaneous sensitization, murine model, IgE, *Staphylococcus aureus* δ-toxin, IL-1α

Introduction

The prevalence of food allergy is increasing, particularly in western countries, thereby poising a critical public health problem. Generally, the sensitization to food allergen via several routes, accompanied by the production of food allergen-specific immunoglobulin E (IgE) antibody (Ab), plays an important role in the development of food allergy, which is caused by the oral intake of the same allergen. The occurrence of food allergy is characterized by diarrhea and anaphylaxis in severe cases. These symptoms are caused by chemical mediators mainly released from mast cells, which are activated by the engagement of high affinity IgE receptor (FcεRI) on their surfaces with food allergen and its specific IgE (1–6). Recent advances highlight that epicutaneous sensitization to food allergen is important in developing food allergy (7–11). Food allergy is associated with atopic dermatitis, in which skin barrier dysfunctions are critical pathogenic factors (1–3, 12, 13). Further, the skin of patients with atopic dermatitis is frequently colonized by *Staphylococcus aureus* (*S. aureus*), which is known to produce several exotoxins. Among them, δ-toxin (also called phenol-soluble modulin (PSM)-γ), which belongs to the peptide toxin family of PSM, directly activates mast cells and promotes T helper 2 (Th2) type skin inflammation with increased IgE production (12–14). The specific receptor of δ-toxin and its function in immune cells are not completely understood (12). PSMα peptides (PSMα1-4) are highly cytotoxic to a variety of cells, while other PSM, including δ-toxin, exhibits limited cytotoxic activity (12–14). In murine model of epicutaneous infection of S. aureus infection, PSMα induces the release of interleukin (IL)-1α and IL-36α that orchestrate IL-17-dependent skin inflammation (15, 16). Furthermore, IL-36α also directly stimulates B cells to enhance IgE production (17). In the skins of patients with atopic dermatitis, expression of IL-1α, IL-1β, IL-18, IL-33, and IL-36α, which belong to IL-1 family, is known to be upregulated in keratinocytes (18–21). However, the context-dependent functions of these cytokines in epicutaneous sensitization is not yet clear.

Mouse models have been used to study food allergy induced by oral challenges with ovalbumin (OVA) following intraperitoneal sensitization with OVA plus alum adjuvant. Mice with food allergy exhibited high serum levels of OVA-specific IgE and mast cell protease 1 (MCPT1), which is a mucosal mast cell activation marker, and intestinal mast cell hyperplasia. In these models, mast cells and OVA-specific IgE are indispensable for the induction of food allergy (4–6). Alternatively, several models of food allergy in mice after epicutaneous sensitization have been developed recently (8–11). Epicutaneous treatment with OVA on the tape-stripped skins of mice induces Th2/T follicular helper (Tfh) responses through several mechanisms. These mechanisms involve epithelial cell-derived cytokines such as thymic stromal lymphopoietin (TSLP) and IL-33 and skin immune cells such as conventional dendritic cell 2 (cDC2). Among several antigen-presenting cells, cDC2 is justified as the most prominent in inducing Th2 responses. Under inflammatory conditions, cDC2 uptakes food allergen in the dermis and moves to the draining lymph node (LN), where it presents the food antigens to naïve $CD4^+$ T cells to induce Th2/Tfh responses (22–26). Recent reports have shown that tape-stripping alone causes the release of IL-33 in skin, which induces the expansion of intestinal mast cells via keratinocyte-derived IL-33-intestinal type 2 innate lymphoid cell (ILC2) axis and enhances IgE-mediated food allergic responses (7, 8).

The present study aimed to investigate whether δ-toxin of *S. aureus* is implicated in the development of food allergy following epicutaneous sensitization of steady-state skin. To remove the effects of tape stripping on immune responses and to clarify the exact roles of δ-toxin on skin with normal barrier function, we used murine model of food allergy after epicutaneous treatment of the non-tape-stripped skin.

Abbreviations: Abs, antibodies; ANOVA, analysis of variance; APC, allophycocyanin; BMMCs, bone marrow-derived mast cells; BV, Brilliant Violet; cDC2, conventional dendritic cell 2; Cy, cyanine; FcεRI, high-affinity IgE receptor; FITC, fluorescein isothiocyanate; IL, interleukin; ILC2, type 2 innate lymphoid cell, IgE, immunoglobulin E; LDH, lactate dehydrogenase; LN, lymph node; MCPT1, mast cell protease 1; OVA, ovalbumin; PBS, phosphate buffer saline; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PSM, phenol-soluble modulin; S. aureus, Staphylococcus aureus; SD, standard deviation; Tfh, T follicular helper; Th2, T helper 2; TSLP, thymic stromal lymphopoietin; WT. wild-type.

Materials and methods

Mice

We used wild-type (WT) BALB/c mice (Japan SLC, Hamamatsu, Japan) and $Kit^{W\text{-}sh/W\text{-}sh}$ and ST2-deficient mice on the BALB/c background were used (6, 27–29). All animal

experiments were approved by the ethical committee of Juntendo University (approval numbers 310050 and 310051).

Antibodies and other reagents

The following antibodies (Abs) were used: Fluorescein isothiocyanate (FITC)-anti- FcεRIα (MAR-1) (eBioscience), MHC Class II (M5/114.15.2) (BioLegend), phycoerythrin (PE)-anti-CD24 (M1/69) and CD103 (2E7) (BioLegend), PE-cyanin 7 (Cy7)-anti-CD64 (X54-5/7.1) and EpCAM (G8.8) (BioLegend), Allophycocyanin (APC)-c-Kit (2B8) (eBioscience), APC-Cy7-CD45 (30-F11) and Zombie (B279801) (BioLegend), Peridinin Chlorophyll Protein Complex (PerCP)-Cy5.5-anti-CD45 (30-F11) and CD11b (M1/70) (BioLegend), Streptavidinanti (BioLegend), Brilliant Violet (BV) 421-anti-CD11c (N418), and CD11b (M1/70) (BioLegend), Biotin anti-CD11c (N418), CD19 (1D3), CD3 (145-2C11), and CD11b (M1/70) (Tonbo Biosciences), Biotin anti-F4/80 (RME-1), FcεRIα (MAR-1), CD49b (DX5), and Ly-6G/Ly-6C (Gr-1) (BioLegend). Cytokines were purchased from R&D Systems. OVA (Grade V) was purchased from Sigma. Alexa Fluor 647-conjugated OVA (OVA-AF647) was generated by labeling OVA with Alexa Fluor^{TM} 647-NHS Ester (Thermo Fisher Scientific) following manufacturer's instructions. The toxins δ-toxin (MAQDIISTIGDLVKWIIDTVNKFTKK) and PSMα3 (MEFVAKLFKF FKDLLGKFLG NN) were synthesized (GL Biochem, Shanghai, China).

Cells

Axillary LNs were isolated from the mice and single-cell suspensions of these LNs were prepared. Bone marrow-derived mast cells (BMMCs) were generated as previously described (6, 27). Briefly, murine BM cells were incubated in RPMI 1640 medium including 10% FCS in the presence of 10 ng/mL IL-3. Five weeks after incubation, more than 95% pure population of FcεRI^{+}c-kit^{+} mast cells (BMMCs) were generated. Alternatively, murine BM cells were incubated in RPMI 1640 medium including 10% FCS in the presence of 20 ng/mL GM-CSF for 10 days to generate CD11b^{+}CD11c^{+} BM-derived dendritic cells (BMDCs). Murine keratinocytes were isolated as described previously (30, 31). To separate the epidermis from the dermis, skins of newborn mice were treated with 5 mg/mL DISPASE II (FUJIFILM) overnight at 4 °C. The mechanically-separated epidermis was washed with phosphate-buffered saline (PBS) and incubated with CnT-ACCUTASE-100 (CELLNTEC) for 20 min at room temperature to obtain the keratinocytes. Murine keratinocytes were cultured in CnT-Prime, Epitherial Culture Medium (CELLNTEC) using 1.2 mM $CaCl_2$ on collagen-coated plates to induce keratinocyte differentiation.

Mouse model of food allergy following epicutaneous sensitization

Epicutaneous sensitization was performed as previously described, with a few modifications (7, 10, 11, 13). Female mice aged 8-10 weeks were anesthetized, and depilatory cream was applied on their abdominal skins. Thereafter, OVA (200 μg in 100 μL saline) and δ-toxin (200 μg in 100 μL saline) or 100 μL saline were applied once a week for six weeks (on days 0, 7, 14, 21, 28, and 35) to the abdominal skins of the mice that had not been subjected to tape stripping. One week after the final epicutaneous treatment, the mice were intragastrically gavaged with OVA (50 mg in 200 μL saline) every 2 d for a total seven to twelve times. Diarrhea was assessed by visually monitoring mice for up to 30 minutes after intragastric challenge of OVA. Mice excreting loose or liquid stools were recorded as mice with diarrhea. The frequency of diarrhea (%) means the percentage of the mice with diarrhea among all the mice tested (6).

ELISA measurements for cytokines, MCPT-1, IgE, and OVA-specific IgE

ELISA kits for IL-4, IL-13, IL-33, IL-1α, IL-1β, IL-25, and TSLP (R&D Systems), mast cell protease-1 (MCPT-1) (eBioscience), and high mobility group box 1 (HMGB1) (Promega) were used to measure their concentrations in serum, culture supernatants, and skin tissue homogenates. ATP levels in culture supernatants and skin tissue homogenates were measured using the CellTiter-Glo Assay (Promega) About 10 mg skin samples were minced using scissors and placed in 200 μL of PBS that contains protease inhibitor cocktail (FUJIFILM) at 4°C for 30 min. Thereafter, it was centrifuged at 12000 g at 4°C for 30 min, and the supernatants were used for cytokine detection. The concentrations of OVA-specific IgE and IgG1 were determined with luminescence ELISA by using OVA, anti-IgE Ab (R35-118) (BD Pharmingen), anti-IgG1 Ab (1070–08) (Southern Biotech), streptavidin-horseradish peroxidase (HRP), and TMB substrate solution (BD Biosciences), as previously described (6, 29).

Histology

Sections of mice jejunum were obtained approximately 10 cm from the pyloric sphincter and fixed in 10% formalin and embedded in paraffin. These paraffin-embedded sections of the jejunum and skin were stained with chloroacetate esterase for the quantification of mast cells, as previously described (6, 27, 29). Alternatively, paraffin sections of the skin were stained with Hematoxylin and Eosin (27).

Ex vivo Th2 responses

Single-cell suspensions of axillary LN cells (2×10^6) were cultured in the presence of 25 μg/mL OVA for 4 d to measure cytokines (IL-4 and IL-13) in the supernatants of the cultures suspensions (6, 29).

Real-time PCR

RNA extraction, cDNA synthesis, and real-time PCR were performed as previously described (27). The jejunum tissues were

homogenized using the tissueLyser (Qiagen), and total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the ReverTra Ace qPCR RT kit (Toyobo). Real-time PCR was performed with the Step One Plus Real-Time PCR System (Thermo Fisher Scientific) using the SYBR Green PCR Master Mix (Applied Biosystems, Life technologies). The primers used were shown (Supplementary Table 1). The mRNA expression levels were quantified with the comparative method using StepOne Software. The housekeeping gene 18S rRNA was used for normalization.

Flow cytometry

Flow cytometric analysis was performed with FACSVerse (BD Biosciences), as previously described (6, 27–29), and the obtained data were analyzed using FlowJo software (Tree Star). cDC2 were identified as $CD45^+CD11c^+$ MHC class II^+ $CD11b^+CD103^-$ $EpCAM^-$, as suggested by some recent studies (22, 32–36).

Measurements of the percentage of OVA-AF647-positive cells among cDC2 in the skin or axillary LN or among MHC Class II^{high} BMDCs

To measure the percentages of OVA-AF647-positive cells *in vivo*, depilatory cream was applied on the abdominal skins of the mice. Thereafter, OVA-AF647 (200 μg in 100 μL saline) and δ-toxin (200 μg in 100 μL saline) or 100 μL saline were applied on days 0 and 7 to the abdominal skins of the mice that had not been subjected to tape stripping. Twenty-four hours after the last epicutaneous treatment, the percentage of OVA-AF647 among skin cDC2 or axillary LN cDC2 obtained from the mice were measured using flow cytometry, as previously described (32). BMDCs were cultured in the presence of 0 or 10 ng/mL IL-1α for 12 h, and then were incubated with 0, 100, or 300 ng/mL OVA-AF647 for 1 h to measure the percentages of OVA-AF647 among $CD11b^+CD11c^+$MHC Class II^{high} BMDCs using flow cytometry.

Evaluation of cytotoxicity

The number of the non-viable cells were estimated by CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI), which is a lactate dehydrogenase (LDH) release-based assay that uses culture supernatants.

Statistical analyses

Results are expressed as means ± standard deviation (SD). Ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons was used in Figures 1–5, and Supplementary Figure 1. Welch's *t*-test was used in Figures 6, 7; Supplementary Figures 2-4. Differences were compared between groups, and $*p < 0.05$ or $**p < 0.01$ was considered statistically significant.

Results

δ-toxin present on the non-tape-stripped skin strongly induced food allergic responses following epicutaneous sensitization to food allergens in a murine model

To investigate whether an epicutaneous treatment with *S. aureus* δ-toxin on steady-state skin contributes to the development of food allergy, we used a murine model of OVA-induced food allergy. We avoided any impact of tape stripping on the immune cells of skin and small intestine. For this, Balb/c mice that had not been subjected to tape stripping were selected, and OVA was applied to their abdominal skin either with or without δ-toxin once a week for six weeks. Between days 42 and 55, these mice were intragastrically administered OVA every 2 days for a total of seven times (Figure 1A). We found that the epicutaneous treatment with OVA in the presence of δ-toxin induced more frequent diarrhea after OVA administration than that with OVA alone did (Figure 1B). In contrast, OVA administration without prior epicutaneous treatment did not induce diarrhea at all (Figure 1B). We also measured the serum levels of OVA-specific IgE and IgG1 as well as MCPT-1 in the mice after the final intragastric administration of OVA. The results revealed that epicutaneous treatment with OVA together with δ-toxin significantly increased all levels compared to those observed following treatment with OVA alone (Figures 1C–E). Histological examination revealed that the numbers of jejunum mast cells were higher in the mice subjected to epicutaneous treatment with OVA and δ-toxin than in those treated with OVA alone (Figures 1F, G). In addition, real time-PCR analysis showed that mRNA levels of the Th2 cytokine IL-4 and MCPT-1 in mouse jejunum tissues were higher in the mice epicutaneously treated with OVA and δ-toxin (Supplementary Figures 1A–D). Thus, δ-toxin present on the skin strongly induced food allergic responses with the Th2 skewing following epicutaneous treatment with OVA even without the procedure of tape stripping (12, 13).

IL-33-ST2 signaling is dispensable for OVA-challenged food allergic responses following epicutaneous treatment with OVA plus δ-toxin on the non-tape-stripped skin

To test whether the OVA-challenged food allergic responses following epicutaneous treatment with OVA plus δ-toxin on the non-tape-stripped skin depend on mast cells, we used the same model in WT and mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. The results showed that intragastric challenges with OVA caused frequent

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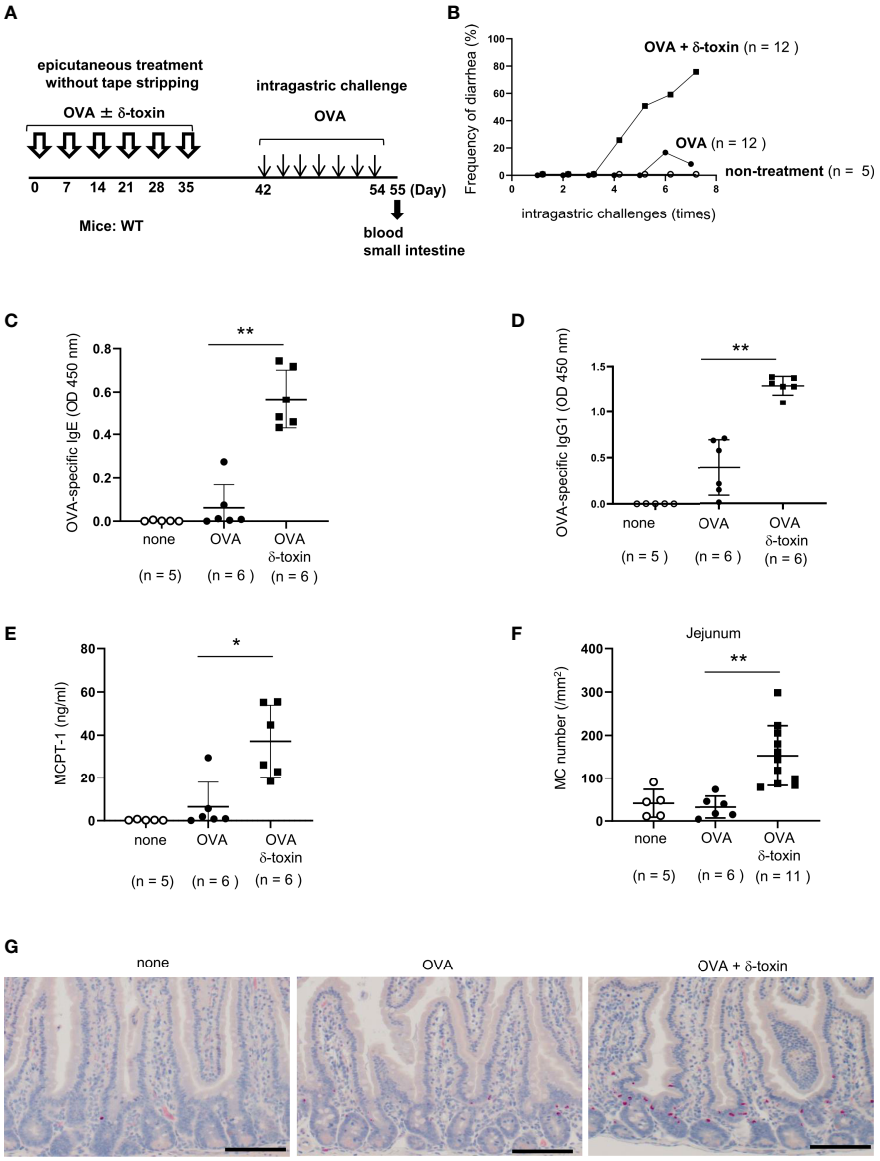

FIGURE 1
δ-toxin present on the non-tape-stripped skin strongly induced food allergic responses following epicutaneous sensitization to food allergens in a murine model. **(A)** Experimental design to investigate the occurrence of for food allergy after intragastric administration of OVA in WT mice that had been epicutaneously treated or not with OVA ± δ-toxin once a week for six weeks. Blood samples were taken, and small intestines were isolated on day 56. **(B)** Frequency of diarrhea in OVA-challenged mice after epicutaneous treatment with OVA ± δ-toxin on the non-tape-stripped skin or after non-treatment. **(C–E)** Serum levels of **(C)** OVA-specific IgE, **(D)** OVA-specific IgG1, and **(E)** MCPT-1 in the mice after the final administration of OVA. **(F)** The numbers of jejunum mast cells of the mice after the final administration of OVA. **(G)** Jejunum sections stained with chloroacetate esterase (scale bars, 100 μm). Mast cells stain red. **(B, F)** Data are pooled from two independent experiments. **(C–E)** Data are representative of two independent experiments. Means ± SD have been plotted. *, $P < 0.05$, **, $P < 0.01$.

diarrhea in WT mice, but not in $Kit^{W\text{-}sh/W\text{-}sh}$ mice, which had no detectable mast cells in the jejunum tissues (Figures 2A, C). However, the serum levels of OVA-specific IgE after final challenges with OVA were comparable between both mice (Figure 2B). Hence, intestinal mast cells are indispensable for OVA-challenged food allergic responses, but mast cells are not necessary for OVA-specific IgE production in this model.

As IL-33 plays an important role in food allergic responses following epicutaneous treatment with OVA on the tape-stripped skin, we also investigated whether ST2, a receptor for IL-33, is involved in δ-toxin-mediated food allergy in our model. We performed the same treatment in WT and ST2-deficient mice, and found that ST2 deficiency failed to influence the frequency of diarrhea after OVA administration (Figure 2D). In addition, no difference was observed in the serum levels of OVA-specific IgE and the numbers of jejunum mast cells between WT and ST2-deficient mice following the OVA challenges (Figures 2E, F). Hence, IL-33/ST2 signaling is not essential for OVA-challenged food allergic responses following epicutaneous treatment with OVA plus δ-toxin on the non-tape-stripped skin.

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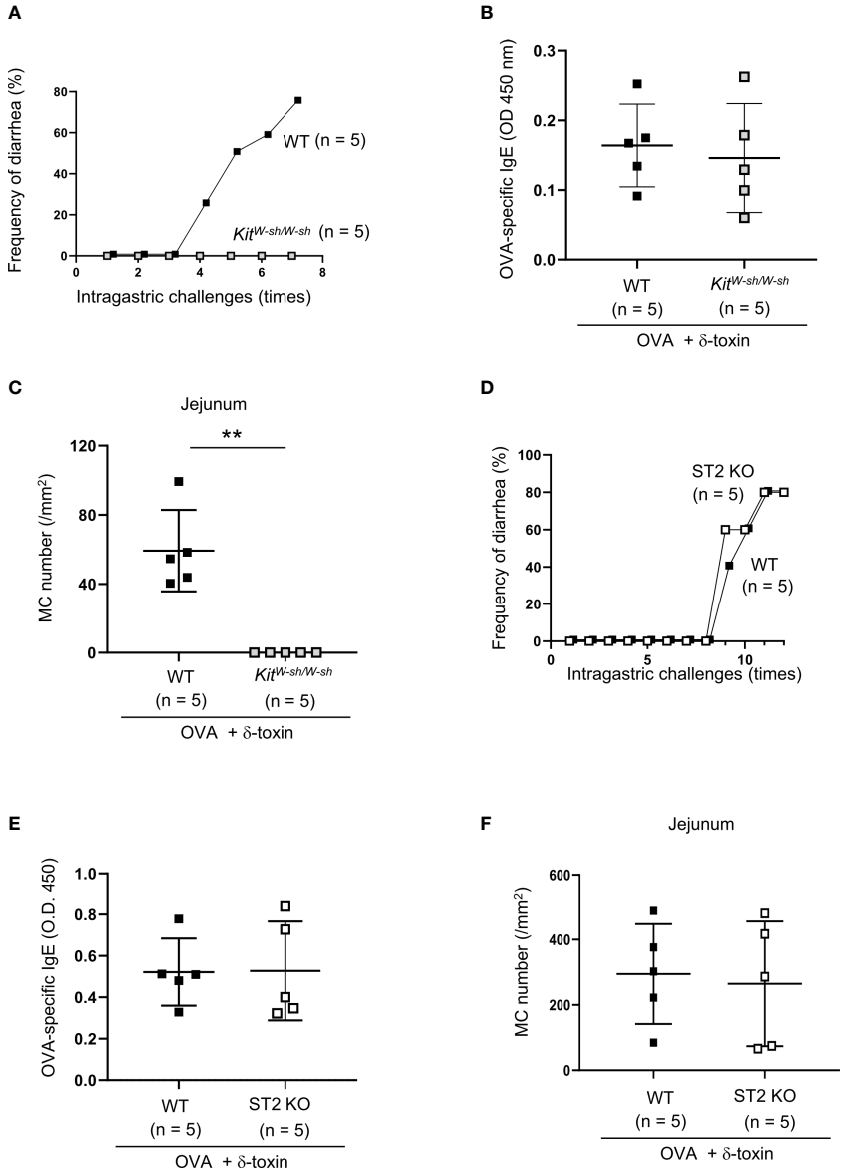

FIGURE 2
IL-33-ST2 signaling is dispensable for OVA-challenged food allergic responses after epicutaneous treatment with OVA and δ-toxin on the non-tape-stripped skin. **(A, D)** Frequency of diarrhea in OVA-administered mice after epicutaneous treatment with OVA ± δ-toxin on the non-tape-stripped skin of **(A)** WT and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and **(D)** WT and ST2 knockout (KO) mice. **(B, E)** Serum levels of OVA-specific IgE in **(B)** WT and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and **(E)** WT and ST2 KO mice after the final administration of OVA. **(C, F)** The numbers of jejunum mast cells in **(C)** WT and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and **(F)** WT and ST2 KO mice after the final administration of OVA. **(A–F)** Data are representative of two independent experiments. **(B, C, E, F)** Means ± SD have been plotted. **, $P < 0.01$.

δ-toxin present on the non-tape-stripped skin enhanced epicutaneous sensitization to food allergens in a murine model

We clarified the role of δ-toxin present on the non-tape-stripped skin by analyzing WT mice on day 41 before intragastric administration. Notably, δ-toxin remarkably increased the serum levels of OVA-specific IgE after the last epicutaneous OVA treatment even without tape-stripping (Figures 3A, B). We also confirmed that mast cell deficiency did not influence δ-toxin-mediated production of OVA-specific IgE in this model (Supplementary Figure 2). In addition, as revealed by histological examination, the mice treated epicutaneously with OVA and δ-toxin exhibited a slight increase in epidermal thickness and mast cell numbers in the skin compared to those that were treated with OVA alone (Figures 3C–E). In contrast, δ-toxin present on the non-tape-stripped skin did not significantly increase jejunum mast cell numbers (Figure 3F). Hence, *S. aureus* δ-toxin present on the non-tape-stripped skin strongly induced epicutaneous sensitization to food allergens independently of mast cells, thereby resulting in the food allergic responses after intragastric challenges with the same allergen in this model.

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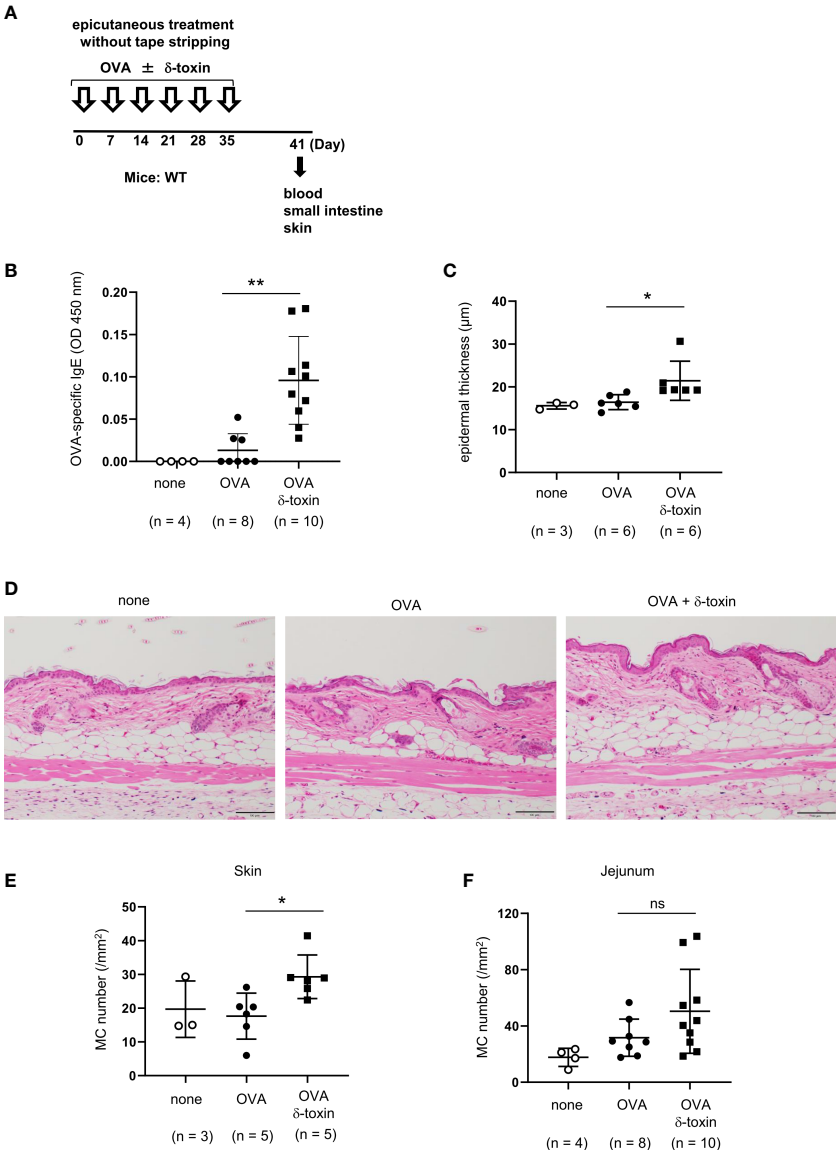

FIGURE 3
δ-toxin present on the non-tape-stripped skin enhanced epicutaneous sensitization to food allergen in a murine model. **(A)** Experimental design for epicutaneous sensitization. WT mice were epicutaneously treated or not with OVA ± δ-toxin once a week for six weeks on the non-tape-stripped abdominal skin. On day 42, blood samples were obtained, and skins and small intestines were isolated. **(B)** Serum levels of OVA-specific IgE. **(C)** The thickness of epidermis. **(D)** Skin sections stained with hematoxylin and eosin (scale bars, 100 μm). **(E, F)** The numbers of mast cells in the **(E)** skin and **(F)** jejunum. **(B, F)** Data are pooled from two independent experiments. **(C, E)** Data are representative of two independent experiments. Means ± SD have been plotted. *, $P < 0.05$, **, $P < 0.01$. ns, not significant.

δ-toxin present on the non-tape-stripped skin strongly induced the translocation of OVA-loaded cDC2 from skin to the draining LN in a murine model

We assessed whether δ-toxin influences the uptake of OVA by skin cDC2 and/or the translocation of OVA-loaded cDC2 to the draining LN. We applied OVA-AF647 with or without δ-toxin on the non-tape-stripped skin on days 0 and 7. About 24 h after the final epicutaneous treatment, we measured the percentages of AF647-positive cells among skin cDC2 and AF647-positive cDC2 numbers in axillary LN (Figure 4A). The percentages of AF647-positive cells among skin cDC2 were higher in those mice that were epicutaneusly treated with AF647-OVA and δ-toxin than in those treated with AF647-OVA alone, although the percentages of skin cDC2 among total skin cells were lower in the former mice than in the latter mice (Figure 4B; Supplementary Figure 3A). In addition, we found a significant increase in cDC2 numbers and AF647-positive cDC2 numbers as well as total cell numbers in axillary LN (Figures 4C, D; Supplementary Figure 3B). It should be noted that the deficiency of mast cells or ST2 did not influence δ-toxin-mediated increase of AF647-positive cDC2 numbers in axillary LN in the same model (Supplementary Figures 3C–E). Moreover, the concentrations of IL-4 and IL-13 were higher in the

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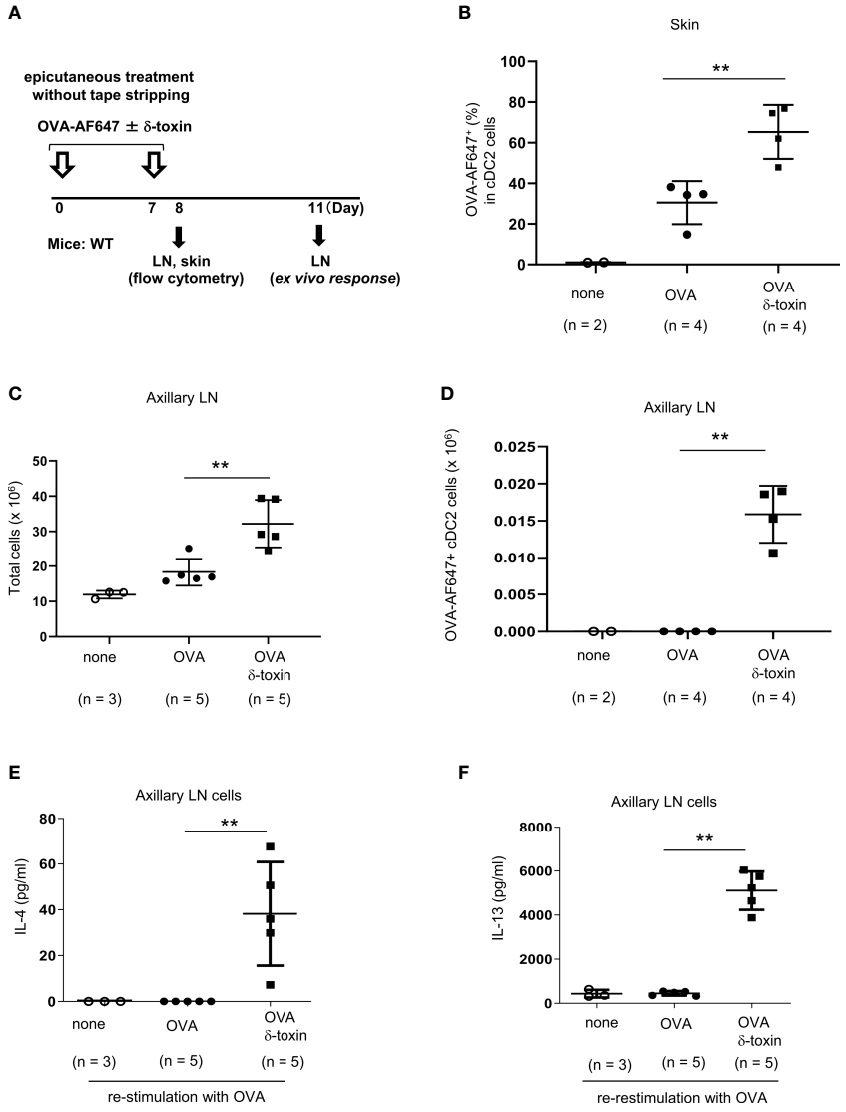

FIGURE 4
δ-toxin present on the non-tape-stripped skin strongly induced the translocation of OVA-loaded cDC2 from skin to draining LN in murine model. **(A)** Experimental design for analyzing dendritic cells in skin and axillary LNs. WT mice were epicutaneously treated or not with OVA-AF647 ± δ-toxin on the non-tape-stripped abdominal skin on days 0 and 7. Samples of skin were isolated on Day 8 and axillary LNs were isolated on Day 8 or 11. **(B)** The percentage of OVA-AF647-positive cells among skin cDC2 from the mice 24 h after the final treatment. **(C, D)**, **(C)** Total cells and **(D)** AF-647-positive cDC2 in axillary LN of mice 24 h after the final treatment. **(E, F)** Axillary LN cells purified from the mice 96 h after the final treatment were re-stimulated with 25 μg/mL OVA for 4 days. Concentrations of **(E)** IL-4 and **(F)** IL-13 in the culture supernatants of axillary LN cells. **(B–F)** Data are representative of two independent experiments. Means ± SD have been plotted. $^{**}P < 0.01$.

supernatants of OVA-restimulated axillary LN cells from the mice that were epicutaneously treated with OVA and δ-toxin compared to those in mice treated with OVA alone (Figures 4E, F). These results indicated that even without tape-stripping, epicutaneously treated δ-toxin enhanced the uptake of OVA from cDC2 in the skin, and enhanced the translocation of OVA-loaded cDC2 from skin to the draining LN, which resulted in enhanced sensitization to OVA.

To examine whether δ-toxin on skin plays a prominent role in the development of food allergy among the peptide toxin family of PSM, we compared the difference in the effects of PSMα3, which is a highly cytotoxic peptide, and δ-toxin in the same model. Analysis of OVA-loaded cDC2 in axillary LN showed that AF647-positive cDC2 numbers were significantly lower in PSMα3-treated mice than in δ-toxin-treated mice (Supplementary Figures 4A, B). Moreover, the serum levels of OVA-specific IgE were also significantly lower in PSMα3-treated mice on day 41 before OVA challenges (Supplementary Figures 4C, D). Consistently, PSMα3-treated mice exhibited less frequent diarrhea as compared with δ-toxin-treated mice (Supplementary Figure 4E). The number of jejunum mast cells after the last OVA administration tended to be lower in PSMα3-treated mice compared to that in δ-toxin-treated mice (Supplementary Figure 4F). Thus, δ-toxin present on the non-tape-stripped skin induced OVA-specific IgE production more strongly than PSMα3. This can likely be due to the enhanced translocation of OVA-loaded cDC2 from skin to the draining LN in this model.

Murine keratinocytes released IL-1α in response to stimulation with δ-toxin

As the major target cells of δ-toxin on the non-tape-stripped skin were likely keratinocytes in the epidermis, we stimulated the murine primary keratinocytes with different concentrations of δ-toxin for 2 h or 24 h. Notably, the concentrations of IL-1α in the culture supernatants increased with an increase in incubation time and δ-toxin concentration (Figure 5A). We found that keratinocytes constitutively released TSLP, whose concentrations did not increase after δ-toxin stimulation (Figure 5B). We could not detect the protein levels of IL-1β, IL-18, IL-25, or IL-33 in the culture supernatants of δ-toxin-stimulated keratinocytes. Real time PCR analysis showed that stimulation with δ-toxin slightly increased the mRNA levels of IL-1α, but it did not of alter those of IL-36α in murine keratinocytes (Figure 5C). However, mRNA levels of putative receptors for δ-toxin, including several formyl peptide receptors, were low in keratinocytes (Supplementary Figure 5A). Instead, stimulation with δ-toxin induced cell death of keratinocytes in a time- and concentration-dependent manner. However, PSMα3 showed higher cytotoxic effect on keratinocytes than δ-toxin (Figure 5D). In accordance with this, stimulation with PSMα3

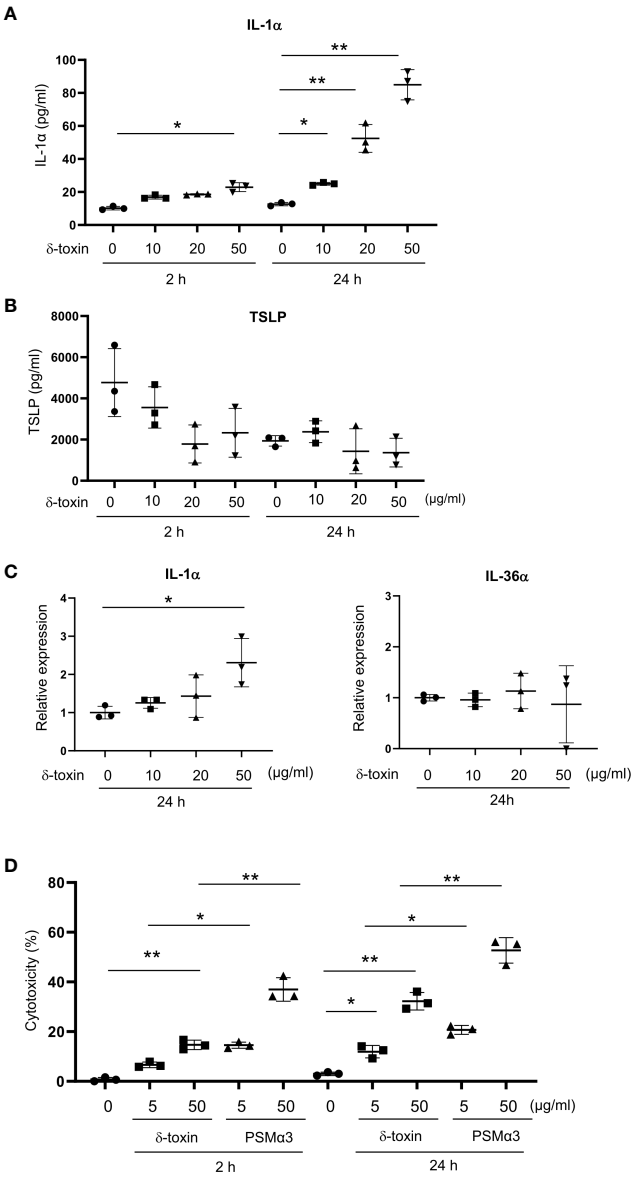

FIGURE 5
Murine keratinocytes released IL-1α in response to stimulation with δ-toxin. Murine keratinocytes were stimulated with different concentrations of δ-toxin for 2 or 24 h, as indicated. **(A, B)** Concentrations of **(A)** IL-1α and **(B)** TSLP in the culture supernatants. **(C)** Relative expression levels of mRNA of IL-1α and IL-36α in the δ-toxin-stimulated keratinocytes. **(D)** The percentage of dead cells. **(A–D)** Data are representative of three independent experiments. Means ± SD have been plotted. *$P < 0.05$ or **$P < 0.01$.

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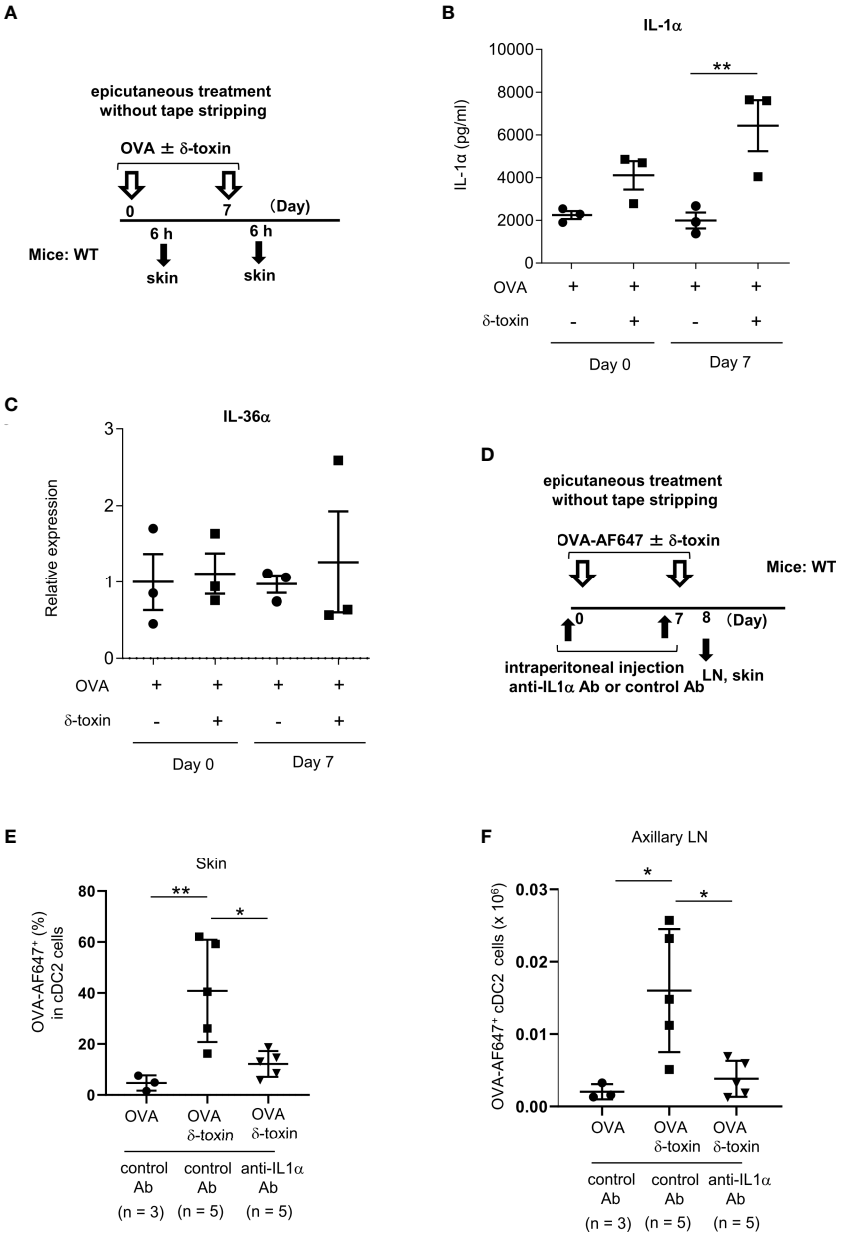

FIGURE 6
Pretreatment with anti-IL-1α Ab decreased the δ-toxin-mediated translocation of OVA-loaded cDC2 from skin to draining LN in murine model. **(A)** Experimental design for analyzing the cytokine levels in skin tissues. **(B, C)** Protein levels of IL-1α in skin tissue homogenates **(B)** and mRNA levels of IL-36α in skin tissues **(C)** obtained from the mice 6 h after the first or second epicutaneous treatment with OVA ± δ-toxin on the non-tape-stripped abdominal skin. **(D)** Experimental design for analyzing dendritic cells in skin and axillary LN. Non-tape-stripped abdominal skin of WT mice were epicutaneously treated with OVA-AF647 ± δ-toxin on days 0 and 7. The effects of intraperitoneal administration of anti-IL-1α Ab or control Ab were examined. Skins and axillary LNs were isolated on day 8. **(E)** The percentage of OVA-AF647-positive cells among skin cDC2 from the mice 24 h after the last treatment. **(F)** AF-647-positive cDC2 in axillary LN from the mice 24 h after the last treatment. **(B, C, E, F)** Data are representative of two independent experiments. Means ± SD have been plotted. *, $P < 0.05$, **, $P < 0.01$.

more strongly induced the release of damage-associated molecular patterns (DAMPs) such as IL-1α, ATP, and HMGB1 than that with δ-toxin (Supplementary Figures 5B–D). In addition, stimulation with IL-1α increased the mRNA levels of IL-1α in murine keratinocytes (Supplementary Figure 5E). Accordingly, it is possible to speculate that δ-toxin induced the release of IL-1α from keratinocytes through passive cell death, which in turn transcriptionally upregulated IL-1α in an autocrine manner.

Pretreatment with anti-IL-1α Ab decreased the δ-toxin-mediated translocation of OVA-loaded cDC2 from skin to the draining LN in a murine model

We measured the protein levels of IL-1α in skin tissues from the mice epicutaneously treated with OVA ± δ-toxin for the indicated periods (Figure 6A). The results showed that IL-1α levels in skin

tissues were higher in the mice treated with OVA plus δ-toxin than those in mice treated with OVA alone, six hours after the second epicutaneous treatment. This suggests that the presence of δ-toxin caused IL-1α production in the local skin even when the skin was not stripped using tape (Figure 6B). However, real time PCR analysis showed that mRNA levels of IL-36α were not up-regulated in δ-toxin-treated skin in the same model (Figure 6C). Notably, IL-1α levels in skin tissues were higher in the mice epicutaneously treated with OVA plus δ-toxin than in those with OVA plus PSMα3, while there was no significant difference in levels of ATP and HMGB1 between the two groups in the same model (Supplementary Figure 6). We found that pretreatment with a blocking Ab against IL-1α, but not with a control Ab, substantially reduced the percentages of AF647-positive cells among skin cDC2 and AF647-positive cDC2 numbers in axillary LN in the mice epicutaneously treated with OVA-AF647 plus δ-toxin (Figures 6D–F). In addition, stimulation with IL-1α increased the uptake of OVA-AF647 in MHC Class II^{high} BMDCs (Supplementary Figure 7). Overall, these results suggested that the δ-toxin-mediated release of IL-1α contributes to the uptake of OVA from skin cDC2 and the translocation of OVA-loaded cDC2 from skin to the draining LN, leading to an efficient sensitization to OVA in this model.

Pretreatment with anti-IL-1α Ab dampened δ-toxin-mediated, OVA-induced food allergic responses in a murine model

To investigate the role of IL-1α in food allergies mediated by δ-toxin present on the steady-state skin, we pretreated the mice with anti-IL-1α Ab or control Ab (Figure 7A). Notably, pretreatment with anti-IL-1α Ab, but not with control Ab, suppressed δ-toxin-mediated, OVA administration-induced diarrhea in our model (Figure 7B). Consistently, pretreatment with anti-IL-1α Ab strongly reduced the serum levels of OVA-specific IgE and MCPT-1 and the numbers of jejunum mast cells (Figures 7C–F) as well as the epidermal thickness (Supplementary Figure 8) in the OVA-administered mice following epicutaneous treatment with OVA plus δ-toxin. Thus, pretreatment with anti-IL-1α Ab suppressed the skin inflammation, the sensitization to food allergen, and subsequent food allergic responses in this model. We concluded that the presence of δ-toxin on the non-tape-stripped skin induced the release of IL-1α from keratinocytes, which promoted the uptake of food allergens by cDC2 in the skin and the subsequent migration of OVA-loaded cDC2 to the draining LN. This, in turn, leads to the efficient sensitization to food allergens in the development of food allergy.

Discussion

To elucidate the mechanisms underlying food allergy following epicutaneous sensitization, tape stripping prior to epicutaneous treatment with food allergen has been widely used in murine models (8–11). Tape stripping mimics mechanical skin injury caused by scratching in patients with atopic dermatitis. However, recent studies have demonstrated that tape stripping alone causes skin epithelial damage, resulting in the local release of IL-33, which stimulates intestinal mast cell hyperplasia via intestinal ILC2 (7). Moreover, tape stripping-derived IL-33 also enhances IgE-dependent food allergic responses via mast cells (8). In addition, when tape-stripped skins of mice were treated with δ-toxin or exposed to δ-toxin-producing *S. aureus*, δ-toxin induced mast cell-dependent Th2 skin inflammation with increased IgE production (12, 13). In models using tape stripping, δ-toxin may directly stimulate the degranulation of mast cells in the dermis. However, the presence of δ-toxin-producing *S. aureus* in humans does not always translate into atopic dermatitis with skin barrier disruption. In most cases, δ-toxin may be present on the normal skin with intact barrier function. In the present study, we aimed to investigate whether δ-toxin present on the steady-state skin contributes to the development of food allergy following epicutaneous sensitization. We used a murine model in which skin without tape stripping was treated with OVA in the presence or absence of δ-toxin prior to intragastrical administration of OVA. It seems therefore that this model is not always a representative of food allergy in patients with atopic dermatitis, but recapitulates food allergy in δ-toxin-producing *S. aureus*-colonized individuals who have suffered from mild atopic dermatitis or have not yet developed atopic dermatitis. Notably, repeated epicutaneous treatment with OVA and δ-toxin on the non-tape-stripped skin induced a mild epidermal thickness and OVA-specific IgE production, leading to the increase in the frequency of diarrhea and number of jejunum mast cells after intragastric OVA challenges. This was not observed in treatments with OVA alone. In addition, analysis of mast cell- and ST2-deficient mice revealed that both mast cells and ST2 are dispensable for OVA-specific IgE production in mice; however, intestinal mast cells, but not ST2, are indispensable for food allergic responses induced after OVA administration. These results indicated that δ-toxin present on the steady-state skin plays a critical role in the epicutaneous sensitization to food allergen, independently of both mast cells and IL-33-ST2 signaling. However, it is unclear how much of δ-toxin present on skin is enough to induce epidermal sensitization. To solve this question, it will be necessary to quantify δ-toxin in the skin of patients with atopic dermatitis or normal controls that are colonized by *S. aureus* in further experiments.

We speculate that epicutaneously treated δ-toxin present on the non-tape-stripped skin stimulates keratinocytes, the most abundant cell type in the epidermis, to release IL-1α. This local release of IL-1α directly or indirectly stimulates the activation of skin cDC2, which is characterized by the uptake of food allergen by cDC2 in the skin and the translocation of cDC2 from the skin to draining LNs. This, in turn, likely results in skewing of Th2/Tfh with food allergen-specific IgE production. Consequently, intragastric OVA administration in δ-toxin-treated mice can result in food allergic responses that are IgE- and mast cell-dependent. These assumptions are potentiated by several findings. *In vitro* stimulation of murine keratinocytes with δ-toxin induced the release of detectable levels of IL-1α, but not of IL-1β, IL-18, IL-33, or IL-25, although these cell types constitutively released TSLP irrespective of δ-toxin stimulation. Furthermore, δ-toxin increased

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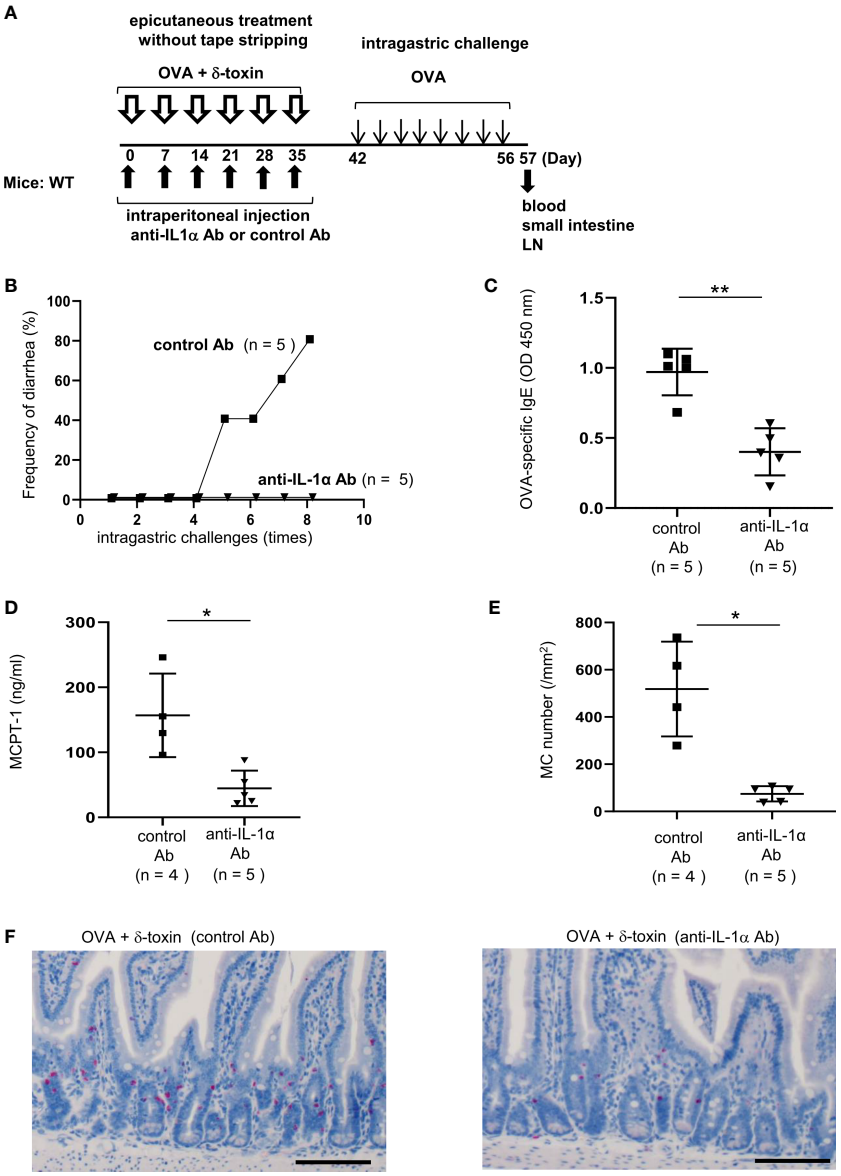

FIGURE 7
Pretreatment with anti-IL-1α Ab dampened δ-toxin-mediated, OVA-induced food allergic responses in murine model. **(A)** Experimental design for food allergy of intragastrically OVA-administered WT mice that had been epicutaneously treated with OVA + δ-toxin once a week for six weeks on the non-tape-stripped abdominal skin. The effects of intraperitoneal administration of anti-IL-1α Ab or control Ab were examined. Blood samples were taken, and small intestines were isolated on day 57 **(B)** Frequency of diarrhea in OVA-administered mice. **(C–E)** The serum levels of **(C)** OVA-specific IgE and **(D)** MCPT-1 in the mice after the last challenge with OVA. **(E)** The numbers of jejunum mast cells of the mice after the last challenge with OVA. **(F)** Jejunum sections stained with chloroacetate esterase (scale bars, 100 μm). Mast cells stain red. **(B-F)** Data are representative of two independent experiments. Means ± SD have been plotted. *$P < 0.05$ or **$P < 0.01$.

protein levels of IL-1α in the epicutaneously treated skin in this model. It should be noted that in this model, δ-toxin decreased the percentages of skin cDC2 among total skin cells but increased the percentage of OVA-AF647-engulfed cDC2 among skin cDC2, while δ-toxin increased the numbers of both total cDC2 and OVA-AF647-engulfed cDC2 in axillary LN. In addition, *in vitro* stimulation with IL-1α increased the uptake of OVA-AF647 in MHC Class II^{high} BMDCs. Most importantly, pretreatment with IL-1α Ab abrogated the activation of skin cDC2, OVA-specific IgE production, and OVA-induced food allergic responses in our model. Nonetheless, taking into consideration the important role of TSLP in epicutaneous sensitization (11, 26, 37, 38), it seemed reasonable that IL-1α cooperates with TSLP in the δ-toxin-mediated epicutaneous sensitization to food allergen. However, further examination will be required to completely understand the mechanisms by which locally released IL-1α causes epicutaneous sensitization via cDC2 in this model.

Although δ-toxin is speculated to directly stimulate several types of immune cells through putative δ-toxin receptors, the expression levels of these receptors are extremely low in murine keratinocytes (12, 39). However, δ-toxin exhibited *in vitro* cytotoxicity on murine keratinocytes. Further, stimulation with IL-1α up-regulated mRNA

levels of IL-1α in murine keratinocytes. Hence, δ-toxin-induced cell death of keratinocytes likely plays a primary role in the release of IL-1α, which transcriptionally up-regulates IL-1α levels in an autocrine manner.

Recent studies using murine models have reported that in epicutaneous infection of *S. aureus*, PSMα induces the release of IL-1α and IL-36α from keratinocytes, leading to IL-17-dependent skin inflammation (15, 16). Furthermore, IL-36α enhances IgE production by directly acting on B cells (17). Although IL-36α expression in skin is strongly up-regulated in previous studies (15, 17), we did not observe it in δ-toxin-treated skin in our model. It should be noted that we were not able to measure protein levels of IL-36α as specific Ab against IL-36α was commercially unavailable. Given that keratinocytes release protein levels of IL-36α in response to the culture supernatants of *S. aureus* (15), PSMα together with other *S. aureus*-derived factors may increase expression levels of IL-36α. Hence, similar mechanisms may be at play to upregulate IL-36α expression in *S. aureus*-colonized skin of patients with atopic dermatitis.

Interestingly, PSMα3-treated mice exhibited weaker food allergic responses with less frequent diarrhea than δ-toxin-treated mice in our model. In accordance with this, the PSMα3-treated mice exhibited weaker activation of skin cDC2 and lower levels of OVA-specific IgE production than δ-toxin-treated mice before OVA administration. Consistent with the finding that PSMα3 exerts stronger cytotoxicity on murine keratinocytes than δ-toxin, stimulation with PSMα3 more strongly induced the release of DAMPs, including IL-1α, ATP, and HMGB1 than that with δ-toxin. However, δ-toxin more strongly increased the levels of IL-1α, but not of ATP and HMGB1, in skin tissues than PSMα3 in murine model. This may partly explain the different responses between the treatments with PSMα3 and δ-toxin in our study. It is also possible to speculate that PSMα on the non-tape-stripped skin cooperates with specific DAMPs locally released to induce inflammation in a different way from δ-toxin. Accordingly, it seems that δ-toxin present on steady-state skin is prone to skew toward Th2/Tfh with antigen-specific IgE production via keratinocyte-derived IL-1α, although PSMα skews toward Th17 in murine model of epicutaneous *S. aureus* infection (15, 16, 40, 41). In any case, we need to further investigate the mechanisms underlying the different effects of δ-toxin and PSMα on epicutaneous sensitization.

In conclusion, epicutaneous treatment of δ-toxin on non-tape-stripped skin strongly promotes epicutaneous sensitization to food allergens, resulting in food allergy after the uptake of the same allergen. This model may recapitulate epicutaneous sensitization in normal skin colonized by δ-toxin-producing *S. aureus*. Hence, keratinocyte-derived IL-1α plays a critical role in the development of food allergy. Therefore, targeting IL-1α may be an appropriate strategy to prevent the development of food allergy in individuals whose skins are colonized by δ-toxin-producing *S. aureus*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

All the procedures using mice were approved by the institutional review boards of Juntendo University (approval No. 310050 and 310051).

Author contributions

HY performed all the experiments and participated in writing the manuscript. AnK, KI, TA, AyK, SU, AM, MK, and RY assisted with the analysis of murine models of food allergy. HW, MN, KM, KU, and NN assisted with the *in vitro* experiments. YO, HO, KO, and TS analyzed the data. JK conceived the project, analyzed the data, and actively participated in manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1173069/full#supplementary-material

References

1. Oyoshi MK, Oettgen HC, Chatila TA, Geha RS, Bryce PJ. Food allergy: insights into etiology, prevention, and treatment provided by murine models. *J Allergy Clin Immunol* (2014) 133(2):309–17. doi: 10.1016/j.jaci.2013.12.1045

2. Tordesillas L, Berin MC, Sampson HA. Immunology of food allergy. *Immunity* (2017) 47(1):32–50. doi: 10.1016/j.immuni.2017.07.004

3. Sampson HA, O'Mahony L, Burks AW, Plaut M, Lack G, Akdis CA. Mechanisms of food allergy. *J Allergy Clin Immunol* (2018) 141(1):1–9. doi: 10.1016/j.jaci.2017.11.005

4. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* (2003) 112(11):1666–77. doi: 10.1172/JCI19785

5. Chen CY, Lee JB, Liu B, Ohta S, Wang PY, Kartashov AV, et al. Induction of interleukin-9-Producing mucosal mast cells promotes susceptibility to IgE-mediated experimental food allergy. *Immunity* (2015) 43(4):788–802. doi: 10.1016/j.immuni.2015.08.020

6. Uchida S, Izawa K, Ando T, Yamada H, Uchida K, Negishi N, et al. CD300f is a potential therapeutic target for the treatment of food allergy. *Allergy* (2019) 75(2):471–4. doi: 10.1111/all.14034

7. Leyva-Castillo JM, Galand C, Kam C, Burton O, Gurish M, Musser MA, et al. Mechanical skin injury promotes food anaphylaxis by driving intestinal mast cell expansion. *Immunity* (2019) 50(5):1262–75. doi: 10.1016/j.immuni.2019.03.023

8. Galand C, Leyva-Castillo JM, Yoon J, Han A, Lee MS, McKenzie ANJ, et al. IL-33 promotes food anaphylaxis in epicutaneously sensitized mice by targeting mast cells. *J Allergy Clin Immunol* (2016) 138(5):1356–66. doi: 10.1016/j.jaci.2016.03.056

9. Tordesillas L, Goswami R, Benedé S, Grishina G, Dunkin D, Järvinen KM, et al. Skin exposure promotes a Th2-dependent sensitization to peanut allergens. *J Clin Invest* (2014) 124(11):4965–75. doi: 10.1172/JCI75660

10. Bartnikas LM, Gurish MF, Burton OT, Leisten S, Janssen E, Oettgen HC, et al. Epicutaneous sensitization results in IgE-dependent intestinal mast cell expansion and food-induced anaphylaxis. *J Allergy Clin Immunol* (2013) 131(2):451–60. doi: 10.1016/j.jaci.2012.11.032

11. Muto T, Fukuoka A, Kabashima K, Ziegler SF, Nakanishi K, Matsushita K, et al. The role of basophils and proallergic cytokines, TSLP and IL-33, in cutaneously sensitized food allergy. *Int Immunol* (2014) 26(10):539–49. doi: 10.1093/intimm/dxu058

12. Nakamura Y, Oscherwitz J, Cease KB, Chan SM, Muñoz-Planillo R, Hasegawa M, et al. Staphylococcus δ-toxin induces allergic skin disease by activating mast cells. *Nature* (2013) 503(7476):397–401. doi: 10.1038/nature12655

13. Matsuo K, Nagakubo D, Komori Y, Fujisato S, Takeda N, Kitamatsu M, et al. CCR4 is critically involved in skin allergic inflammation of BALB/c mice. *J Invest Dermatol* (2018) 138(8):1764–73. doi: 10.1016/j.jid.2018.02.027

14. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, Li M, et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* (2007) 13(12):1510–4. doi: 10.1038/nm1656

15. Nakagawa S, Matsumoto M, Katayama Y, Oguma R, Wakabayashi S, Nygaard T, et al. Staphylococcus aureus virulent PSMα peptides induce keratinocyte alarmin release to orchestrate IL-17-Dependent skin inflammation. *Cell Host Microbe* (2017) 22(5):667–77. doi: 10.1016/j.chom.2017.10.008

16. Liu H, Archer NK, Dillen CA, Wang Y, Ashbaugh AG, Ortines RV, et al. Staphylococcus aureus epicutaneous exposure drives skin inflammation via IL-36-Mediated T cell responses. *Cell Host Microbe* (2017) 22(5):653–66. doi: 10.1016/j.chom.2017.10.006

17. Patrick GJ, Liu H, Alphonse MP, Dikeman DA, Youn C, Otterson JC, et al. Epicutaneous staphylococcus aureus induces IL-36 to enhance IgE production and ensuing allergic disease. *J Clin Invest* (2021) 131(5):e143334. doi: 10.1172/JCI143334

18. Inoue Y, Aihara M, Kirino M, Harada I, Komori-Yamaguchi J, Yamaguchi Y, et al. Interleukin-18 is elevated in the horny layer in patients with atopic dermatitis and is associated with staphylococcus aureus colonization. *Br J Dermatol* (2011) 164(3):560–7. doi: 10.1111/j.1365-2133.2010.10145.x

19. Kezic S, O'Regan GM, Lutter R, Jakasa I, Koster ES, Saunders S, et al. Filaggrin loss-of-function mutations are associated with enhanced expression of IL-1 cytokines in the stratum corneum of patients with atopic dermatitis and in a murine model of filaggrin deficiency. *J Allergy Clin Immunol* (2012) 129(4):1031–9. doi: 10.1016/j.jaci.2011.12.989

20. Savinko T, Matikainen S, Saarialho-Kere U, Lehto M, Wang G, Lehtimäki S, et al. IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. *J Invest Dermatol* (2012) 132(5):1392–400. doi: 10.1038/jid.2011.446

21. Suárez-Fariñas M, Ungar B, Correa da Rosa J, Ewald DA, Rozenblit M, Gonzalez J, et al. RNA Sequencing atopic dermatitis transcriptome profiling provides insights into novel disease mechanisms with potential therapeutic implications. *J Allergy Clin Immunol* (2015) 135(5):1218–27. doi: 10.1016/j.jaci.2015.03.003

22. Henri S, Poulin LF, Tamoutounour S, Ardouin L, Guilliams M, de Bovis B, et al. CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of langerhans cells. *J Exp Med* (2010) 207(1):189–206. doi: 10.1084/jem.20091964

23. Kumamoto Y, Linehan M, Weinstein JS, Laidlaw BJ, Craft JE, Iwasaki A. CD301b$^+$ dermal dendritic cells drive T helper 2 cell-mediated immunity. *Immunity* (2013) 39(4):733–43. doi: 10.1016/j.immuni.2013.08.029

24. Sokol CL, Camire RB, Jones MC, Luster AD. The chemokine receptor CCR8 promotes the migration of dendritic cells into the lymph node parenchyma to initiate the allergic immune response. *Immunity* (2018) 49(3):449–63. doi: 10.1016/j.immuni.2018.07.012

25. Gao Y, Nish SA, Jiang R, Hou L, Licona-Limón P, Weinstein JS, et al. Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* (2013) 39(4):722–32. doi: 10.1016/j.immuni.2013.08.028

26. Oyoshi MK, Larson RP, Ziegler SF, Geha RS. Mechanical injury polarizes skin dendritic cells to elicit a T(H)2 response by inducing cutaneous thymic stromal lymphopoietin expression. *J Allergy Clin Immunol* (2010) 126(5):976–84. doi: 10.1016/j.jaci.2010.08.041

27. Izawa K, Yamanishi Y, Maehara A, Takahashi M, Isobe M, Ito S, et al. The receptor LMIR3 negatively regulates mast cell activation and allergic responses by binding to extracellular ceramide. *Immunity* (2012) 37(5):827–39. doi: 10.1016/j.immuni.2012.08.018

28. Fukase S, Ando T, Matsuzawa M, Kimura M, Sone Y, Izawa K, et al. Pollen shells and soluble factors play non-redundant roles in the development of allergic conjunctivitis in mice. *Ocul Surf* (2021) 22:152–62. doi: 10.1016/j.jtos.2021.08.009

29. Kamei A, Izawa K, Ando T, Kaitani A, Yamamoto R, Maehara A, et al. Development of mouse model for oral allergy syndrome to identify IgE cross-reactive pollen and food allergens: ragweed pollen cross-reacts with fennel and black pepper. *Front Immunol* (2022) 13:945222. doi: 10.3389/fimmu.2022.945222

30. Hirabayashi T, Anjo T, Kaneko A, Senoo Y, Shibata A, Takama H, et al. PNPLA1 has a crucial role in skin barrier function by directing acylceramide biosynthesis. *Nat Commun* (2017) 8:14609. doi: 10.1038/ncomms14609

31. Caldelari R, Müller EJ. Short- and long-term cultivation of embryonic and neonatal murine keratinocytes. *Methods Mol Biol* (2010) 633:125–38. doi: 10.1007/978-1-59745-019-5_10

32. Tordesillas L, Lozano-Ojalvo D, Dunkin D, Mondoulet L, Agudo J, Merad M, et al. PDL2$^+$ CD11b$^+$ dermal dendritic cells capture topical antigen through hair follicles to prime LAP$^+$ tregs. *Nat Commun* (2018) 9(1):5238. doi: 10.1038/s41467-018-07716-7

33. Malissen B, Tamoutounour S, Henri S. The origins and functions of dendritic cells and macrophages in the skin. *Nat Rev Immunol* (2014) 14(6):417–28. doi: 10.1038/nri3683

34. Merad M, Ginhoux F, Collin M. Origin, homeostasis and function of langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol* (2008) 8(12):935–47. doi: 10.1038/nri2455

35. Benck CJ, Martinov T, Fife BT, Chatterjea D. Isolation of infiltrating leukocytes from mouse skin using enzymatic digest and gradient separation. *J Vis Exp* (2016) 107:e53638. doi: 10.3791/53638

36. Kashem SW, Haniffa M, Kaplan DH. Antigen-presenting cells in the skin. *Annu Rev Immunol* (2017) 35:469–99. doi: 10.1146/annurev-immunol-051116-052215

37. Segaud J, Yao W, Marschall P, Daubeuf F, Lehalle C, German B, et al. Context-dependent function of TSLP and IL-1β in skin allergic sensitization and atopic march. *Nat Commun* (2022) 13(1):4703. doi: 10.1038/s41467-022-32196-1

38. Marschall P, Wei R, Segaud J, Yao W, Hener P, German BF, et al. Dual function of langerhans cells in skin TSLP-promoted T_{FH} differentiation in mouse atopic dermatitis. *J Allergy Clin Immunol* (2021) 147(5):1778–94. doi: 10.1016/j.jaci.2020.10.006

39. Weiß E, Kretschmer D. Formyl-peptide receptors in infection, inflammation, and cancer. *Trends Immunol* (2018) 39(10):815–29. doi: 10.1016/j.it.2018.08.005

40. Nakae S, Komiyama Y, Yokoyama H, Nambu A, Umeda M, Iwase M, et al. IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response. *Int Immunol* (2003) 15(4):483–90. doi: 10.1093/intimm/dxg054

41. Dolence JJ, Kobayashi T, Iijima K, Krempski J, Drake LY, Dent AL, et al. Airway exposure initiates peanut allergy by involving the IL-1 pathway and T follicular helper cells in mice. *J Allergy Clin Immunol* (2018) 142(4):1144–58. doi: 10.1016/j.jaci.2017.11.020

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Berberine-containing natural-medicine with boiled peanut-OIT induces sustained peanut-tolerance associated with distinct microbiota signature

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Background: Gut microbiota influence food allergy. We showed that the natural compound berberine reduces IgE and others reported that BBR alters gut microbiota implying a potential role for microbiota changes in BBR function.

Objective: We sought to evaluate an oral Berberine-containing natural medicine with a boiled peanut oral immunotherapy (BNP) regimen as a treatment for food allergy using a murine model and to explore the correlation of treatment-induced changes in gut microbiota with therapeutic outcomes.

Methods: Peanut-allergic (PA) mice, orally sensitized with roasted peanut and cholera toxin, received oral BNP or control treatments. PA mice received periodic post-therapy roasted peanut exposures. Anaphylaxis was assessed by visualization of symptoms and measurement of body temperature. Histamine and serum peanut-specific IgE levels were measured by ELISA. Splenic IgE^+B cells were assessed by flow cytometry. Fecal pellets were used for sequencing of bacterial 16S rDNA by Illumina MiSeq. Sequencing data were analyzed using built-in analysis platforms.

Results: BNP treatment regimen induced long-term tolerance to peanut accompanied by profound and sustained reduction of IgE, symptom scores, plasma histamine, body temperature, and number of IgE^+ B cells (p <0.001 vs Sham for all). Significant differences were observed for *Firmicutes*/*Bacteroidetes* ratio across treatment groups. Bacterial genera positively correlated with post-challenge histamine and PN-IgE included *Lachnospiraceae*, *Ruminococcaceae*, and *Hydrogenanaerobacterium* (all *Firmicutes*) while

Verrucromicrobiacea. *Caproiciproducens*, *Enterobacteriaceae*, and *Bacteroidales* were negatively correlated.

Conclusions: BNP is a promising regimen for food allergy treatment and its benefits in a murine model are associated with a distinct microbiota signature.

KEYWORDS

peanut allergy, IgE, berberine, microbiota, 16S rDNA, oral immunotherapy (OIT) *Angelica sinensis*

1 Introduction

An appreciation for the role of gut microbiota to modulate immune responses (1, 2) has led to intense interest in the relationship between gut microbiota and food allergy (3–5), which continues to be a major health problem worldwide. Food allergy treatments have been elusive and the development of lasting cures for food allergy remains an active area of research (6–9). Several studies in human and animal models of food allergy have demonstrated an association with distinct gut microbiota profiles (3, 10–12). In addition to microbiota characteristics of food-allergic patients, several reports have described changes in gut microbiota that accompany natural resolution (4, 13) or treatment-induced improvement in disease (14–17), suggesting a role for gut microbes in the outcome of food allergy treatment. We have previously demonstrated that botanical medicines derived from Traditional Chinese Medicine provide persistent protection from anaphylaxis and cause long-term reduction of IgE and beneficial re-programming of the T-helper cytokine profile in mice with peanut allergy (18, 19), concomitant peanut/tree nut allergy (20), and multiple food allergies (21). Building upon these studies, we showed that berberine (BBR), a quinolizidine alkaloid present in *Phellodendron chinensis* has the remarkable ability to suppress IgE production in IgE-producing human myeloma cells and peripheral blood mononuclear cells obtained from allergic patients (22). *In vivo* validation of this property and the evaluation of its potential for food allergy treatment has not been possible due to very poor BBR biovailability (23, 24). Using *in vitro* approaches, we observed that BBR bioavailability was enhanced after oral feeding of food allergy herbal formulas FAHF-2 and B-FAHF-2. Subsequent profiling of the individual constituent herbs of FAHF-2 for the ability to enhance BBR uptake by CACO-2 cells led to the identification of *Angelica sinensis* (AS) as one of the component herbs with the ability to increase BBR uptake (25). Roasted peanut powder (flour) oral immunotherapy (OIT) is a current FDA-approved clinical treatment for PN allergy, but it has the potential for side effects, such as gastrointestinal inflammation and immediate allergic symptoms, including anaphylaxis (26–29). Boiled PN has been shown to be less reactive than roasted PN by us and others (30–32). Thus, in the current study, we tested a combined therapy regimen using BBR, water extracts of AS and boiled PN OIT referred to together as BBR-containing Natural-medicine with boiled Peanut immunotherapy (BNP) (33). In addition to investigating the effects of the treatments on allergic responses, we explored whether the BNP regimen would have consequences on the gut microbiota profile and whether observed changes correlated with disease status.

Abbreviations: BBR, Berberine; BNP, BBR-containing natural medicine with boiled peanut-oral immunotherapy; AS, *Angelica sinensis*; PA Mice, Peanut-allergic mice; PN, Peanut; CPE, Crude peanut extract; OTU, Operational Taxonomical Unit.

2 Materials and methods

2.1 Peanut sensitization and challenge and treatment

2.1.1 Peanut sensitization and challenge of mice

Five-week-old female C3H/HeJ mice (PA mice) purchased from the Jackson Laboratory (Bar Harbor, ME) were maintained in pathogen-free facilities at the Mount Sinai vivarium according to standard guidelines for the care and use of animals (34).

C3H/HeJ mice were orally sensitized with roasted PN and cholera toxin as previously published by our research group (35, 36). As shown in Figure 1A, a detailed experimental protocol was established as previously reported with minor modifications (37). Mice were intragastrically (i.g.) sensitized with 10 mg of homogenized roasted peanut in a 0.5 ml PBS containing 75 mg sodium bicarbonate, 10 µg of the mucosal adjuvant cholera toxin (List Laboratories, CA), and 16.5 µl (1.1 µl/g body weight) of 80 proof Stolichnaya Vodka® (a source of food grade ethanol) to neutralize stomach pH and to increase gastrointestinal permeability. A boosting dose of 50 mg PN was given at weeks 6 and 8 using the same gavage solution as above. Naïve mice were not sensitized. Oral challenges with 200 mg roasted PN were given at weeks 30 and 50.

2.1.2 Treatment regimen

The course of oral treatment was started at week 8 after completion of the sensitization and boosting protocol and continued until week 26. This represents a therapeutic protocol as we have shown that mice developed peanut IgE sensitization and exhibited reactions in response to peanut challenge at week 8

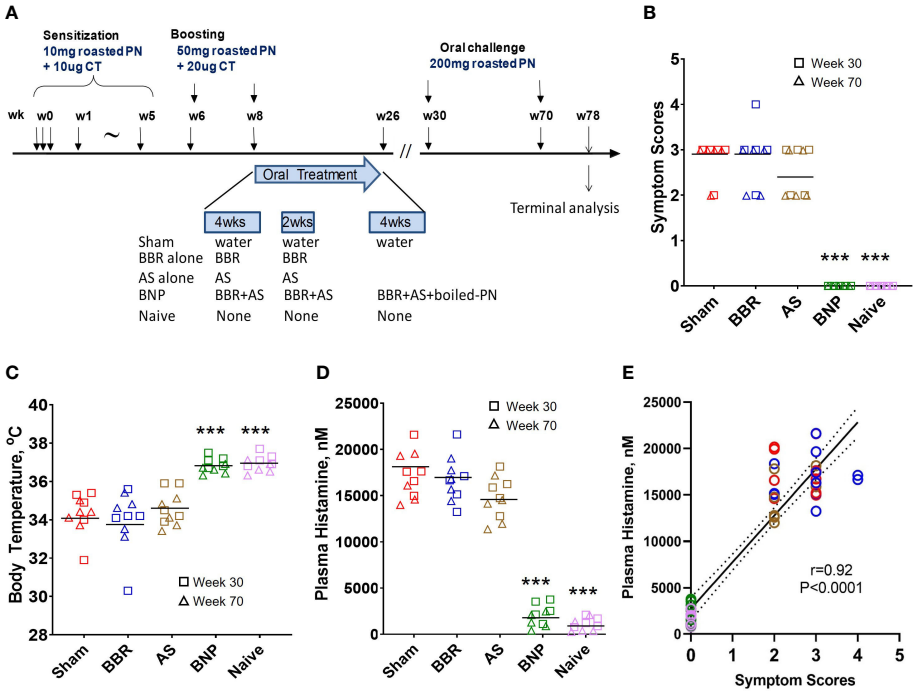

FIGURE 1
Experimental design and anaphylactic reactions at challenge. **(A)** Experimental protocol. *In vivo*, experimental protocol- 6-week-old female C3H/HeJ mice were subjected to oral peanut sensitization in the presence of cholera toxin from week 0 through week 5 and boosted thereafter at week 6 and week 8. Mice were then given BNP therapy between W8-W26 as described in methods. Mice were challenged at W30 (Data shown using square symbols) and W70 (Data shown using triangle symbols). The experiment was terminated at W78. **(B)** Symptom scores were assigned 30 minutes after challenge using criteria described in methods. **(C)** Body temperature was measured by rectal probe after assessment of symptom scores. **(D)** Plasma histamine levels were measured by duplicate ELISA of individual plasma samples harvested from blood collected 30 minutes after the measurement of body temperatures. **(E)** Analysis of correlation between symptom scores and plasma histamine at W30 and W70 challenge time points. Color key for symbols: Red-Sham, Blue-BBR, Brown-AS, Green-BNP, Pink-Naïve. Bars in B are group medians and in C and D are group means. In E solid line represents the regression line and the dashed line represents the 95% confidence interval. Red color represents sham, blue color represents BBR, tan color represents AS, green color represents BNP, and purple color represents naïve. N=5 mice/group. Data represent 10 readouts from a combination of W30 (square symbols) and W70 (triangle symbols) challenges. ***$p<0.001$ vs Sham.

(20, 38). During the 18-week treatment regimen, PN allergic mice were orally treated with a combination of daily BBR and AS extract in three courses. Two off-treatment intervals of 2 and 4 weeks were included to assess the potential for off-therapy IgE rebound. Boiled peanut OIT was added for the final 4 weeks of the BBR+AS therapy, altogether referred to as BNP. Control groups included PA mice given either 2 mg BBR/day (Sigma-Aldrich), AS water extract (10 mg/day), sham-treated PA mice, and naïve mice. Water extract of AS was prepared by Sanmenxia Shanshui Fangzheng Biotechnology Ltd, Sanmenxia, China as follows. Verified AS raw herb was cut to obtain 1-2 cm pieces and soaked in excess water (1:10 v/v) overnight. The water/AS mixture was then boiled for 2 hours. The aqueous phase was collected through a filtration process and the residue was subjected to a second round of hot water extraction (1:8 v/v). The two decoctions were collected and concentrated to a density of 1.15-1.20 g/ml. The condensed extract was then dried at 65 °C under vacuum. Dried AS extract was subsequently ground to a fine powder for further use. The product quality control was conducted by HPLC fingerprint. BBR was purchased from Sigma-Aldrich and the ratio of BBR: AS was determined by previous *in vitro* uptake studies and estimation of BBR in the daily dose of 12 mg B-FAHF-2 for mice (25). Raw peanuts without shells but with skin were boiled in water for 30 minutes and subsequently homogenized in phosphate-buffered saline. Daily boiled peanut OIT was given at a dose of 10 mg/day. All treatments were given by oral gavage dissolved in drinking water at a volume of 0.5ml/gavage.

2.2 Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30-40 minutes after oral PN challenge as described previously (37). The severity of observed symptoms on a scale of 0 (no reactions)-5 (death due to anaphylaxis was scored utilizing the scoring system described previously (37). 0 - no symptoms; 1 - scratching and rubbing around the snout and head; 2 - puffiness around the eyes and snout, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3 - wheezing, labored respiration, cyanosis around the mouth and the tail; 4 - no activity after prodding, or tremor and convulsion; 5 - death. Cage identities were concealed during the visual assessment of anaphylactic symptoms. Rectal temperatures were measured using a rectal probe (Harvard Apparatus, NJ, USA).

2.3 Measurement of plasma histamine levels and PN-specific IgE

ELISA measurements for plasma histamine levels and PN-specific IgE have been detailed as described previously, plasma was harvested within 20 minutes after blood collection at challenge and stored at -80 °C until used (20, 37). Histamine was measured using an enzyme immunoassay kit (Fisher Scientific, NJ) as described by the manufacturer. Peanut-specific IgE levels in serum were measured as reported previously (20). Briefly, microtiter plates were coated with crude peanut extract (CPE) (39) or standard of purified anti-mouse IgE (BDBiosciences, CA, USA) and held overnight at 4 °C. After washing, plates were blocked with 2% Bovine Serum Albumin-PBS. Washed plates were incubated with samples overnight at 4 °C and developed using biotinylated anti-IgE detection antibodies (BD Biosciences, CA, USA), avidin-peroxidase, and ABTS substrate (KPL, MN, USA).

2.4 Flow cytometry analysis

A single-cell suspension of splenocytes was suspended in ice-cold staining buffer (PBS including 0.5 mM EDTA, 0.05 mM Sodium Azide, and 0.5% BSA). First, surface staining was performed by incubating cells with unlabeled anti-IgE (to block membrane IgE), BV605 anti-B220, BV711-anti-CD3, anti-CD16/32 (Fc-block), all from BD Biosciences, CA). Live-dead discriminating dye (Live-Dead Aqua, Invitrogen, CA) was also included at this point. Cells were incubated in the dark for 30 minutes on ice. Cells were then washed 3 times with a staining buffer. Cells were then incubated with fixation/permeabilization buffer for 15 minutes. Cells were washed with permeabilization buffer and incubated with FITC-anti IgE, in permeabilization buffer for 30 minutes in the dark on ice. Cells were then again washed 3 times with a staining buffer. Cells were treated with Cytofix buffer for 15 minutes for post-fixation. Then they were resuspended in 200 µl staining buffer for cell acquisition on an LSRII flow cytometer (Becton Dickinson, CA). Flow cytometry analysis was performed using Flow jo (Tree Star) as follows. Live cells were selected by excluding Live-Dead Aqua-positive cells. Of live cells, singlet staining was selected on the basis of FSC-A/FSC-H profile. Singlet cells were then analyzed for IgE+ B cells (FITC-IgE +; BV605-B220+ cells).

2.5 Fecal microbiota analysis

Approximately 7-10 fecal pellets from individual mice were collected prior to terminal analyses at week 52 post-therapy. Fecal pellets were aseptically collected and stored at -20 °C. Samples were shipped to Utah State University where sample processing and microbiota studies were performed. Briefly, isolated total community DNA was isolated from the fecal samples using a QIAGen QIAamp DNA stool mini kit according to the instructions provided by the manufacturer. Upon obtaining total DNAs, samples were sent to Idaho State University (ISU) Molecular Core Facility for Illumina MiSeq next-generation sequencing (https://www.isu.edu/research/centers-and-institutes/molecular-research-core-facility/services/). Briefly, first-stage PCR was performed to amplify the V3-V4 region of 16S rRNA from total DNA. Subsequently, the PCR products for each sample were cleaned using Ampure XP beads. Then, a second-stage PCR was performed for Illumina indexing. Upon running Illumina MiSeq for the samples, the raw fastaq files were hosted at the site managed by Idaho State University and their sequencing facility to perform the bioinformatics analysis using Mothur software package and we received processed data files. These files were uploaded to microbiomeanalyst.ca for selected analyses using Mothur output files and SILVA taxonomy as the reference 16S rDNA database.

2.6 Statistical analysis

Data were analyzed using GraphPad prism 8 software. Symptom scores, plasma histamine, body temperature, and IgE data were analyzed by One-Way ANOVA on ranks followed by Kruskal Wallis post-test for symptom scores and One-Way ANOVA followed by Dunnett's post-test for histamine, temperature, and IgE data. Secondary statistical analyses of microbiota data were performed through Welches ANOVA and multiple T-tests using the Holmes-Sidak method to correct for multiple comparisons, generate Spearman R values and graphs of linear regression, and stacked bar and donut charts of microbiota data. p values < 0.05 were considered significant.

3 Results

3.1 Oral BNP treatment confers persistent protection from anaphylactic reactions to oral peanut challenge

As described in Figure 1A, oral roasted peanut challenges were given at weeks 30 and 70 of the experimental protocol. At each challenge, mice were evaluated for anaphylactic symptoms, drop in body temperature, and plasma histamine levels. Mice given BNP treatment were completely protected from anaphylaxis as no mouse at either challenge displayed symptoms of anaphylaxis following oral challenge (Figure 1B). Groups given BBR alone or AS alone were similar to mice in the Sham group with respect to median symptom scores. Protection from systemic anaphylaxis in the BNP treatment group was also evidenced by a lack of temperature drop after peanut challenges (Figure 1C). Mean body temperature was similar to naïve mice and significantly higher than mice in the Sham group (p<0.001 vs Sham). Plasma histamine levels in mice in the BNP mice were significantly lower than those observed for Sham mice (p<0.001 vs Sham, Figure 1D). Aggregate evaluation of plasma histamine levels of all mice and correlation with symptom scores (r=0.92, p<0.0001, Figure 1E) was found to be of robust strength and statistical significance underscoring the central role of histamine with anaphylactic severity in our model and its

suitability as a marker of anaphylaxis used for further analysis in our study.

3.2 BNP-treated mice show rapid, profound, and sustained decline of PN-specific IgE that was accompanied by reduction of IgE$^+$ B cells

Within 2-4 weeks after commencing treatment, PN-specific IgE levels were observed to decline, as shown in Figure 2A. Reduction in PN-specific IgE was rapid, achieving nearly 70% reduction within 2 weeks of starting treatment. PN-specific IgE was reduced by nearly 80% in the BNP group by the end of treatment at week 26. Importantly no significant increases in PN-specific IgE were observed with the introduction of boiled peanut OIT during the final 4 weeks of BNP treatment and no symptoms were observed. Reduction in peanut-specific IgE was sustained over the remainder of the protocol post-therapy, which included oral roasted peanut challenges at weeks 30 and 70. Overall, as shown by the comparison of AUC values in Figure 2B, PN-specific IgE was markedly reduced in BNP-treated mice (p<0.0001). Groups given BBR alone or AS alone showed no significant reduction of IgE over the course of the experiment compared with the sham-treated group. Mice were sacrificed at week 78 (52 weeks after stopping therapy) for terminal analyses. At this time IgE$^+$ B (IgE$^+$B220$^+$) cells were evaluated in spleens of mice using flow cytometry. BNP-treated mice showed significantly reduced percentages of IgE$^+$ B cells and IgE-plasma cells (Figures 2C, D, p<0.01-0.001 vs Sham). Taken together treatment of peanut-allergic mice with the BNP led to profound and sustained reduction of peanut-specific IgE and IgE$^+$B cells.

3.3 Gut microbiota of allergic mice given BNP significantly differs from sham allergic mice and is more similar to naïve controls

In light of recent reports that demonstrate the association of gut microbiota signatures with food allergy in humans (4) and mice (16, 17, 40), we investigated the impact of different treatment groups evaluated in this study for an effective food allergy treatment on the gut microbiota in mice. We performed 16S rDNA sequencing analysis of fecal pellets collected at the end of the study. Overall,

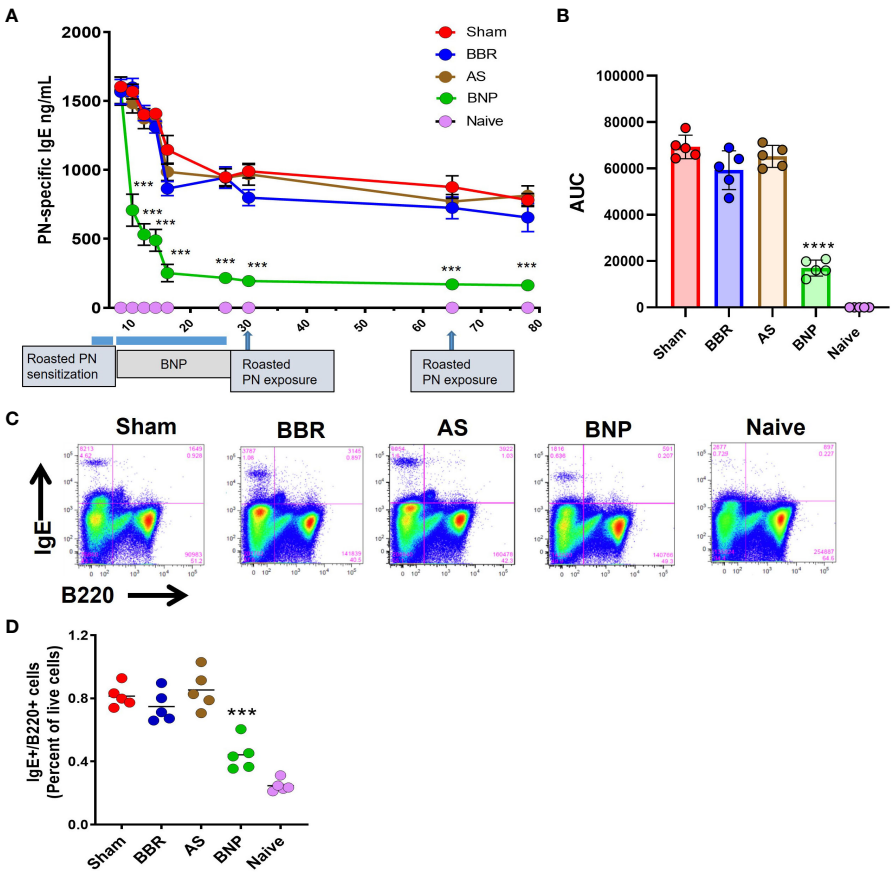

FIGURE 2
Peanut-specific IgE and IgE$^+$ B cells. **(A)** Peanut-specific IgE levels measured by duplicate ELISA of individual samples. **(B)** Differences in PN-specific IgE expressed as AUC values. **(C)** Flow cytometry panels showing percentages of IgE$^+$ B cells in the spleen of representative mice for each group. Splenocytes from individual mice were processed for flow cytometry staining to evaluate IgE$^+$ B cells using FITC-IgE and BV605-B220 antibodies **(D)** Scatter graph of data for the percentage of IgE$^+$ B cells in each group. Data in A shown as group Means± SEM. Bars in B and C are group Means. N=5 mice/group. ***, **** represent p<0.001, 0.0001 vs Sham.

469 operational taxonomic units (OTUs) were identified in the samples across all groups.

Principal Coordinate Analysis (PCoA) at the OTU level (Figure 3) indicated a closer relation between OTU profiles of mice treated with BNP and Naïve control mice, than with Sham mice or mice given BBR alone or AS alone. However, the richness of the microbiota at the OTU level was not different across experimental groups (data not shown). Together these data suggest that experimental groups in our study come to acquire distinct microbiota signatures and that mice given the BNP treatment regimen display a microbiota profile that is more closely related to naïve mice.

3.4 Phylum composition of gut microbiota in mice treated with the BNP regimen shows a lower *Firmicutes*/*Bacteroidetes* ratio and a higher abundance of phyla associated with beneficial metabolic status

Evaluation of actual abundance at the phylum level showed that a major portion of the gut microbiome in all experimental groups in our study was accounted for by the *Firmicutes* and *Bacteroidetes* (Figure 4A). A high *Firmicutes*/*Bacteroidetes* ratio is reported to be correlated with inflammation, metabolic dysregulation, and autism spectrum accompanied by gastric disturbances (41–43). In our study, the Sham group displayed higher *Firmicutes* to *Bacteroidetes* ratio compared to the Naïve group (p<0.0001, Figure 4B). Mice in the BNP treatment group had lower *Firmicutes* : *Bacteroidetes* ratios compared to all other groups except naïve mice (p<0.0001-0.05, Figure 4B). Interestingly, *Verrucromicrobiacea* was noticeably increased in the group given BNP (Figure 4A). *Verrucromicrobiacea* has been reported to be associated with improved metabolic status in humans and mice. Its abundance is also correlated with the integrity of the intestinal epithelium. To understand the relationship between observed phylum and disease status in our model, we evaluated correlations between phylum abundances and levels of plasma histamine at final challenge. Histamine is a central mediator of anaphylactic responses, and the histamine level is strongly correlated with the severity of symptoms in this model, (Figure 1D) as demonstrated in our previous studies. We found that plasma histamine levels were strongly correlated with *Firmicutes* abundance (Figures 4C, D) and strongly but inversely correlated with *Bacteroidetes* (Figures 4C, E). Inverse correlations with histamine, albeit of moderate strength were also observed for *Verricumicrobia* (Figure 4C). These results suggested that enhancement of the phyla *Bacteroidetes* and *Verrucomicrobia* might be linked to the induction of peanut tolerance by BNP treatment.

3.5 *Bacteroidales*, *Tannerellacea*, and *Clostridale* Family XIII_ge were found to be negatively correlated with plasma histamine and IgE

A more in-depth understanding of microbiota perturbations in experimental groups was possible by evaluation of taxa abundance at the genus level. As shown in the heat map in Figure 5, several regions of the heat map show taxa enrichment profiles unique to the experimental groups in our study. *Ruminococcaceae_UGC_014* and *Peptococcacea* showed highly differential enrichment profiles between Sham and Naïve groups with high enrichment in Sham and lower in Naïve mice. In contrast, *Bacteroidales_unclassified* were most enriched in Naïve and BNP groups relative to Sham.

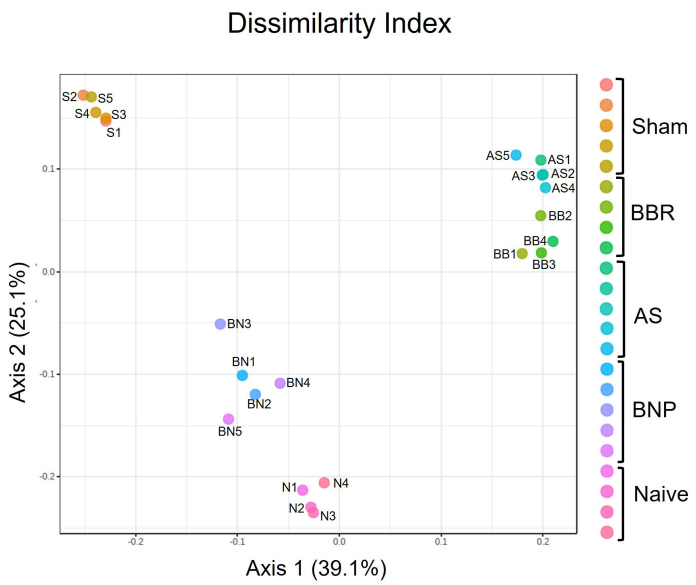

FIGURE 3
Principle Coordinate analysis of OTUs generated using PERMANOVA. N=4-5 mice/group. Symbols on the graph are as follows: S-Sham, BB-BBR, BN-BNP, AS-AS, and N-Naïve.

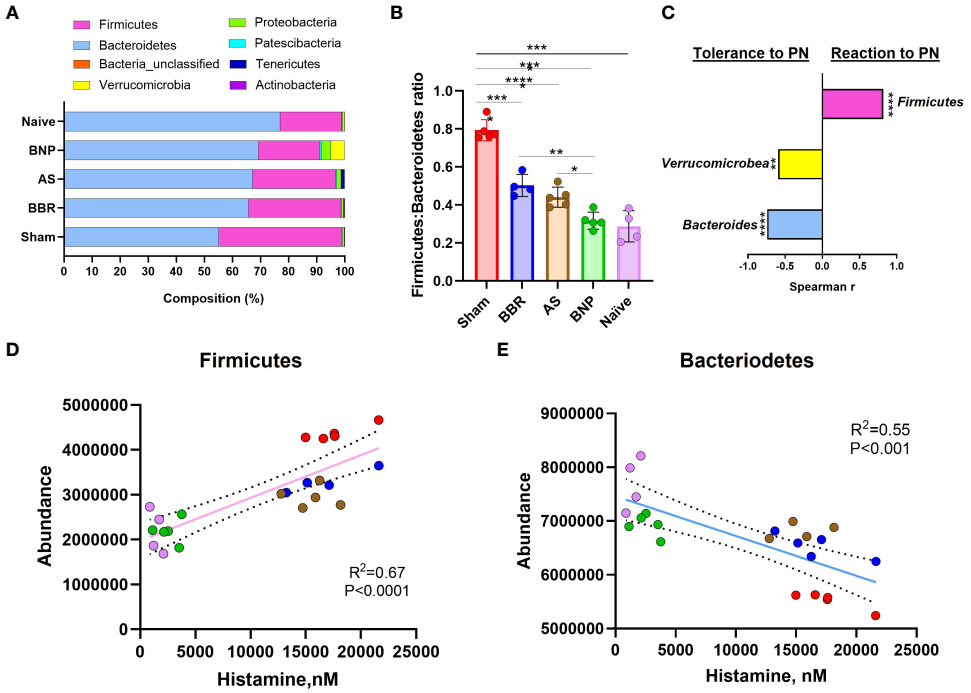

FIGURE 4
Phylum Abundance data. **(A)**. Actual Phylum abundance values obtained using Mothur outputs were used to generate phylum composition data. **(B)**. *Firmicutes : Bacteroidetes* ratio. **(C)**. Spearman Correlation Index (r) using GraphPad Prism. **(D)** & **(E)** are linear regression plots for phylum abundance of *Firmicutes* and *Bacteroidetes* respectively against Plasma histamine levels at week 70 challenge. Color key for symbols in D & E: Red-Sham, Blue-BBR, Brown-AS, Green-BNP, Pink-Naive. N=4-5 mice/group *p<0.05; **p<0.01; ****p<0.0001 vs Sham.

Bacteroides and *Erysepaloclostridium* were the most decreased in the Sham group. *Verrucomicrobiae_unclassified* were only enriched in the BNP group.

We then evaluated the differential composition of those bacterial genera which had abundances that were significantly different from the sham group and at least one of the other experimental groups (Figure 6) as assessed by Welch's ANOVA (P value thresh hold set to <0.05). The majority of taxa belonged either to *Firmicutes* (indicated in shades of pink) or *Bacteroidetes* (indicated in shades of blue). The greatest changes were observed for the *Bacteroides* and *Bacteroidales_unclassified*, which were increased in BNP and Naïve groups compared to the Sham group. Specifically, *Bacteroides_unclassified* representation in Naïve and BNP groups were essentially similar and increased compared to all other experimental groups. *Lachnospiraceae_unclassified* (a *Firmicutes* member) were more abundant in the Sham, BBR, and AS groups relative to Naïve and BNP mice.

To determine whether changes in the abundance of specific bacterial genera were associated with treatment benefits, we evaluated the correlation of genera in Figure 6 (among those with significantly different abundance compared to sham) with plasma histamine and IgE levels at the time of final challenge. We also noted the phylum of each candidate (*Firmicutes*-Pink, *Bacteroidetes*- Blue, *Proteobacteria*- Green, and *Verrucomicrobia*- Yellow). Results of these analyses (Figure 7) showed that *Caproiciproducens* (Firmicutes), Family XIII_ge (Firmicutes), *Tannerellaceae* (*Bateroidetes*), and *Bacteroidales unclassified* (*Bacteroidetes*) had a strong inverse correlation with plasma histamine (Figure 7A) and IgE (Figure 7B) suggesting that these taxa may be associated with protection. Conversely, *Firmicutes* members *Lachnospiraceae_unclassified* and *Ruminococcaceae_UGC_014* were strongly and positively associated with these disease markers implying a possible role in food allergy pathology. *Verrucomicrobia* showed a moderate but significant inverse correlation with both IgE and histamine. Statistically significant differences in actual abundance values between Sham and BNP groups were found for *Ruminococcaceae_UGC_014*, (Figure 8A) which were reduced in the BNP group, and *Verrucomicrobia* and *Bacteroidales* which were significantly increased (Figures 8B, C). Further research to identify specific bacterial species and transfer experiments to prove causation are needed to validate the functionality of these findings.

4 Discussion

The findings of our current study showed that the design of a food allergy treatment regimen containing the medicinal natural compound BBR is efficacious in a murine model of peanut allergy and is associated with a distinct gut microbiota signature. Our observation that BNP is therapeutically equivalent to and with regard to IgE reduction, even superior to parent TCM formulas FAHF-2 and E-B-FAHF-2 represents (Yang et al. Manuscript in preparation) a major achievement in our research efforts to develop an orally available BBR-centered food allergy treatment. We were the first to identify the IgE-lowering abilities of BBR *in vitro* (22),

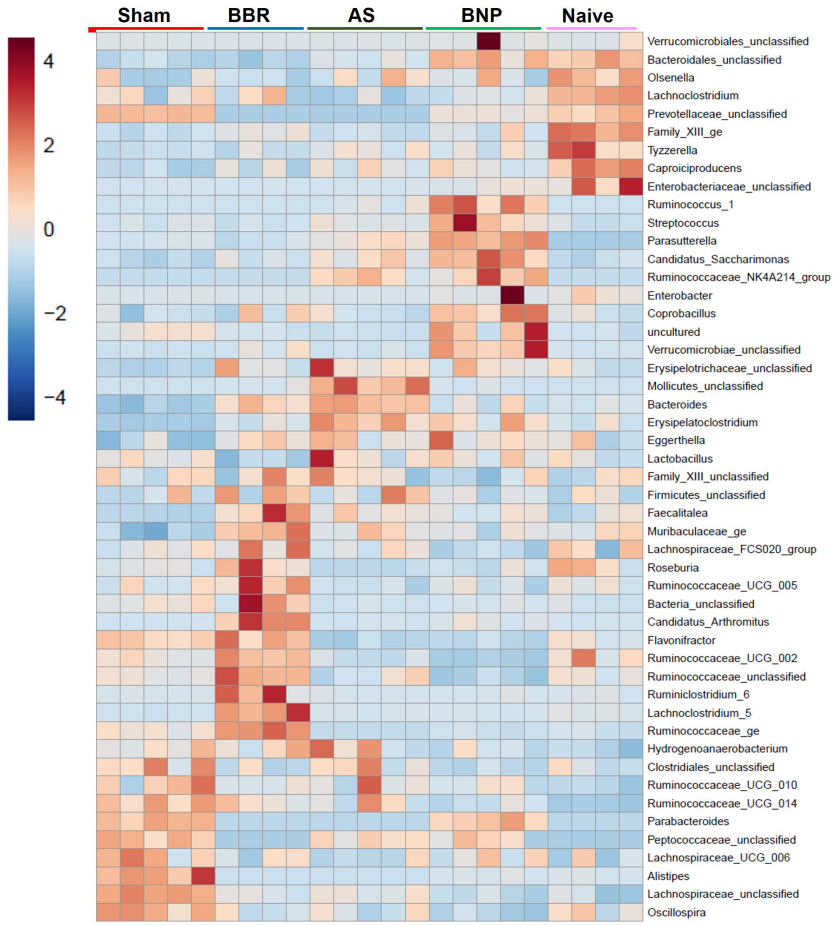

FIGURE 5
Genus Heat map. Heat map generated by Mothur output of genus abundance data using the built-in method at microbiomeanalyst.edu.ca.

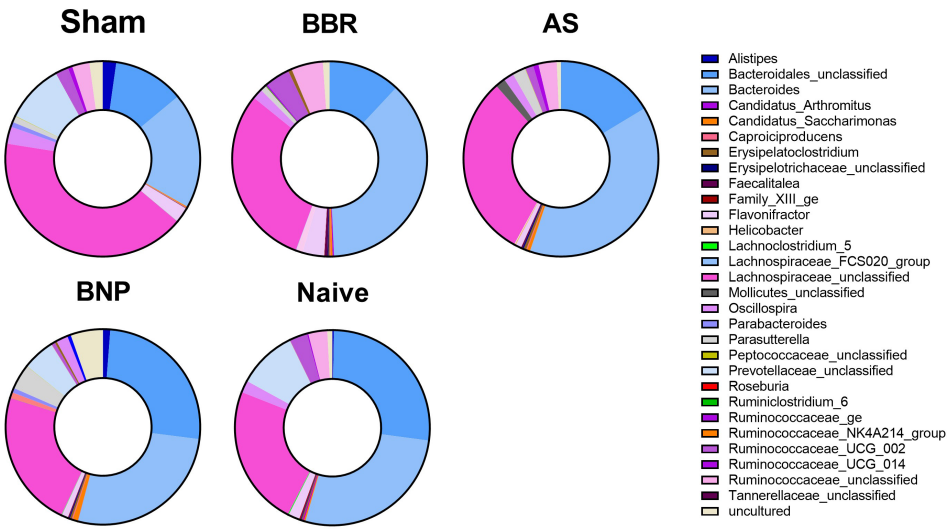

FIGURE 6
Composition of taxa with significantly different abundance levels compared to the Sham group. Donut plots of actual genus abundance data for taxa that were statistically significant ($p<0.05$) for any experimental group vs Sham using Welch's ANOVA. Taxa belonging to *Firmicutes* are shown in shades of pink and those belonging to *Bacteroidetes* are indicated in shades of blue. N=4-5 mice/group.

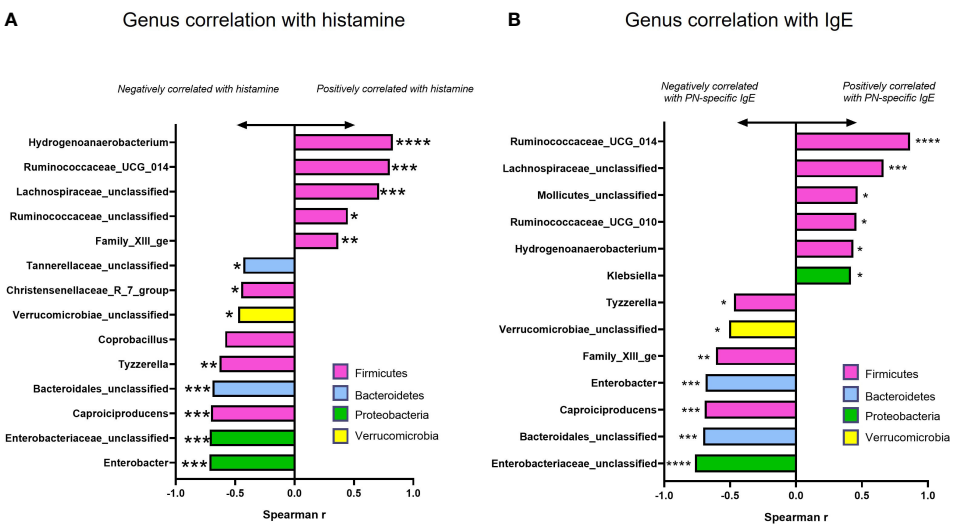

FIGURE 7
Correlation and abundance data among taxa with significantly different abundance values compared to Sham. Spearman Correlation Index (r) for actual genus abundances for taxa with statistically different abundance compared to Sham against plasma histamine levels **(A)** and IgE **(B)** at week 70 challenge using GraphPad Prism. Color of bars indicates Phylum assignment. Color key: Pink-*Firmicutes*, Blue-*Bacteroidetes*, Green-*Proteobacteria*, Yellow-*Verrucomicrobia*. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs Sham.

but poor bioavailability was an obstacle to its *in vivo* application as a potential medicine to treat food allergies. Research efforts to solve this problem led us to the identification of AS, one of the component herbs of FAHF-2 and B-FAHF-2, which we found to enhance BBR uptake in CACO-2 cells (25). Our current data support these earlier findings and shows that BBR-uptake-enhancing natural medicine AS (*Angelica* species) is necessary for the translation of the IgE- lowering effect of BBR *in vivo*. AS have been shown to contain natural inhibitors of p-glycoprotein (p-Gp) (44–46). Since p-Gp has been shown to promote intestinal efflux of BBR driving down its uptake (47–49), potential inhibition by AS likely enhances BBR bioavailability in our system. BBR has been used in Traditional Chinese Medicine as a treatment for diarrhea (50) and more recently it has been used as medicine for diabetes (51, 52), metabolic syndrome (52, 53), and hyperlipidemia (54, 55). As a result, there is extensive interest in BBR-modulation of gut microbiota as these diseases are intimately linked to gut microbiota responses to diet. Several publications have reported alteration of the gut microbiota by BBR in various disease models and humans (56–59). In light of this and the growing appreciation for the role of gut microbiota in food allergy, we were interested in exploring potential alterations in gut microbiota in our food allergy model and the relationship of these changes to the therapeutic benefits of BNP.

Analysis of 16S rDNA sequences in fecal pellets obtained from mice at the time of final peanut exposure in our study showed that PA mice had higher a *Firmicutes*/*Bacteroidetes* ratio than Naïve mice, although microbiota richness at the OTU level was not significantly different. Mice treated with BNP regimens were more similar to Naïve mice in this regard. A lower *Firmicutes*/*Bacteroidetes* ratio is generally associated with healthier metabolic status and a high *Firmicutes*/*Bacteroidetes* ratio is observed in obesity, autistic children with gastric disturbances (41) (60), and in murine models of allergic asthma (61, 62) whereas a higher *Firmicutes*/*Bacteroidetes* ratio is considered beneficial in the setting of autoimmune inflammatory conditions such as colitis and IBD

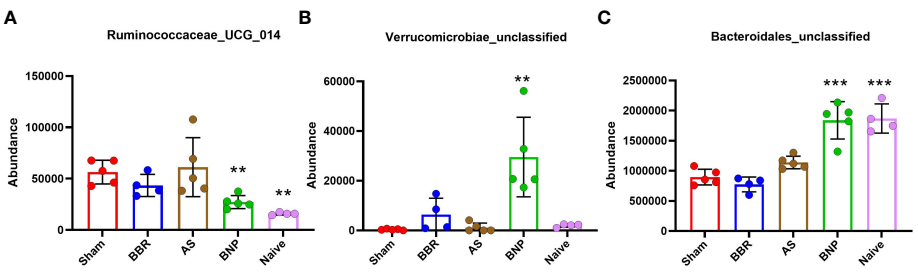

FIGURE 8
Actual abundance values of 3 bacterial genera (**A** Ruminococcaceae_UCG_014, **B** Verrucomicrobiae_unclassified, **C** Bacteroidales_unclassified) with positive or negative correlation with plasma histamine and IgE at week 70 challenge that were also significantly different from Sham. N=4-5 mice/group. **p<0.01; ***p<0.001 vs Sham.

(63–65). No strong evidence has been reported for the impact of *Firmicutes*/*Bacteroidetes* ratio on food allergy. Using plasma histamine levels at challenge, a biomarker of food allergy reactions, for correlation analysis, we found that *Firmicutes* abundance was strongly and positively correlated with histamine levels at challenge whereas *Bacteroidetes* had a strong negative correlation. The negative association of *Bacteroidetes* abundance with allergic reactions in our murine study was in line with findings in humans as reviewed by Shu et al. (66), where the lower levels of *Bacteroides* subsequent to maternal intrapartum antibiotic exposure were implicated in higher sensitization rates in cesarean born children.

Although at the Phylum level, *Firmicutes* abundance was positively correlated with allergy in our study, deeper analyses revealed that at the genus level, *Firmicutes* members exhibited both positive and negative correlations with histamine and PN-IgE levels. This is consistent with previous reports of *Firmicutes* members such as certain *Clostridiales* to be beneficial in the context of food allergy (13, 67).

In our study, genus-level analysis revealed that some *Firmicutes* members such as *Lachnospiraceae_unclassified* and *Ruminococcaceae_UGC_014* were strongly and positively correlated with histamine levels and IgE. Enrichment for *Lachnospiraceae* and *Ruminococcacea* members has been reported in patients with cow's milk and egg allergy and in murine models of peanut allergy (11, 14, 15). In contrast, the *Firmicutes* member *Caproiciproducens*, belonging to the order *Clostridiales* had robust negative correlations with histamine and IgE. Other taxa negatively associated with histamine levels and IgE were *Bacteroidales* and *Verrucomicrobia*. *Bacteroidales* have been associated with improved integrity of the intestinal epithelium and *Verrucomicrobia* phylum has been shown to be associated with restoration of metabolic health (68, 69). Knowledge about gut-inhabiting *Verrucumicrobia* appears to be limited to *Akkermansia muciniphila* (70, 71). *A. muciniphila*, a mucus-degrading gut bacterium has recently received much attention due to its association with improved metabolic health (72), anti-inflammatory profile (73, 74) and ability to promote the integrity of the intestinal epithelium (69, 75, 76). More research is needed to definitively understand whether a BNP-induced shift in microbiota drives an early and rapid decline of IgE or whether these changes are more indicative of a gut microbial community reprogramming as a result of BBR-induced reduction of allergic responses. Comparing microbiota findings in our study to other food allergy studies described previously has proven to be difficult due to published studies being drawn from varying settings of food allergy. Gut microbiota studies in allergy need to be controlled for several aspects such as patient age, IgE vs non-IgE, and specific foods. Murine model data also is drawn from models using various strains, antigens and adjuvants, and routes of sensitization. More in-depth analysis of specific strains followed by functional evaluation using microbiota transfer and screening of fecal metabolites is needed for definitive conclusions. At the very least, however, we believe that enrichment for microbiota members *Verrucomicrobia*, *Bacteroidales*, and *Caproiciproducens* in BNP-treated mice contributes to sustained suppression of allergy even in the setting of repeated peanut exposure.

A theme that has emerged from our data is that sustained lowering of allergic disease status is potentially benefitted by gut microbiota enriched for bacteria with known benefits for metabolic health. The parallel rise of obesity and allergy and their increased prevalence in Western societies hints at a potential relationship (77, 78). The Western diet and urban environment have been implicated in higher rates of both allergy and obesity. In a recent study, Hussain et al. used a murine model of allergy to ovalbumin in conjunction with a high-fat diet to show that mice made obese on a high-fat diet were more susceptible to food allergy and this susceptibility was transferrable *via* gut microbiota to non-obese mice (40). Interestingly, in this study, *Verrucomicrobia* were enriched in the gut of obese allergic mice but were decreased in the recipient mice that were rendered allergic post-transfer. Instead, recipient mice showed increased *Lachnospiracea* abundance. Mechanistic understanding of the interrelationships between allergy and metabolic status is currently a subject of intense investigation. Key pathways that intersect the fields of metabolism and immunity implicate a pivotal role in fatty acid utilization and mTOR signaling, which is a nutrient-sensing pathway (79–81). As benefits of OIT alone on protection from anaphylaxis were transient, the loss of therapeutic effects was just a few weeks after stopping OIT (82). Our previous study suggested that reactions in the OIT alone group were extremely severe including loss of mice due to death from anaphylaxis (20), it was prudent at the time to not subject Sham, BBR, and AS groups to OIT. Though allergen challenge may disrupt the gut microbiome shortly after the challenge. We collected fecal samples 8 weeks after the final oral challenge. We believe this time period is sufficient for regaining the stable status of the gut microbiome and represents a time point that provides information regarding lasting changes. Nevertheless, limitations in this study lie in no boiled PN OIT alone group and only BBR + AS group, which makes data interpretation more complicated. Further research into how BNP-induced shift in gut microbiota contributes to IgE-reduction, studies of microbiome change at different time points, and the consequence of food allergy protection *via* immunometabolism regulation is needed. Further study is also needed to determine total IgE and other total isotype antibodies (total IgG2a, IgG1, total IgA) as well as other isotypes of peanut-specific antibodies (peanut-specific IgG2a, IgG1, and IgA) at different time points to monitor if there is an association between these antibodies and gut microbiome changes. An additional limitation of this study is that in the third round of treatment, we did not add the boiled peanut to other groups but only the BBR/AS group. Since our goal is to investigate an intervention that will be available for individuals with established peanut allergy, we tested in mice if their therapeutic effect such as reduced peanut-specific IgE has been established, adding boiled peanut would not alter the established effect. Since neither the AS nor BBR group showed any therapeutic effect, we did not add boiled peanut to those groups. Our previous study should that boiled peanut was not able to sufficiently sensitize mice compared to roasted peanut (Srivastava et al. unpublished data). Our hypothesis is that adding boiled peanut would not interfere with the BBR/AS-established IgE

reduction. This hypothesis is consistent with the finding of a previous publication that boiled peanuts reduced the capability to induce allergic responses in mice (83). Our data showed that adding the third component of the treatment regimen i.e. boiled peanut did not alter the BBR/AS-established IgE-reduction effect. For the same reason, since neither BBR nor AS alone group showed any therapeutic effect compared to Sham-treated peanut allergic mice, we did not pursue an additional course of treatment to those control groups to BBR and AS alone groups. However, in future research design, we should add boiled peanut to other control groups and compare with both testing and control groups that are not adding boiled peanut for comparison to provide additional evidence to support our hypothesis (83). We should also add additional treatment courses to BBR and AS alone groups even if they had not shown therapeutic effects to learn that additional courses of treatment may not have significant changes.

In summary, we found that oral therapy with natural medicines containing BBR induced profound and lasting reduction of IgE and IgE-producing B cells leading to tolerance of peanut in peanut-allergic mice. Distinct microbiota profiles were observed in peanut-allergic mice and those rendered tolerant after treatment with BNP. Identified bacterial taxa in this study with known action to increase intestinal epithelial integrity were strongly and inversely correlated with post-challenge plasma histamine and specific IgE. This study provides insight into important biological markers of food allergy for future mechanistic and therapeutic investigation.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: 10.6084/m9.figshare.23708382.

Ethics statement

The animal study was reviewed and approved by Mount Sinai vivarium.

Author contributions

KS, OF, NY and YS performed the experiment and data analyses. MC contributed to analysis and manuscript preparation. MM, AN-W and HS revised the manuscript. JZ and X-ML funded the study, and X-ML contributed to the conception of the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

X-ML received research support to her institution from the National Institutes of Health NIH/National Center for Complementary and Alternative Medicine NCCAM#1P01AT002644725-01" Center for Chinese Herbal Therapy CHT for Asthma", and grant #1 R01AT001495-01A1 and 2R01AT001495-05A2, NIH/NIAIDR43AI148039, Food Allergy Research and Education FARE,Winston Wolkoff Integrative Medicine Fund for Allergies and Wellness, the Parker Foundation and Henan University of Chinese Medicine; received consultancy fees from FARE and Johnson & Johnson Pharmaceutical Research & Development, L.L.C. received travel expenses from the NCCAM and FARE; shares US patent US7820175B2 FAHF-2, US10500169B2 XPP, US10406191B2 S. Flavescens, US10028985B2 WL; US11351157B2 nanoBBR: takes compensation from her practice at Center for Integrative Health and Acupuncture PC; US Times Technology Inc is managed by her related party; is a member of General Nutraceutical Technology LLC and Health Freedom LLC. NY received research support from the National Institutes of Health NIH/National Center for Complementary and Alternative Medicine NCCAM, NIH/NIAID R43AI148039; shares US patent: US10500169B2 XPP, US10406191B2 S. Flavescens, US10028985B2 WL; and is a member of General Nutraceutical Technology LLC and Health Freedom LLC. HS shares US patents US7820175B2 FAHF-2, US10500169B2 XPP, and US10406191B2 S. Flavescens. KS shares the patent US11351157B2 nanoBBR. Author KS was a member of General Nutraceutical Technology LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Haahtela T, Biodiversity Hypothesis A. *Allergy* (2019). doi: 10.1111/all.13763

2. Kataoka K. The intestinal microbiota and its role in human health and disease. *J Med Invest* (2016) 63(1-2):27–37. doi: 10.2152/jmi.63.27

3. Zhao W, Ho HE, Bunyavanich S. The gut microbiome in food allergy. *Ann Allergy Asthma Immunol* (2019) 122(3):276–82. doi: 10.1016/j.anai.2018.12.012

4. Marrs T, Sim K. Demystifying dysbiosis: can the gut microbiome promote oral tolerance over IgE-mediated food allergy? *Curr Pediatr Rev* (2018) 14(3):156–63. doi: 10.2174/1573396314666180507120424

5. Shu SA, Yuen AWT, Woo E, Chu KH, Kwan HS, Yang GX, et al. Microbiota and Food Allergy. *Clin Rev Allergy Immunol* (2019) 57(1):83–97. doi: 10.1007/s12016-018-8723-y

6. Renz H, Allen KJ, Sicherer SH, Sampson HA, Lack G, Beyer K, et al. Food allergy. *Nat Rev Dis Primers* (2018) 4:17098. doi: 10.1038/nrdp.2017.98

7. Koplin JJ, Peters RL, Allen KJ. Prevention of food allergies. *Immunol Allergy Clin North Am* (2018) 38(1):1–11. doi: 10.1016/j.iac.2017.09.001

8. Burbank AJ, Sood P, Vickery BP, Wood RA. Oral immunotherapy for food allergy. *Immunol Allergy Clin North Am* (2016) 36(1):55–69. doi: 10.1016/j.iac.2015.08.007

9. Sampson HA. Food allergy: past, present and future. *Allergol Int* (2016) 65 (4):363–9. doi: 10.1016/j.alit.2016.08.006

10. Pascal M, Perez-Gordo M, Caballero T, Escribese MM, Lopez Longo MN, Luengo O, et al. Microbiome and allergic diseases. *Front Immunol* (2018) 9:1584. doi: 10.3389/fimmu.2018.01584

11. Fazlollahi M, Chun Y, Grishin A, Wood RA, Burks AW, Dawson P, et al. Early-life gut microbiome and egg allergy. *Allergy* (2018) 73(7):1515–24. doi: 10.1111/all.13389

12. Prince BT, Mandel MJ, Nadeau K, Singh AM. Gut microbiome and the development of food allergy and allergic disease. *Pediatr Clin North Am* (2015) 62 (6):1479–92. doi: 10.1016/j.pcl.2015.07.007

13. Bunyavanich S, Shen N, Grishin A, Wood R, Burks W, Dawson P, et al. Early-life gut microbiome composition and milk allergy resolution. *J Allergy Clin Immunol* (2016) 138(4):1122–30. doi: 10.1016/j.jaci.2016.03.041

14. Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, et al. Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. *ISME J* (2016) 10(3):742–50. doi: 10.1038/ismej.2015.151

15. Andreassen M, Rudi K, Angell IL, Dirven H, Nygaard UC. Allergen immunization induces major changes in microbiota composition and short-chain fatty acid production in different gut segments in a mouse model of lupine food allergy. *Int Arch Allergy Immunol* (2018) 177(4):311–23. doi: 10.1159/000492006

16. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE, et al. Dietary fiber and bacterial SCFA enhance oral tolerance and protect against food allergy through diverse cellular pathways. *Cell Rep* (2016) 15(12):2809–24. doi: 10.1016/j.celrep.2016.05.047

17. Noval Rivas M, Burton OT, Wise P, Zhang YQ, Hobson SA, Garcia Lloret M, et al. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. *J Allergy Clin Immunol* (2013) 131(1):201–12. doi: 10.1016/j.jaci.2012.10.026

18. Srivastava KD, Kattan JD, Zou ZM, Li JH, Zhang L, Wallenstein S, et al. The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. *J Allergy Clin Immunol* (2005) 115(1):171–8. doi: 10.1016/j.jaci.2004.10.003

19. Song Y, Qu C, Srivastava K, Yang N, Busse P, Zhao W, et al. Food allergy herbal formula 2 protection against peanut anaphylactic reaction is via inhibition of mast cells and basophils. *J Allergy Clin Immunol* (2010) 126(6):1208–17 e3. doi: 10.1016/j.jaci.2010.09.013

20. Srivastava KD, Song Y, Yang N, Liu C, Goldberg IE, Nowak-Węgrzyn A, et al. B-FAHF-2 plus oral immunotherapy (OIT) is safer and more effective than OIT alone in a murine model of concurrent peanut/tree nut allergy. *Clin Exp Allergy* (2017). doi: 10.1111/cea.12936

21. Srivastava KD, Bardina L, Sampson HA, Li XM. Efficacy and immunological actions of FAHF-2 in a murine model of multiple food allergies. *Ann Allergy Asthma Immunol* (2012) 108(5):351–358 e1. doi: 10.1016/j.anai.2012.03.008

22. Yang N, Wang J, Liu C, Song Y, Zhang S, Zi J, et al. Berberine and limonin suppress IgE production by human b cells and peripheral blood mononuclear cells from food-allergic patients. *Ann Allergy Asthma Immunol* (2014) 113(5):556–564 e4. doi: 10.1016/j.anai.2014.07.021

23. Wang K, Feng X, Chai L, Cao S, Qiu F. The metabolism of berberine and its contribution to the pharmacological effects. *Drug Metab Rev* (2017) 49(2):139–57. doi: 10.1080/03602532.2017.1306544

24. Chen W, Miao YQ, Fan DJ, Yang SS, Lin X, Meng LK, et al. Bioavailability study of berberine and the enhancing effects of TPGS on intestinal absorption in rats. *AAPS PharmSciTech* (2011) 12(2):705–11. doi: 10.1208/s12249-011-9632-z

25. Yang N, Srivastava K, Song Y, Liu C, Cho S, Chen Y, et al. Berberine as a chemical and pharmacokinetic marker of the butanol-extracted food allergy herbal formula-2. *Int Immunopharmacol* (2017) 45:120–7. doi: 10.1016/j.intimp.2017.01.009

26. Bird JA, Spergel JM, Jones SM, Rachid R, Assa'ad AH, Wang J, et al. Efficacy and safety of AR101 in oral immunotherapy for peanut allergy: results of ARC001, a randomized, double-blind, placebo-controlled phase 2 clinical trial. *J Allergy Clin Immunol Pract* (2018) 6(2):476–485 e3. doi: 10.1016/j.jaip.2017.09.016

27. Wang J. Advances in the management of peanut allergy (oral immunotherapy and epicutaneous immunotherapy). *Allergy Asthma Proc* (2020) 41(1):5–9. doi: 10.2500/aap.2020.41.190011

28. Nowak-Wegrzyn A, Sato S, Fiocchi A, Ebisawa M. Oral and sublingual immunotherapy for food allergy. *Curr Opin Allergy Clin Immunol* (2019) 19(6):606–13. doi: 10.1097/ACI.0000000000000587

29. Lee TH, Chan JKC, Lau PC, Luk WP, Fung LH. Peanut allergy and oral immunotherapy. *Hong Kong Med J* (2019) 25(3):228–34. doi: 10.12809/hkmj187743

30. Turner PJ, Mehr S, Sayers R, Wong M, Shamji MH, Campbell DE, et al. Loss of allergenic proteins during boiling explains tolerance to boiled peanut in peanut allergy. *J Allergy Clin Immunol* (2014) 134(3):751–3. doi: 10.1016/j.jaci.2014.06.016

31. Kim J, Lee JY, Han Y, Ahn K. Significance of ara h 2 in clinical reactivity and effect of cooking methods on allergenicity. *Ann Allergy Asthma Immunol* (2013) 110 (1):34–8. doi: 10.1016/j.anai.2012.10.011

32. Beyer K, Morrow E, Li XM, Bardina L, Bannon GA, Burks AW, et al. Effects of cooking methods on peanut allergenicity. *J Allergy Clin Immunol* (2001) 107(6):1077–81. doi: 10.1067/mai.2001.115480

33. Grzeskowiak LE, Tao B, Aliakbari K, Chegeni N, Morris S, Chataway T. Oral immunotherapy using boiled peanuts for treating peanut allergy: an open-label, single-arm trial. *Clin Exp Allergy* (2023) 53(3):327–36. doi: 10.1111/cea.14254

34. Institute of Laboratory Animal Resources Commission of Life Sciences NRC. *Guide for the care and use of laboratory animals*. National Academic Press (1996).

35. Srivastava K, Yang N, Chen Y, Lopez-Exposito I, Song Y, Goldfarb J, et al. Efficacy, safety and immunological actions of butanol-extracted food allergy herbal formula-2 on peanut anaphylaxis. *Clin Exp Allergy* (2011) 41(4):582–91. doi: 10.1111/j.1365-2222.2010.03643.x

36. Srivastava KD, Seifert A, Fahmy TM, Caplan MJ, Li X-M, Sampson HA. Investigation of peanut oral immunotherapy using CpG/Peanut-nanoparticles in a murine model of peanut allergy. *J Allergy Clin Immunol* (2016) 135(2):AB235. doi: 10.1016/j.jaci.2014.12.1701

37. Srivastava KD, Siefert A, Fahmy TM, Caplan MJ, Li XM, Sampson HA. Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. *J Allergy Clin Immunol* (2016) 138(2):536–43.e4. doi: 10.1016/j.jaci.2014.12.1701

38. Qu C, Srivastava K, Ko J, Zhang TF, Sampson HA, Li XM. Induction of tolerance after establishment of peanut allergy by the food allergy herbal formula-2 is associated with up-regulation of interferon-gamma. *Clin Exp Allergy* (2007) 37(6):846–55. doi: 10.1111/j.1365-2222.2007.02718.x

39. Burks AW, Williams LW, Helm RM, Thresher W, Brooks JR, Sampson HA. Identification of soy protein allergens in patients with atopic dermatitis and positive soy challenges; determination of change in allergenicity after heating or enzyme digestion. *Adv Exp Med Biol* (1991) 289:295–307. doi: 10.1007/978-1-4899-2626-5_22

40. Hussain M, Bonilla-Rosso G, Kwong Chung CKC, Bariswyl L, Rodriguez MP, Kim BS, et al. High dietary fat intake induces a microbiota signature that promotes food allergy. *J Allergy Clin Immunol* (2019) 144(1):157–170.e8. doi: 10.1016/j.jaci.2019.01.043

41. Williams BL, Hornig M, Buie T, Bauman ML, Cho Paik M, Wick I, et al. Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. *PloS One* (2011) 6(9):e24585. doi: 10.1371/journal.pone.0024585

42. Melli LC, do Carmo-Rodrigues MS, Araujo-Filho HB, Sole D, de Morais MB. Intestinal microbiota and allergic diseases: a systematic review. *Allergol Immunopathol (Madr)* (2016) 44(2):177–88. doi: 10.1016/j.aller.2015.01.013

43. Ling Z, Li Z, Liu X, Cheng Y, Luo Y, Tong X, et al. Altered fecal microbiota composition associated with food allergy in infants. *Appl Environ Microbiol* (2014) 80 (8):2546–54. doi: 10.1128/AEM.00003-14

44. Liao ZG, Tang T, Guan XJ, Dong W, Zhang J, Zhao GW, et al. Improvement of transmembrane transport mechanism study of imperatorin on p-Glycoprotein-Mediated drug transport. *Molecules* (2016) 21(12):1606. doi: 10.3390/molecules21121606

45. Liang XL, Zhang J, Zhao GW, Li Z, Luo Y, Liao ZG, et al. Mechanisms of improvement of intestinal transport of baicalin and puerarin by extracts of radix angelicae dahuricae. *Phytother Res* (2015) 29(2):220–7. doi: 10.1002/ptr.5242

46. Chen C, Wu C, Lu X, Yan Z, Gao J, Zhao H, et al. Coniferyl ferulate, a strong inhibitor of glutathione s-transferase isolated from radix angelicae sinensis, reverses multidrug resistance and downregulates p-glycoprotein. *Evid Based Complement Alternat Med* (2013) 2013:639083. doi: 10.3390/molecules21121606

47. Zhang YT, Yu YQ, Yan XX, Wang WJ, Tian XT, Wang L, et al. Different structures of berberine and five other protoberberine alkaloids that affect

p-glycoprotein-mediated efflux capacity. *Acta Pharmacol Sin* (2019) 40(1):133–42. doi: 10.1038/s41401-018-0183-7

48. Zhang X, Qiu F, Jiang J, Gao C, Tan Y. Intestinal absorption mechanisms of berberine, palmatine, jateorhizine, and coptisine: involvement of p-glycoprotein. *Xenobiotica* (2011) 41(4):290–6. doi: 10.3109/00498254.2010.529180

49. Shan YQ, Zhu YP, Pang J, Wang YX, Song DQ, Kong WJ, et al. Tetrandrine potentiates the hypoglycemic efficacy of berberine by inhibiting p-glycoprotein function. *Biol Pharm Bull* (2013) 36(10):1562–9. doi: 10.1248/bpb.b13-00272

50. Lv Z, Peng G, Liu W, Xu H, Su J. Berberine blocks the relapse of clostridium difficile infection in C57BL/6 mice after standard vancomycin treatment. *Antimicrob Agents Chemother* (2015) 59(7):3726–35. doi: 10.1128/AAC.04794-14

51. Ma X, Chen Z, Wang L, Wang G, Wang Z, Dong X, et al. The pathogenesis of diabetes mellitus by oxidative stress and inflammation: its inhibition by berberine. *Front Pharmacol* (2018) 9:782. doi: 10.3389/fphar.2018.00782

52. Pirillo A, Catapano AL. Berberine, a plant alkaloid with lipid- and glucose-lowering properties: from *in vitro* evidence to clinical studies. *Atherosclerosis* (2015) 243 (2):449–61. doi: 10.1016/j.atherosclerosis.2015.09.032

53. Sirtori CR, Pavanello C, Calabresi L, Ruscica M. Nutraceutical approaches to metabolic syndrome. *Ann Med* (2017) 49(8):678–97. doi: 10.1080/07853890.2017.1366042

54. Yan HM, Xia MF, Wang Y, Chang XX, Yao XZ, Rao SX, et al. Efficacy of berberine in patients with non-alcoholic fatty liver disease. *PloS One* (2015) 10(8): e0134172. doi: 10.1371/journal.pone.0134172

55. Lan J, Zhao Y, Dong F, Yan Z, Zheng W, Fan J, et al. Meta-analysis of the effect and safety of berberine in the treatment of type 2 diabetes mellitus, hyperlipemia and hypertension. *J Ethnopharmacol* (2015) 161:69–81. doi: 10.1016/j.jep.2014.09.049

56. Jia X, Jia L, Mo L, Yuan S, Zheng X, He J, et al. Berberine ameliorates periodontal bone loss by regulating gut microbiota. *J Dent Res* (2019) 98(1):107–16. doi: 10.1177/0022034518797275

57. Tian Y, Cai J, Gui W, Nichols RG, Koo I, Zhang J, et al. Berberine directly affects the gut microbiota to promote intestinal farnesoid X receptor activation. *Drug Metab Dispos* (2019) 47(2):86–93. doi: 10.1124/dmd.118.083691

58. Zhu L, Zhang D, Zhu H, Zhu J, Weng S, Dong L, et al. Berberine treatment increases akkermansia in the gut and improves high-fat diet-induced atherosclerosis in apoe(-/-) mice. *Atherosclerosis* (2018) 268:117–26. doi: 10.1016/j.atherosclerosis.2017.11.023

59. Guo Y, Zhang Y, Huang W, Selwyn FP, Klaassen CD. Dose-response effect of berberine on bile acid profile and gut microbiota in mice. *BMC Complement Altern Med* (2016) 16(1):394. doi: 10.1186/s12906-016-1367-7

60. Kraneveld AD, Szklany K, de Theije CG, Garssen J. Gut-to-Brain axis in autism spectrum disorders: central role for the microbiome. *Int Rev Neurobiol* (2016) 131:263–87. doi: 10.1016/bs.irn.2016.09.001

61. Ather JL, Chung M, Hoyt LR, Randall MJ, Georgsdottir A, Daphtary NA, et al. Weight loss decreases inherent and allergic methacholine hyperresponsiveness in mouse models of diet-induced obese asthma. *Am J Respir Cell Mol Biol* (2016) 55 (2):176–87. doi: 10.1165/rcmb.2016-0070OC

62. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep* (2012) 13(5):440–7. doi: 10.1038/embor.2012.32

63. West CE, Renz H, Jenmalm MC, Kozyrskyj AL, Allen KJ, Vuillermin P, et al. The gut microbiota and inflammatory noncommunicable diseases: associations and potentials for gut microbiota therapies. *J Allergy Clin Immunol* (2015) 135(1):3–13. doi: 10.1016/j.jaci.2014.11.012

64. Zeng MY, Inohara N, Nunez G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol* (2017) 10(1):18–26. doi: 10.1038/mi.2016.75

65. Thorburn AN, Macia L, Mackay CR. Diet, metabolites, and "western-lifestyle" inflammatory diseases. *Immunity* (2014) 40(6):833–42. doi: 10.1016/j.immuni.2014.05.014

66. Shu SA, Yuen AWT, Woo E, Chu KH, Kwan HS, Yang GX, et al. Microbiota and food allergy. *Clin Rev Allergy Immunol* (2019) 57(1):83–97. doi: 10.1007/s12016-018-8723-y

67. Bunyavanich S. Food allergy: could the gut microbiota hold the key? *Nat Rev Gastroenterol Hepatol* (2019) (4):201–2. doi: 10.1038/s41575-019-0123-0

68. Kuhn KA, Schulz HM, Regner EH, Severs EL, Hendrickson JD, Mehta G, et al. Bacteroidales recruit IL-6-producing intraepithelial lymphocytes in the colon to promote barrier integrity. *Mucosal Immunol* (2018) 11(2):357–68. doi: 10.1038/mi.2017.55

69. Reunanen J, Kainulainen V, Huuskonen L, Ottman N, Belzer C, Huhtinen H, et al. Akkermansia muciniphila adheres to enterocytes and strengthens the integrity of the epithelial cell layer. *Appl Environ Microbiol* (2015) 81(11):3655–62. doi: 10.1128/AEM.04050-14

70. Derrien M, Belzer C, de Vos WM. Akkermansia muciniphila and its role in regulating host functions. *Microb Pathog* (2017) 106:171–81. doi: 10.1016/j.micpath.2016.02.005

71. Ottman N, Geerlings SY, Aalvink S, de Vos WM, Belzer C. Action and function of akkermansia muciniphila in microbiome ecology, health and disease. *Best Pract Res Clin Gastroenterol* (2017) 31(6):637–42. doi: 10.1016/j.bpg.2017.10.001

72. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci U.S.A.* (2013) 110(22):9066–71. doi: 10.1073/pnas.1219451110

73. Zhao S, Liu W, Wang J, Shi J, Sun Y, Wang W, et al. Akkermansia muciniphila improves metabolic profiles by reducing inflammation in chow diet-fed mice. *J Mol Endocrinol* (2017) 58(1):1–14. doi: 10.1530/JME-16-0054

74. Anhe FF, Roy D, Pilon G, Dudonne S, Matamoros S, Varin TV, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased akkermansia spp. *population gut microbiota mice Gut* (2015) 64(6):872–83. doi: 10.1136/gutjnl-2014-307142

75. de Vos WM. Microbe profile: akkermansia muciniphila: a conserved intestinal symbiont that acts as the gatekeeper of our mucosa. *Microbiology* (2017) 163(5):646–8. doi: 10.1099/mic.0.000444

76. Ottman N, Reunanen J, Meijerink M, Pietila TE, Kainulainen V, Klievink J, et al. Pili-like proteins of akkermansia muciniphila modulate host immune responses and gut barrier function. *PloS One* (2017) 12(3):e0173004. doi: 10.1371/journal.pone.0173004

77. Boulet LP. Obesity and atopy. *Clin Exp Allergy* (2015) 45(1):75–86. doi: 10.1111/cea.12435

78. Baumann S, Lorentz A. Obesity - a promoter of allergy? *Int Arch Allergy Immunol* (2013) 162(3):205–13. doi: 10.1159/000353972

79. Ray JP, Staron MM, Shyer JA, Ho PC, Marshall HD, Gray SM, et al. The interleukin-2-mTORc1 kinase axis defines the signaling, differentiation, and metabolism of T helper 1 and follicular b helper T cells. *Immunity* (2015) 43(4):690–702. doi: 10.1016/j.immuni.2015.08.017

80. Ramiscal RR, Parish IA, Lee-Young RS, Babon JJ, Blagih J, Pratama A, et al. Attenuation of AMPK signaling by ROQUIN promotes T follicular helper cell formation. *Elife* (2015) 4:e08698. doi: 10.7554/eLife.08698

81. Lam WY, Bhattacharya D. Metabolic links between plasma cell survival, secretion, and stress. *Trends Immunol* (2018) 39(1):19–27. doi: 10.1016/j.it.2017.08.007

82. Leonard SA, Martos G, Wang W, Nowak-Wegrzyn A, Berin MC. Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa. *J Allergy Clin Immunol* (2012) 129(6):1579–1587 e1. doi: 10.1016/j.jaci.2012.04.009

83. Zhang T, Shi Y, Zhao Y, Tang G, Niu B, Chen Q. Boiling and roasting treatment affecting the peanut allergenicity. *Ann Transl Med* (2018) 6(18):357. doi: 10.21037/atm.2018.05.08

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Murine model identifies tropomyosin as IgE cross-reactive protein between house dust mite and coho salmon that possibly contributes to the development of salmon allergy

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Background: Recently, we have developed a method to identify IgE cross-reactive allergens. However, the mechanism by which IgE cross-reactive allergens cause food allergy is not yet fully understood how. In this study, we aimed to understand the underlying pathogenesis by identifying food allergens that cross-react with house dust mite allergens in a murine model.

Material and methods: Allergenic protein microarray analysis was conducted using serum from mice intraperitoneally injected with *Dermatophagoides pteronyssinus* (Der p) extract plus alum or alum alone as controls. Der p, *Dermatophagoides farinae* (Der f), coho salmon extract-sensitized and control mice were analyzed. Serum levels of IgE against Der p, Der f, coho salmon extract, protein fractions of coho salmon extract separated by ammonium sulfate precipitation and anion exchange chromatography, and recombinant coho salmon tropomyosin or actin were measured by an enzyme-linked immunosorbent assay. A murine model of cutaneous anaphylaxis or oral allergy syndrome (OAS) was established in Der p extract-sensitized mice stimulated with coho salmon extract, tropomyosin, or actin.

Results: Protein microarray analysis showed that coho salmon-derived proteins were highly bound to serum IgE in Der p extract-sensitized mice. Serum IgE from Der p or Der f extract-sensitized mice was bound to coho salmon extract,

whereas serum IgE from coho salmon extract-sensitized mice was bound to Der p or Der f extract. Analysis of the murine model showed that cutaneous anaphylaxis and oral allergic reaction were evident in Der p extract-sensitized mice stimulated by coho salmon extract. Serum IgE from Der p or Der f extract-sensitized mice was bound strongly to protein fractions separated by anion exchange chromatography of coho salmon proteins precipitated with 50% ammonium sulfate, which massively contained the approximately 38 kDa protein. We found that serum IgE from Der p extract-sensitized mice was bound to recombinant coho salmon tropomyosin. Der p extract-sensitized mice exhibited cutaneous anaphylaxis in response to coho salmon tropomyosin.

Conclusion: Our results showed IgE cross-reactivity of tropomyosin between *Dermatophagoides* and coho salmon which illustrates salmon allergy following sensitization with the house dust mite *Dermatophagoides*. Our method for identifying IgE cross-reactive allergens will help understand the underlying mechanisms of food allergies.

KEYWORDS

food allergy, IgE cross-reactivity, house dust mite, salmon, mast cell, murine model

Introduction

The incidence of allergic diseases, such as food allergies, atopic dermatitis, and allergic rhinitis, is increasing worldwide. Food allergies, including oral allergy syndrome (OAS), are an increasing public health concern. Food allergen-specific immunoglobulin E (IgE) production, also known as IgE sensitization to food allergens, often precedes the development of food allergies. Intake of the same food allergen induces the crosslinking of its specific IgE-bound high-affinity IgE receptor (FcεRI) with the same allergen in mast cells. Consequently, mast cells degranulate and release various chemical mediators, leading to food allergy symptoms, such as diarrhea, abdominal pain, and anaphylaxis in severe cases (1–8). In the case of OAS, mast cell degranulation in the oral and pharyngeal tissues causes itching and/or angioedema of the lip, tongue, and palate. Pollen food allergy syndrome (PFAS) is a pollinosis-associated OAS; IgE cross-reactive allergens between certain foods (e.g., fruits and vegetables) and pollens (e.g., birch and ragweed pollens) play critical roles in the development of PFAS (9–15). Understanding how some people become sensitized to specific food allergens is important for the prevention of food allergies. We recently developed a method for the comprehensive identification of foods that may cross-react with pollen, using murine models of sensitization to pollen and allergic protein microarray technology (15). This encouraged us to apply our method to identify IgE cross-reactivity among various environmental allergens, including food allergens.

House dust mites (HDM) are well-known allergens that contribute to the development of allergic diseases, such as atopic dermatitis, allergic rhinitis, and allergic asthma with specific IgEs against HDM allergens. *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) are the most known HDM species and have been reported to include up to 40 distinct allergens (16, 17). Among them, mite tropomyosin Der p 10 or Der f 10 shows IgE cross-reactivity with shrimp tropomyosin, which is thought to be responsible for shrimp allergy (18, 19). However, a causal relationship between HDM and food allergies is not yet fully understood.

Seafood can be classified into three different groups: arthropods, mollusks and vertebrates. Crustaceans (e.g., shrimp and crab) are arthropods, while cephalopods (e.g., squid and octopus) and bivalves (e.g., clams and oysters) are mollusks. Fish (e.g., salmon and tuna) are aquatic vertebrates. Fish allergy is also an IgE-mediated food allergy, and its prevalence has increased, particularly in countries with high fish consumption. Parvalbumin, a fish pan-allergen, is a low-molecular-weight protein abundant in fish muscle. Enolase, aldolase, and gelatin are recognized fish allergens. A recent study using serum from patients with salmon or catfish allergies identified tropomyosin as an important allergen, indicating that tropomyosin, in addition to parvalbumin, should be considered an important fish allergen to improve the diagnosis of fish allergy (20–22).

In this study, we identified coho salmon as a food that may cross-react with Der p extract using a murine model of sensitization to Der p extract and protein microarray technology (15). *In vitro* and *in vivo* analyses demonstrated IgE cross-reactivity between Der p and coho salmon extracts. Tropomyosin was identified as an IgE

Abbreviations: ANOVA, analysis of variance; CBB, Coomassie Brilliant Blue; Der f, *Dermatophagoides farinae*; Der p, *Dermatophagoides pteronyssinus*; ELISA, Enzyme-linked immunosorbent assay; FcεRI, high-affinity IgE receptor; HDM, house dust mite; IgE, immunoglobulin E; OAS, oral allergy syndrome; PBS, phosphate-buffered saline; PFAS, pollen food allergy syndrome; SD, standard deviation.

cross-reactive protein that mediates local anaphylactic reactions in a murine model of cutaneous allergy and OAS. Our method will be useful for clarifying the pathogenesis of food allergies caused by IgE cross-reactivity between environmental proteins.

Materials and methods

Mice

Female BALB/c mice (aged 8–12 weeks) were used in this study. All the procedures were approved by the Institutional Review Committee of Juntendo University (approval numbers: 2021188, 2022099, and 2023129).

Reagents

The Der p extract (Biostir Inc., Kobe, Japan) was used, as shown in Figures 1, 2, 4. Der f extract (Greer Laboratories, Lenoir, NC) or Der p extract (Greer Laboratories, Lenoir, NC) was used, as shown in Figures 3, 5, 6. A fillet of commercially available coho salmon in Japan was used to prepare coho salmon (*Oncorhynchus kisutch*) extract.

Allergenic protein microarray

BALB/c mice were intraperitoneally injected with 30 μg Der p extract plus 2 mg alum or with phosphate-buffered saline (PBS) plus 2 mg alum as a control six times at 1-week intervals to generate Der p

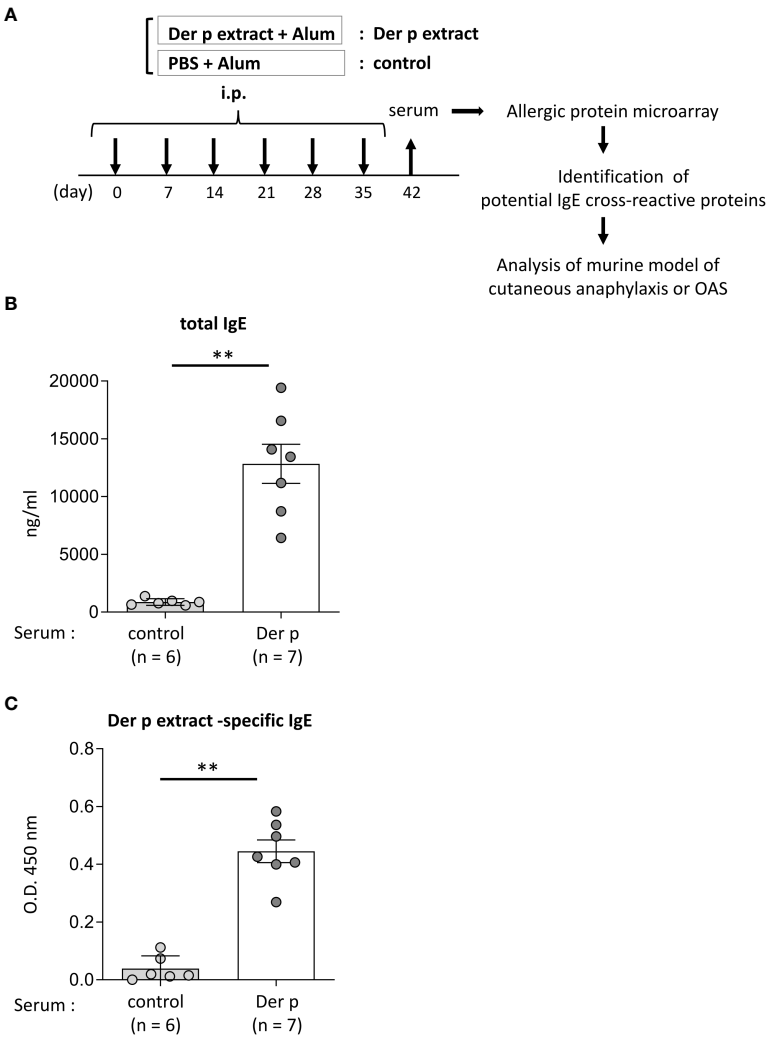

FIGURE 1
Identification of foods that may cross-react with Der p extract by using a murine model of sensitization with Der p extract and allergenic protein microarray technology.**(A)** Allergenic protein microarray analysis was performed using serum from BALB/c mice that had been intraperitoneally injected with Der p extract plus alum (Der p extract) or PBS plus alum as a control (control) six times at a 1- week interval, prior to analysis of the murine model of cutaneous anaphylaxis or OAS. **(B, C)** Serum levels of total IgE **(B)** and Der p extract-specific IgE **(C)** from mice on day 42. Data are representative of two independent experiments. Means ± SD have been plotted. $^{**}P < 0.01$.

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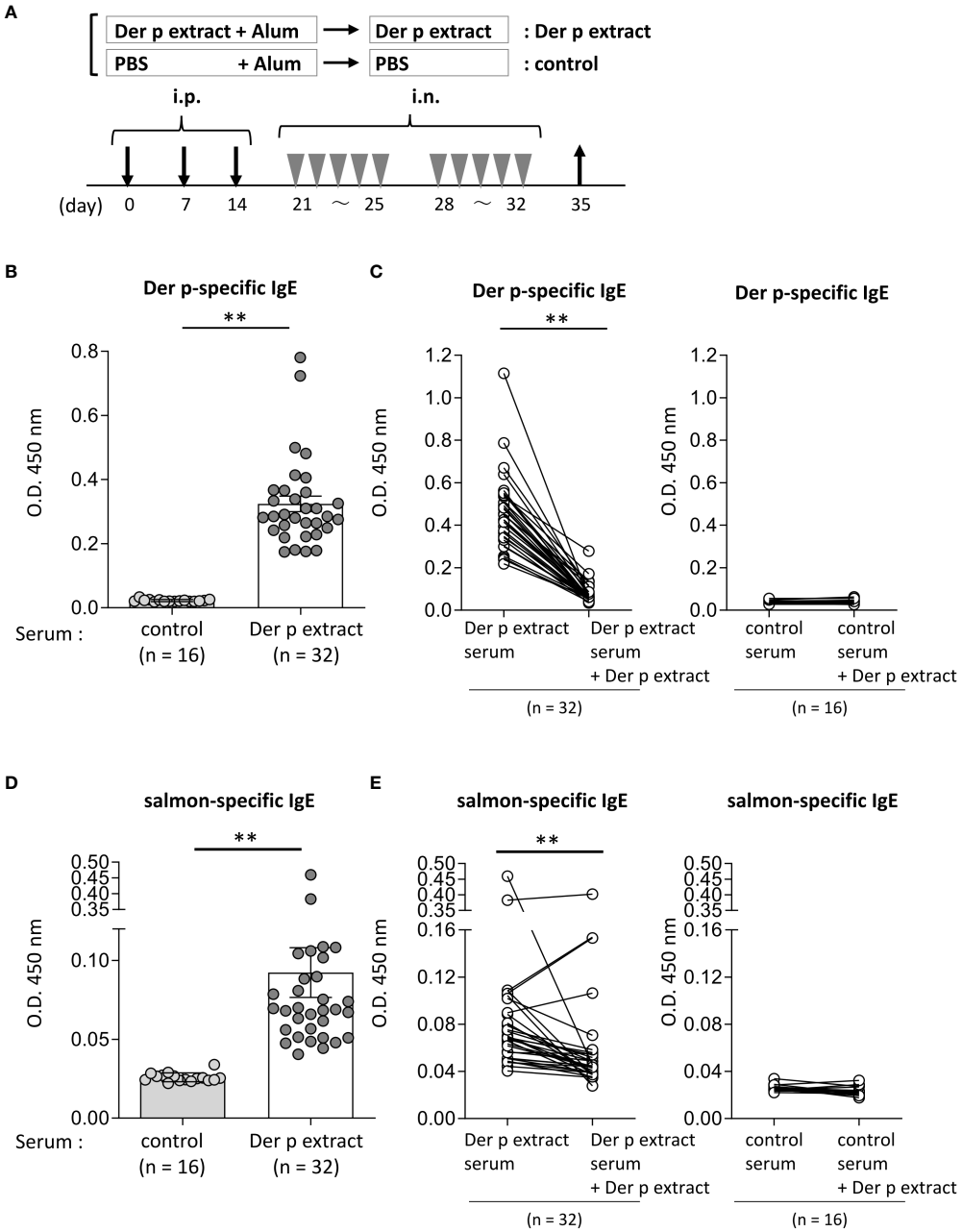

FIGURE 2
Serum IgE from mice sensitized with the Der p extract was significantly bound to the salmon extract. **(A)** A schematic representation of mouse sensitization with Der p extract. Mice were intraperitoneally injected with Der p extract plus alum or PBS plus alum alone followed by intranasal administration of Der p extract or PBS to generate Der p extract-sensitized mice (Der p extract) or control mice (control), respectively. On day 35, blood samples were obtained. **(B, D)** Serum levels of specific IgE from Der p extract-sensitized mice or control mice against **(B)** Der p extract and **(D)** coho salmon extract. **(C, E)** Serum levels of specific IgE from Der p extract-sensitized mice or control mice against **(D)** Der p extract and **(F)** coho salmon extract after serum preincubation with Der p extract or PBS as a control. **(B–E)** Data were pooled from three independent experiments. Means ± SD have been plotted. $^{**}P < 0.01$.

extract-sensitized or control mice, respectively. The serum obtained from these mice on day 42 was incubated on microarray plates coated with 1178 types of crude allergenic protein extracts (Fukushima Translational Research Project, Fukushima, Japan), including plants (e.g., vegetables and fruits), animals, processed foods (e.g., fish, shellfish, insects, ticks, parasites, meats, eggs, cheeses, and yogurts), and microorganisms (e.g., bacteria and fungi). *Oncorhynchus kisutch* (coho salmon), *Scomber japonicus* (Chub mackerel), *Gadus macrocephalus* (Pacific cod), *Sardinops melanostictus* (sardine), and *Thunnes orientalis* (Pacific bluefin tuna) were used as fish. Alexa Fluor 647-conjugated anti-mouse IgE antibody was added to the wells before the microarray plates were scanned with a GenePix 4000 B scanner (Molecular Devices, San Jose, CA, USA) to measure fluorescence intensity. The relative binding intensity of serum IgE to each protein extract was calculated by subtracting the IgE binding intensity of control mice from that of the Der p extract-sensitized mice (15, 23).

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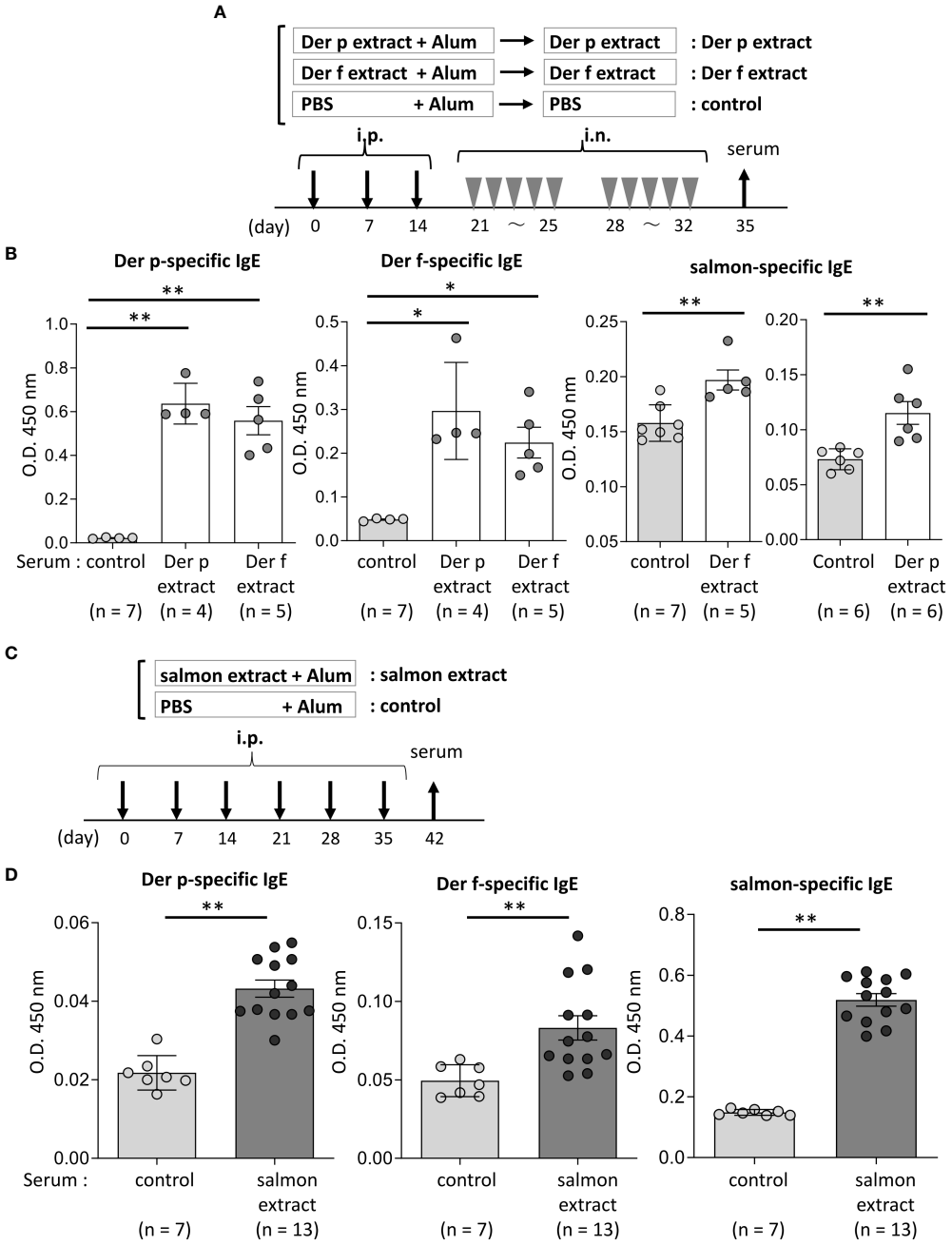

FIGURE 3
Serum IgE from mice sensitized with coho salmon extract was significantly bound to Der p and Der f extract. **(A)** A schematic representation of mouse sensitization with Der p or Der f extract. Mice were intraperitoneally injected with Der p or Der f extract plus alum or PBS plus alum alone followed by intranasal administration of Der p or Der f extract or PBS to generate Der p or Der f extract-sensitized mice (Der p extract or Der p extract) or control mice (control), respectively. On day 35, blood samples were obtained. **(B)** Serum levels of specific IgE from Der p or Der f extract-sensitized mice or control mice against Der p extract (left), Der f extract (middle), or coho salmon extract (right). **(C)** A schematic representation of mouse sensitization with coho salmon extract. Mice were intraperitoneally injected with coho salmon extract plus alum or PBS plus alum six times at a 1- week interval to generate coho salmon extract-sensitized mice (salmon extract) or control mice (control), respectively. On day 42, blood samples were obtained. **(D)** Serum levels of specific IgE from salmon extract-sensitized mice or control mice against Der p extract (left), Der f extract (middle), or coho salmon extract (right). **(B, D)** Data are representative of two independent experiments. Means ± SD have been plotted. $*P < 0.05$ or $**P < 0.01$.

Production of recombinant protein of coho salmon tropomyosin or actin

The amino acid sequences of *Oncorhynchus kisutch* (coho salmon) tropomyosin and actin were retrieved from UniProt (tropomyosin 1, accession: A0A8C7KTN0, https://www.uniprot.org/uniprotkb/A0A8C7KTN0/entry; and actin, alpha skeletal muscle 2-like, accession: A0A8C7G2G1, https://www.uniprot.org/uniprotkb/A0A8C7G2G1/entry). The sequences were reverse-translated into codon-optimized DNA sequences for *Escherichia*

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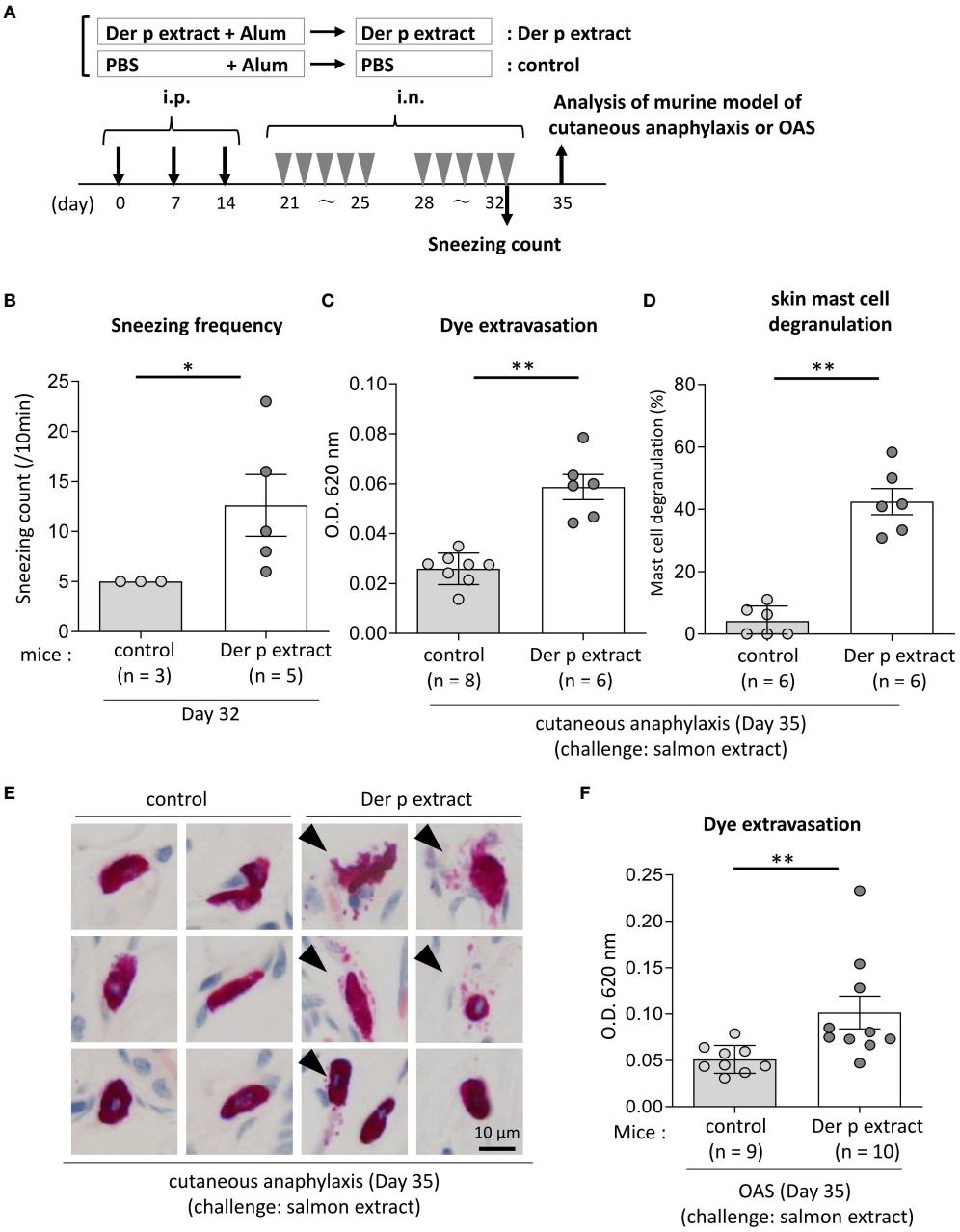

FIGURE 4
Both cutaneous anaphylaxis and oral allergy were evident in Der p extract-sensitized mice after administration of coho salmon extract. **(A)** A schematic representation of the murine model of cutaneous anaphylaxis or OAS. Mice were intraperitoneally injected with Der p extract plus alum or PBS plus alum followed by intranasal administration of Der p extract or PBS to generate Der p extract-sensitized mice (Der p extract) or control mice (control), respectively. On day 35, mice were challenged with coho salmon extract. **(B)** The frequency of sneezing during 30 min after nasal administration of Der p extract or PBS on day 32. **(C–E)** The ear skin was removed from Der p extract-sensitized mice or control mice stimulated by coho salmon extract in murine model of cutaneous anaphylaxis. **(C)** Quantification of the Evans blue dye. **(D)** Percentages of degranulated mast cells. **(E)** Representative images of chloroacetate esterase-stained mast cells in tissue sections. The arrowhead indicates the degranulated mast cell. **(F)** Quantification of the Evans blue dye that extravasated into the neck skin from Der p extract-sensitized mice or control mice stimulated by coho salmon extract in the murine model of OAS. **(B–F)** Data are representative of two independent experiments. Means ± SD have been plotted. $*P < 0.05$ or $**P < 0.01$.

coli expression. N-terminal Strep-II-tagged and C-terminal His6-tagged sequences were synthesized at Eurofins Genomics (Tokyo, Japan). The DNA fragments were cloned into the pET24(+) vector using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, MA, U.S.A.) according to the manufacturer's instructions. The recombinant proteins were expressed in SHuffle T7 Express lysY- competent *Escherichia coli* (New England Biolabs, MA, USA) according to the manufacturer's instructions. The cells

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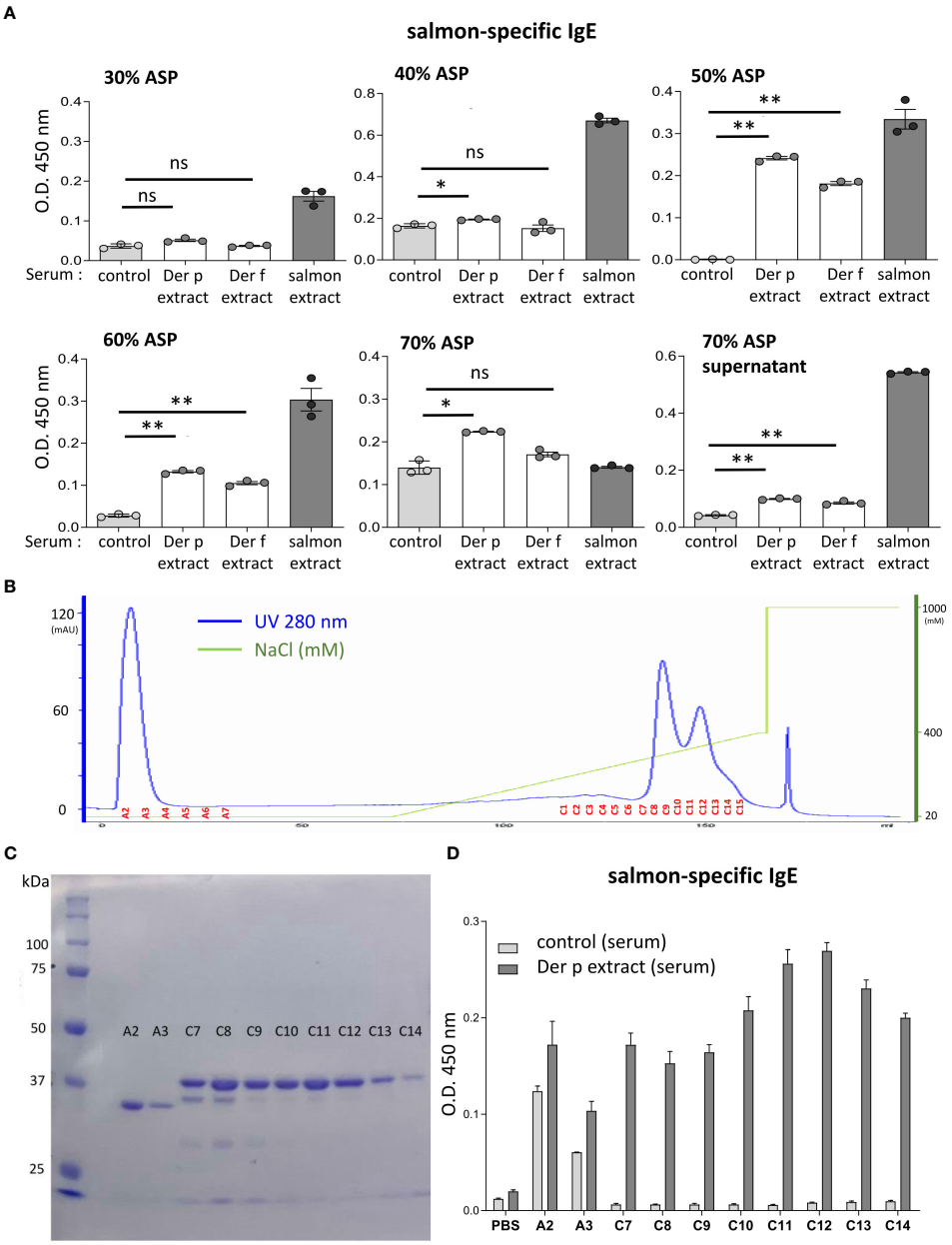

FIGURE 5
The molecular weight of the approximately 38-kDa protein included in coho salmon extract was possibly recognized by serum IgE from Der p extract-sensitized mice. **(A)** Serum levels of specific IgE from Der p or Der f extract-sensitized mice (Der p extract or Der f extract), control mice (control), or coho salmon extract-sensitized mice (salmon extract) against coho salmon proteins precipitated with 30%, 40%, 50%, 60%, or 70% ammonium sulfate (30% ASP, 40% ASP, 50% ASP, 60% ASP, 70% ASP) or soluble in 70% ammonium sulfate (70% ASP supernatant). **(B)** Coho salmon proteins precipitated with 50% ammonium sulfate were subjected to anion exchange chromatography. Spectra of anion exchange chromatography fractions are shown. The blue line indicates a UV 280 nm signal. The green line indicates NaCl concentration in the buffer. **(C)** Equivalent amounts of total protein in each fraction of A2, A3, and C7 to C14 were subjected to SDS-PAGE before CBB staining. **(D)** Serum levels of specific IgE from Der p extract-sensitized mice or control mice against proteins of each fraction (A2, A3, and C7 to C14), salmon extracts, or vehicle (PBS). **(A–D)** Data are representative of two independent experiments. **(A)** Means ± SD have been plotted. $*P < 0.05$ or $**P < 0.01$. ns, not significant.

were harvested by centrifugation and lysed by sonication in 100 mM Tris-HCl (pH 8.0) buffer containing 150 mM NaCl, 200 mM EDTA 200 mM, 1% Triton X-100, 1 mM PMSF, and 1 mg/mL lysozyme (Merck, Darmstadt, Germany). The protein was purified using Strep-Tactin®XT 4Flow® gravity flow column (IBA Lifesciences, Germany) according to the manufacturer's instructions.

Separation of proteins from coho salmon extract

The coho salmon (*Oncorhynchus kisutch*) meat was homogenized in PBS or in 1% NP40 lysis buffer containing 1 M Tris-HCl (pH 7.4), 10% glycerol, 137 mM NaCl, and Halt Protease

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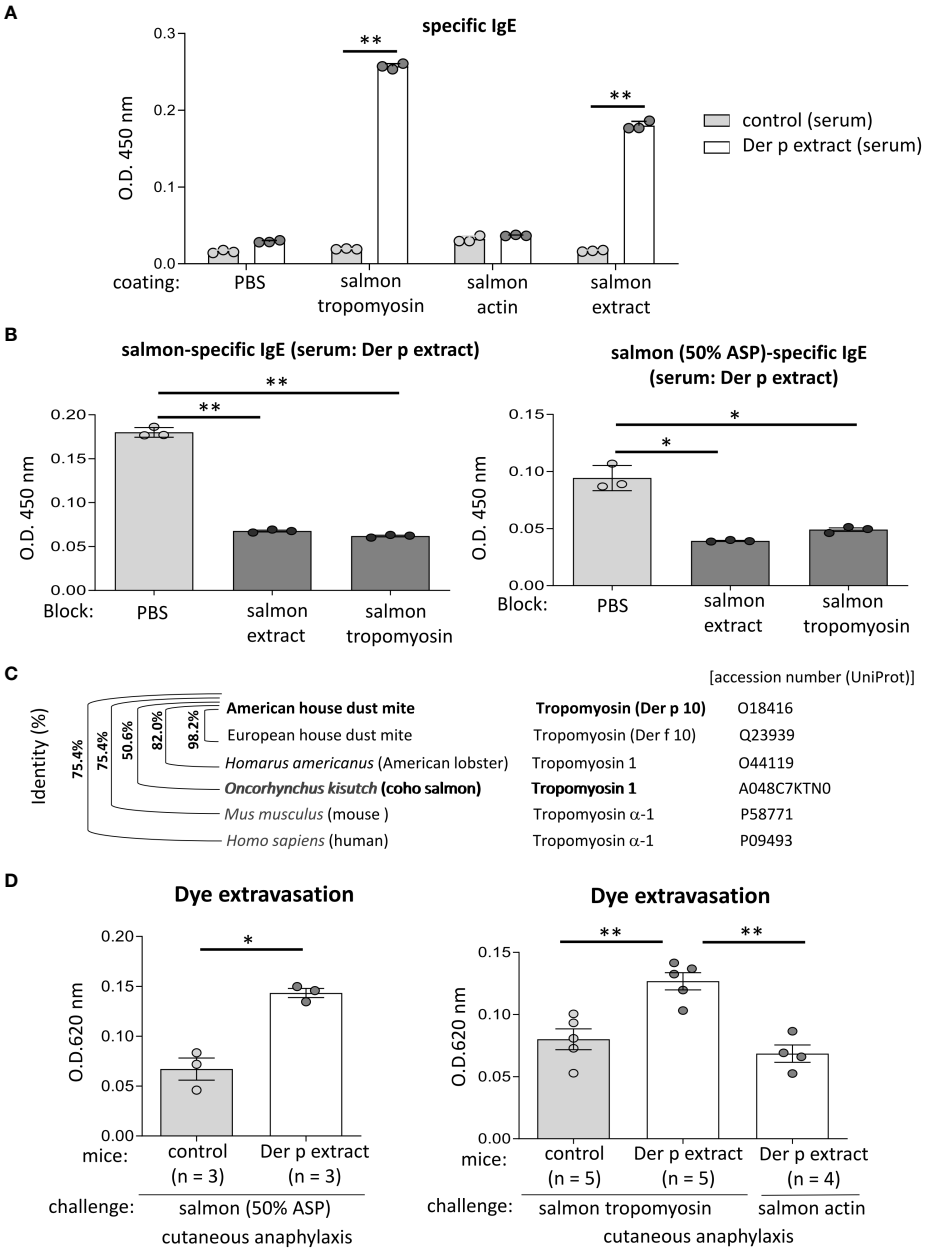

FIGURE 6
Tropomyosin was identified as IgE cross-reactive protein between Der p and coho salmon extract. **(A)** Serum levels of specific IgE from Der p extract-sensitized mice (Der p extract) or control mice (control) against recombinant coho salmon tropomyosin or actin, coho salmon extracts, or vehicle (PBS). **(B)** Serum levels of specific IgE from Der p extract-sensitized mice after serum preincubation with coho salmon extract, recombinant coho salmon tropomyosin, or PBS. **(C)** Percentages of amino acid sequence identity of Der p 10/tropomyosin from American house dust mite with Der f 10/tropomyosin from European house dust mite, tropomyosin 1 from *Homarus americanus* (American lobster), tropomyosin 1 from *Oncorhynchus kisutch* (coho salmon), tropomyosin α-1 from *Mus musculus* (mouse), or tropomyosin α-1 from *Homo sapiens* (human). The accession number (UniProt) is shown. **(D)** Quantification of the Evans blue dye that extravasated into the ear skin from Der p extract-sensitized mice or control mice stimulated by coho salmon proteins precipitated with 50% ammonium sulfate (left panel) or recombinant coho salmon tropomyosin or actin (right panel) in murine model of cutaneous anaphylaxis. **(A, B, D)** Data are representative of two independent experiments. Means ± SD have been plotted. *$P < 0.05$ or **$P < 0.01$.

Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation, the supernatant was serially treated with two concentrations of ammonium sulfate; the fractions soluble in 20%, 30%, 40%, 50%, 60% ammonium sulfate but insoluble in 30%, 40%, 50%, 60%, 70% ammonium sulfate, respectively, were dissolved in deionized water. The solution was dialyzed against PBS and the protein concentration of each sample was measured using the Pierce BCA Protein Assay (Thermo Fisher Scientific). To further separate coho salmon proteins by anion exchange chromatography, five milligrams of coho salmon proteins precipitated with 50% ammonium sulfate was dissolved in 40 mL buffer A (20 mM Tris-HCl (pH 7.5)/20 mM NaCl) and loaded onto RESOURCE Q anion exchange column (GE Healthcare Life Sciences, Marlborough, MA). After elution with buffer A, proteins were eluted by a linear gradient

from 0 to 40% buffer B (20 mM Tris-HCl (pH 7.5)/1M NaCl) at a flow rate of 6 mL/min to collect fractions (500 µL each one). The protein concentration of each fraction was measured using the Pierce BCA Protein Assay (Thermo Fisher Scientific). Equal amounts of protein in each fraction were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie Brilliant Blue (CBB) staining.

Evaluation of total or specific IgE

Enzyme-linked immunosorbent assay (ELISA) kits for total IgE (eBioscience, San Diego, CA, USA) were used. Specific IgE against Der p, Der f, or coho salmon extract, and recombinant proteins of coho salmon tropomyosin or actin were determined using luminescence ELISA as previously described (15, 24, 25). BALB/c mice were intraperitoneally injected with 30 µg of Der p or Der f extract plus 2 mg alum or with PBS plus 2 mg alum three times at 1-week intervals (days 0, 7, and 14), followed by intranasal administration of 20 µg (20 µL) of Der p extract or PBS (20 µL) five times a week for two weeks (days 21-25 and 28-32), to generate Der p or Der f extract-sensitized mice or control mice, respectively. Alternatively, BALB/c mice were intraperitoneally injected with 30 µg coho salmon extract plus 2 mg alum or PBS plus 2 mg alum six times at 1-week intervals (days 0, 7, 14, 21, 28, and 35) to generate coho salmon extract-sensitized mice or control mice, respectively. ELISA plates were coated with 20 µg/mL of Der p, Der f, or coho salmon extract, recombinant coho salmon tropomyosin or actin, and blocked with a blocking buffer containing 10% bovine serum albumin before adding serial dilutions of serum samples. For competitive ELISA, serum samples were preincubated with 1 mg/mL Der p extract, coho salmon extract, recombinant coho salmon tropomyosin, or PBS. After the plate incubation, a biotinylated anti-IgE antibody (R35-118) (BD Pharmingen, San Diego, CA, USA) and 3,3',5,5'-tetramethylbenzidine substrate solution were added. After adding a stop solution were added (BD Biosciences, San Jose, CA, USA), the absorbance was measured at a wavelength of 450 nm wavelength using a microplate reader (15).

Murine model of cutaneous anaphylaxis or OAS

A murine model of cutaneous anaphylaxis was established by slightly modifying a murine model of passive cutaneous anaphylaxis (15, 26–29). Briefly, Der p extract-sensitized mice or control mice were intradermally injected with 3 µg of coho salmon extract, recombinant coho salmon tropomyosin, or recombinant coho salmon actin in the ears immediately before intravenous injection of 0. 2 mL of 0.1% Evans blue dye. Thirty minutes later, the ears were removed to evaluate the amount of extravasated dye. Alternatively, the frequency of sneezing, which is the major symptom of allergic rhinitis in murine models, was analyzed for 10 min after the last challenge with Der p extract or PBS in Der p extract-sensitized mice or control mice, respectively (30, 31). A murine model of oral allergy was established as previously described (15). Der p extract-sensitized mice or control mice were administered an injection of 1 µg coho salmon extract inside the lower lip right immediately before intravenous injection of 0. 2 mL of 0.1% Evans blue dye. Thirty minutes later, the skin on the neck was removed to evaluate the amount of extravasated dye. In both models, the removed tissue was cut into small pieces that were incubated in 0.3 mL of 1N KOH overnight at 37°C with shaking. Then, 0.15 mL of 1N phosphoric acid and 0.39 mL of acetone were added to the solution. After mixing by inversion and centrifugation at 700 g for 15 min, 0.2 mL of the supernatant was added to a 96-well microplate. The absorbance was measured at 620 nm using a 96-well microplate luminometer to evaluate the amount of extravasated dye (15, 26–29).

Histological analyses

Histological analyses were performed as described previously (15). Sections of ear skin were stained with toluidine blue or chloroacetate esterase to calculate the percentage of degranulated mast cells among the total mast cells in ear skin.

Statistical analyses

The results are expressed as means ± standard deviation (SD). The Mann-Whitney test results are shown in Figures 1B, C, 2B, D, 3D, 4B–D, F. The results of the paired t-test are shown in Figure 2C. The Wilcoxon matched-pairs signed-rank test results are shown in Figure 2E. The results of a paired t-test with Welch's correction are shown in Figures 3B (right panel) and 6D (left panel). The results of Brown-Forsythe and Welch analysis of variance (ANOVA) tests With Dunnett's T3 multiple comparisons test are shown in Figures 3B (left and middle panels), 5A, 6B, D (right panel). The results of two-way ANOVA with Sidak's multiple comparisons test are shown in Figure 6A. Differences between groups were compared, and $^{*}p < 0.05$ or $^{**}p < 0.01$ was considered statistically significant.

Results

Identification of foods that may cross-react with Der p extract

To identify foods that may cross-react with the Der p extract, we used a murine model which we had recently established (15). To this end, BALB/c mice were intraperitoneally injected with Der p extract plus alum or PBS plus alum as a control six times at 1-week intervals (days 0, 7, 14, 21, 28, and 35). Serum levels of total IgE and Der p extract-specific IgE on day 42 after the sixth injection of Der p extract plus alum were significantly higher than those after the injection of alum alone (Figures 1A–C). We then conducted allergenic protein microarray analyses using serum from the mice after the sixth injection with Der p extract plus alum or PBS plus alum to analyze the binding affinity of serum IgE to 1178 types of

crude protein extracts deriving from a variety of plants (vegetables and fruits), animals, processed foods (fish, shellfish, meats, eggs, insects, and ticks), and microorganisms (bacteria and fungi). The relative binding affinities to each protein extract of serum IgE from Der p extract-sensitized mice versus control mice were calculated. The top 22 protein extracts, that were highly bound to serum IgE from Der p extract-sensitized mice, are shown (Table 1). It seemed reasonable to select the edible animals or plants to further analyze out of top around 20 protein extracts. We found that two protein extracts, ranked fourteenth and sixteenth, were derived from house dust mite Der p. Ten protein extracts, including those ranked first, were derived from bacteria and fungi, among which seven were from *Escherichia coli*, suggesting that these microorganism-derived proteins may be included in the Der p extract. Notably, the second, seventh, and twenty-second-ranked species were derived from *Oncorhynchus kisutch* (coho salmon), a member of the *Salmonidae* family. The extract from coho salmon among fish tested, including *Scomber japonicus* (Chub mackerel), *Gadus macrocephalus* (Pacific cod), *Sardinops melanostictus* (sardine), and *Thunnes orientalis* (Pacific bluefin tuna), was most highly bound to serum IgE from Der p extract-sensitized mice. The twelfth-ranked was a mixture of several types of shellfish. The eighteenth and twenty-first-ranked were from *Sus scrofa domesticus* (pork) and *Gallus gallus domesticus* (chicken), respectively. Here, we focused on *Oncorhynchus kisutch* (coho salmon), an edible fish that may contain proteins that cross-react with Der p allergens.

Serum IgE from mice sensitized with Der p extract was significantly bound to coho salmon extract

To test whether Der p extract showed IgE cross-reactivity with the coho salmon extract, mice were intraperitoneally injected with Der p extract plus alum or with alum alone three times at 1-week intervals, followed by intranasal administration of Der p extract or PBS, respectively, five times a week for two weeks (Figure 2A). On day 35, the mice administered the Der p extract exhibited higher levels of specific IgE against the Der p extract in serum compared to control mice that had not been administered the same extract

TABLE 1 Relative binding intensity of serum IgE to the respective protein extracts.

	ID	Classification-1	Classification-2	Allergen	Intensity
1	000094	Bacteria	*Enterobacteriaceae*	*Escherichia coli serotype O45:H2*	1.79
2	001274	fish and shellfish	*Salmonidae*	*Oncorhynchus kisutch* (coho salmon)	1.71
3	000505	Plant	*Calophyllaceae*	*Calophyllum inophyllum.*	1.67
4	000098	Bacteria	*Enterobacteriaceae*	*Escherichia coli serotype O111:H8*	1.57
5	000849	Plant	*Lamiaceae*	*Salvia officinalis*	1.52
6	000097	Bacteria	*Enterobacteriaceae*	*Escherichia coli serotype O104:H12*	1.42
7	000564	fish and shellfish	*Salmonidae*	*Oncorhynchus kisutch* (coho salmon)	1.41
8	000113	Bacteria	*Streptococcaceae*	*Streptococcus pyogenes*	1.32
9	000957	Fungus	*Aureobasidium*	*Aureobasidium pullulans*	1.32
10	000072	Plant	*Cupressaceae*	*Chamaecyparis obtusa*	1.25
11	000095	Bacteria	*Enterobacteriaceae*	*Escherichia coli serotype O91*	1.16
12	000817	fish and shellfish	Shellfish Mix	clam, crab, oyster, scallops, and shrimp	1.15
13	000110	Bacteria	*Salmonella enterica*	*Salmonella Typhimurium*	1.08
14	000031	Mite	*Dermatophagoidinae*	*Dermatophagoides pteronyssinus*	1.07
15	000104	Bacteria	*Enterobacteriaceae*	*Escherichia coli serotype O145:H2*	1.04
16	000086	mite	*Dermatophagoidinae*	*Dermatophagoides pteronyssinus*	0.95
17	000105	bacteria	*Enterobacteriaceae*	*Escherichia coli serotype O157:H7*	0.92
18	000623	animal	*Suidae*	*Sus scrofa domesticus* (pork)	0.88
19	000103	bacteria	*Enterobacteriaceae*	*Escherichia coli serotype O121:H19*	0.88
20	000111	bacteria	*Shigella*	*Shigella*	0.88
21	000652	animal	*Phasianidae*	*Gallus gallus domesticus* (chicken)	0.84
22	000565	fish and shellfish	*Salmonidae*	*Oncorhynchus kisutch* (coho salmon)	0.82

The relative binding intensity of serum IgE from Der p-sensitized mice to the respective protein extracts (ID, Classification-1, Classification-2, and Allergen) was estimated by subtracting the IgE binding intensity of serum from mice injected with PBS plus alum from the IgE binding intensity of serum from mice injected with Der p extract plus alum. The shellfish mix included a mixture of clams, crabs, oysters, scallops, and shrimp.

(Figure 2B). The high levels of serum IgE binding to Der p extract were profoundly lowered by pre-incubating these sera with Der p extract before ELISA, confirming that serum IgE from Der p extract-administered mice was specifically bound to the Der p extract (Figure 2C). It should be noted that the mice administered Der p extract exhibited significantly higher levels of serum IgE against the self-prepared coho salmon extract than the control mice (Figure 2D), which were also significantly decreased by preincubation of these sera with Der p extract prior to ELISA (Figure 2E). These results indicated that IgE cross-reactive proteins exist between Der p and coho salmon extracts.

Serum IgE from mice sensitized with coho salmon extract was significantly bound to Der p and Der f extract

We then tested whether the Der p or Der f extract, manufactured by a different company, also cross-reacted with the salmon extract. Similarly, Der p or Der f extract-treated mice and control mice were prepared (Figure 3A). The results showed that Der p or Der f extract-administered mice exhibited high levels of specific IgE against both Der p and Der f extracts in serum, while serum IgE from these mice was bound significantly to the salmon extract compared to serum from control mice (Figure 3B). To further verify the IgE cross-reactivity between Der p and coho salmon extract, mice were intraperitoneally injected with salmon extract plus alum or PBS plus alum as a control six times at 1-week intervals. The results showed that coho salmon extract-administered mice exhibited high levels of serum IgE against the coho salmon extract, whereas serum IgE from these mice was bound more strongly to both Der p and Der f extracts than serum IgE from control mice (Figures 3C, D). Taken together, these results suggested a possible IgE cross-reactivity between Der and coho salmon.

Mice that had been sensitized intraperitoneally and intranasally with Der p extract exhibited evident dye extravasation in response to stimulation with coho salmon extract in the murine model of cutaneous anaphylaxis or OAS

To test whether IgE cross-reactivity between Der p and coho salmon extract causes allergic reaction in mice, we used the murine model of cutaneous anaphylaxis or OAS. Mice were intraperitoneally injected with Der p extract plus alum or alum alone three times at 1-week intervals, followed by intranasal administration of Der p extract or PBS, respectively, five times a week for two weeks (Figure 4A). We analyzed the frequency of sneezing during 30 min after the nasal administration of Der p extract on day 32. The results showed that Der p extract-treated mice exhibited higher numbers of sneezes than control mice, indicating that the former suffered from Der p extract-mediated allergic rhinitis in murine model (Figure 4B). When these mice were intradermally injected in ears with coho salmon extract just prior to intravenous injection of Evans blue dye on day 35, higher amounts of dye extravasation and more frequently degranulated mast cells in the ear skin were evident in Der p extract-administered mice but not in control mice (Figures 4C–E). In addition, when these mice were intravenously injected with Evans blue dye after the injection of coho salmon extract inside the lower lip on day 35, we found significantly higher amounts of dye extravasation in the neck skin of Der p extract-treated mice than in control mice (Figure 4F). These results indicated that the challenge with coho salmon extract induced mast cell-dependent anaphylactic responses in Der p extract-sensitized mice, presumably due to IgE cross-reactivity between Der p and coho salmon extract.

The approximately 38-kDa protein present in the coho salmon extract was possibly bound to serum IgE from Der p extract-sensitized mice

To identify the IgE cross-reactive proteins between Der p and coho salmon extracts, coho salmon extract proteins were separated by ammonium sulfate precipitation. Notably, coho salmon proteins that had not been precipitated with 40% ammonium sulfate but precipitated with 50% ammonium sulfate were bound most highly by serum IgE from both Der p and Der f extract-sensitized mice as compared with serum IgE from control mice. Serum IgE from both Der p and Der f extract-sensitized mice also bound to coho salmon proteins that had not been precipitated with 50% ammonium sulfate but precipitated with 60% ammonium sulfate (Figure 5A). Then, coho salmon proteins precipitated with 50% ammonium sulfate were subjected to anion exchange chromatography to separate proteins based on their net surface charge, showing that proteins were abundantly included in fractions A2 to A3 and C7 to C14 (Figure 5B). Equivalent amounts of total protein in each fraction were subjected to SDS-PAGE, and the protein bands were stained with CBB. We found approximately 34 kDa or 38 kDa proteins as the major protein bands in the A2–A3 fractions or C7–C14 fractions, respectively (Figure 5C). In addition, we examined the binding capacity of serum IgE from Der p-sensitized mice and control mice to wells coated with equal amounts of total protein from each fraction. The results showed that serum IgE from Der p-sensitized mice was bound strongly to the proteins included in the C7–C14 fractions compared to serum IgE from control mice (Figure 5D). These results suggest that the approximately 38 kDa protein of coho salmon in fractions C7 to C14 was strongly bound by serum IgE from Der p extract-sensitized mice.

Tropomyosin was identified as the IgE cross-reactive protein between Der p and coho salmon extract

To identify the approximately 38 kDa protein, that was bound strongly to serum IgE from Der p extract-sensitized mice, we performed N-terminal amino acid sequence analysis of an approximately 38 kDa band using the Edman degradation method; however, we could not do so, presumably because of the

chemically modified N-terminus of this protein. Instead, we considered tropomyosin as a candidate coho salmon protein of approximately 38 kDa, because tropomyosin is known to show IgE cross-reactivity between house dust mites and crustaceans (16–19). After producing, recombinant proteins of coho salmon tropomyosin and coho salmon actin as a control, we tested whether serum from Der p extract-sensitized mice was bound to recombinant coho salmon tropomyosin or actin. The results showed that the recombinant coho salmon tropomyosin protein, but not coho salmon actin, was strongly bound by the serum IgE from Der p extract-sensitized mice (Figure 6A). In addition, the binding of serum IgE from Der p extract-sensitized mice to coho salmon extract or coho salmon proteins precipitated with 50% ammonium sulfate was substantially inhibited by serum preincubation with coho salmon tropomyosin (Figure 6B). According to UniProt sequences, tropomyosin of American house dust mite (Der p 10) shares 98.2%, 82.0%, and 50.6% amino acid sequence identity with tropomyosin of European house dust mite (Der f 10), tropomyosin 1 of *Homarus americanus* (American lobster), and tropomyosin 1 of *Oncorhynchus kisutch* (coho salmon), respectively (Figure 6C). These results indicate that tropomyosin is one of the major proteins responsible for IgE cross-reactivity between house dust mite Der and coho salmon. It should be noted that Der p 10 shares 75.4% amino acid sequence identity with Tropomyosin α-1 of *Mus musculus* (mouse) and Tropomyosin α-1 of *Homo sapiens* (human) (Figure 6C). To finally verify this IgE cross-reactivity *in vivo*, Der p extract-sensitized mice or control mice were intradermally injected with coho salmon proteins precipitated with 50% ammonium sulfate or coho salmon tropomyosin in ears before intravenous injection of Evans blue dye. Der p extract-sensitized mice exhibited higher amounts of dye extravasation than the control mice (Figure 6D). However, Der p extract-sensitized coho salmon actin-stimulated mice did not exhibit evident dye extravasation in their ears (Figure 6D). Taken together, our murine model identified tropomyosin as an IgE cross-reactive protein between house dust mite Der p and coho salmon.

Discussion

Many questions remain regarding the development of food allergies; however, IgE cross-reactivity between environmental allergens may partly explain the mechanisms underlying food allergies subsequent to other allergic diseases, including atopic dermatitis, allergic rhinitis, allergic conjunctivitis, and allergic asthma (1–8). Recent studies indicate that exposure of food allergens on skin with barrier dysfunction strongly induces IgE sensitization, thereby causing food allergic responses following intake of the same allergens (32–34). However, sensitization to environmental allergens (e.g., pollen and HDM allergens) through various organs (e.g., the nose, eyes, lungs, and skin) may be responsible for food-related allergic responses after the intake of specific foods containing IgE cross-reactive allergens. This is also the case for PFAS (9–15). When IgE cross-reactive allergens are present in different foods, it is possible that an allergy to a specific food will subsequently cause another food allergy. This may be the case in patients with allergies to multiple, seemingly irrelevant foods. Nonetheless, IgE cross-reactivity between environmental allergens is not fully understood. Therefore, we attempted to identify food allergens that might cross-react with HDM allergens, which are known to trigger several allergic diseases, by improving a previously developed method using a murine model of sensitization and allergic protein microarray technology (15, 23). Analysis of Der p extract-sensitized mice versus control mice using allergic protein microarray technology identified *Oncorhynchus kisutch* (coho salmon) as a food containing allergens cross-reactive with Der p. In fact, serum IgE from Der p extract-sensitized mice was bound to the commercially available coho salmon extract, whereas serum IgE from coho salmon extract-sensitized mice was bound to the Der p extract. Importantly, administration of coho salmon extract induced an increase in local vascular permeability through IgE-mediated mast cell degranulation in Der p extract-sensitized mice, but not in control mice, in murine models of both cutaneous anaphylaxis and OAS (15). These *in vitro* and *in vivo* results corroborated the IgE cross-reactivity between Der p and coho salmon extract. Surprisingly, serum IgE from Der p extract-sensitized mice was preferentially bound to the extracts derived from *Escherichia coli*, which was not observed in a previous study using serum IgE from ragweed pollen-sensitized mice (15). We speculate that proteins from *Escherichia coli* may be present in the Der p extract; that is, *Escherichia coli* exists in the microbiome of HDM. Accordingly, it may be speculated that exposures to HDM may induce the production of IgG/IgE against *Escherichia coli*, that requires further verification. On the other hand, when using serum from mice sensitized with crude extracts from animals or plants in our model, we need to keep in mind that their microbiome may influence the protein microarray data. Alternatively, microarray plates are coated with various crude protein extracts. Each sample has a variety of allergens with different quantities and stabilities, which can be affected by cultivar, climates, or chemical and heat treatments (15). Therefore, the part and condition of each plant or animal sample used and the procedures of protein extraction can also impact the protein microarray data. In any case, we need to carefully analyze the protein microarray data after taking these factors into consideration.

Importantly, the approximately 38 kDa protein present in the coho salmon extract was identified as one of the major IgE cross-reactive proteins between Der p and coho salmon extracts. This finding was guided by several experiments, including protein separation from coho salmon extract by ammonium sulfate precipitation and anion exchange chromatography, visualization of proteins separated by SDS-PAGE, and measurement of the binding affinity of serum IgE from Der p extract-sensitized mice versus control mice to separate protein fraction-coated plates by ELISA. According to recent reports, salmon tropomyosin is an allergenic protein with approximately 38 kDa molecular weight. In addition, IgE cross-reactivity between HDM tropomyosin (e.g., Der p 10 and Der f 10) and shrimp tropomyosin is often responsible for shrimp allergy found in patients with HDM allergies. Accordingly, it seems plausible that tropomyosin acts as an IgE cross-reactive protein between Der p and salmon extracts. Preincubation with coho salmon tropomyosin strongly suppressed the binding of serum IgE from Der p-sensitized mice to the plate-coated coho salmon extract. Moreover, the administration of coho salmon tropomyosin induced a local anaphylactic response in Der p-

sensitized mice. Although N-terminal amino acid sequencing of the corresponding band using the Edman degradation method was unsuccessful in this case, this method and/or protein mass spectrometry will be useful for identifying unknown proteins of interest included in the condensed fraction.

Because crustaceans (e.g., shrimp and crab) and arachnids (e.g., HDM) are arthropods, it is reasonable that IgE cross-reactive tropomyosin between crustaceans and HDM plays an important role in crustacean allergy (16–19). Serum IgE from Der p-sensitized mice was bound strongly to a mixture of clams, crabs, oysters, scallops, and shrimp. Although a recent report has pointed to the significance of tropomyosin as a fish allergen (20–22), our murine model showed a possible causal relationship between HDM and salmon allergies. It may be speculated that patients with HDM allergy develop salmon allergy; however, further clinical investigation is required to clarify the significance of this relationship. Interestingly, protein microarray analysis showed that the extract from coho salmon among fish tested was most highly bound to serum IgE from Der p extract-sensitized mice. On the other hand, American house dust mite tropomyosin shares 82.0%, 75.4% or 50.6% amino acid sequence identity with American lobster tropomyosin 1, mouse and human Tropomyosin α-1, and coho salmon tropomyosin 1, respectively. Accordingly, one possible explanation is that tropomyosin from coho salmon among tropomyosin-containing lives may contain conformational IgE epitope similar to arthropod tropomyosin. In any case, further examination will be required to completely understand the relevant molecular mechanisms.

In conclusion, our murine model identified tropomyosin as an IgE cross-reactive protein between HDM and coho salmon, illustrating salmon allergy following HDM allergy. Our method for identifying IgE cross-reactive allergens will help clarify the unknown mechanisms underlying the development of food allergies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

All the procedures were approved by the Institutional Review Committee of Juntendo University (approval numbers: 2021188, 2022099, and 2023129). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RY performed all the experiments and participated in writing the manuscript. KI assisted with the analysis of murine model and the *in vitro* experiments, analyzed the data, and actively participated in manuscript writing. TA assisted with the *in vitro* experiments and statistical analysis and analyzed the data. AT assisted with protein purification. ST, AM, HW, MN, and NNe assisted with the *in vitro* experiments. AK, HY, SU, AY, YK, and NNa assisted with the *in vivo* experiments. NE, TS, HO, and KO analyzed the data. JK conceived the project, analyzed the data, and actively participated in manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Valenta R, Hochwallner H, Linhart B, Pahr S. Food allergies: the basics. *Gastroenterology* (2015) 148(6):1120–31.e4. doi: 10.1053/j.gastro.2015.02.006

2. Nakano N, Kitaura J. Mucosal mast cells as key effector cells in food allergies. *Cells* (2022) 11(3):329. doi: 10.3390/cells11030329

3. Kitaura J, Murakami M. Positive and negative roles of lipids in mast cells and allergic responses. *Curr Opin Immunol* (2021) 72:186–95. doi: 10.1016/j.coi.2021.06.001

4. Oyoshi MK, Oettgen HC, Chatila TA, Geha RS, Bryce PJ. Food allergy: Insights into etiology, prevention, and treatment provided by murine models. *J Allergy Clin Immunol* (2014) 133(2):309–17. doi: 10.1016/j.jaci.2013.12.1045

5. Tordesillas L, Berin MC, Sampson HA. Immunology of food allergy. *Immunity* (2017) 47(1):32–50. doi: 10.1016/j.immuni.2017.07.004

6. Sampson HA, O'Mahony L, Burks AW, Plaut M, Lack G, Akdis CA. Mechanisms of food allergy. *J Allergy Clin Immunol* (2018) 141(1):1–9. doi: 10.1016/j.jaci.2017.11.005

7. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* (2003) 112(11):1666–77. doi: 10.1172/JCI19785

8. Chen CY, Lee JB, Liu B, Ohta S, Wang PY, Kartashov AV, et al. Induction of interleukin-9-producing mucosal mast cells promotes susceptibility to IgE-mediated experimental food allergy. *Immunity* (2015) 43(4):788–802. doi: 10.1016/j.immuni.2015.08.020

9. Mari A, Ballmer-Weber BK, Vieths S. The oral allergy syndrome: improved diagnostic and treatment methods. *Curr Opin Allergy Clin Immunol* (2005) 5(3):267–73. doi: 10.1097/01.all.0000168793.27948.b0

10. Muluk NB, Cingi C. Oral allergy syndrome. *Am J Rhinol Allergy* (2018) 32(1):27–30. doi: 10.2500/ajra.2018.32.4489

11. Katelaris CH. Food allergy and oral allergy or pollen-food syndrome. *Curr Opin Allergy Clin Immunol* (2010) 10(3):246–51. doi: 10.1097/ACI.0b013e32833973fb

12. Ma S, Sicherer SH, Nowak-Wegrzyn A. A survey on the management of pollen-food allergy syndrome in allergy practices. *J Allergy Clin Immunol* (2003) 112(4):784–8. doi: 10.1016/s0091-6749(03)02008-6

13. Egger M, Mutschlechner S, Wopfner N, Gadermaier G, Briza P, Ferreira F. Pollen-food syndromes associated with weed pollinosis: an update from the molecular point of view. *Allergy* (2006) 61(4):461–76. doi: 10.1111/j.1398-9995.2006.00994.x

14. Carlson G, Coop C. Pollen food allergy syndrome (PFAS): A review of current available literature. *Ann Allergy Asthma Immunol* (2019) 123(4):359–65. doi: 10.1016/j.anai.2019.07.022

15. Kamei A, Izawa K, Ando T, Kaitani A, Yamamoto R, Maehara A, et al. Development of mouse model for oral allergy syndrome to identify IgE cross-reactive pollen and food allergens: ragweed pollen cross-reacts with fennel and black pepper. *Front Immunol* (2022) 13:945222. doi: 10.3389/fimmu.2022.945222

16. Becker S, Gröger M, Canis M, Pfrogner E, Kramer MF. Tropomyosin sensitization in house dust mite allergic patients. *Eur Arch Otorhinolaryngol* (2012) 269(4):1291–6. doi: 10.1007/s00405-011-1826-1

17. Huang FL, Liao EC, Yu SJ. House dust mite allergy: Its innate immune response and immunotherapy. *Immunobiology* (2018) 223(3):300–2. doi: 10.1016/j.imbio.2017.10.035

18. Asero R, Pravettoni V, Scala E, Villalta D. House dust mite-shrimp allergen interrelationships. *Curr Allergy Asthma Rep* (2020) 20(4):9. doi: 10.1007/s11882-020-0902-2

19. Boquete M, Iraola V, Morales M, Pinto H, Francisco C, Carballás C, et al. Seafood hypersensitivity in mite sensitized individuals: is tropomyosin the only responsible allergen? *Ann Allergy Asthma Immunol* (2011) 106(3):223–9. doi: 10.1016/j.anai.2010.11.014

20. Ruethers T, Taki AC, Nugraha R, Cao TT, Koeberl M, Kamath SD, et al. Variability of allergens in commercial fish extracts for skin prick testing. *Allergy* (2019) 74(7):1352–63. doi: 10.1111/all.13748

21. Ruethers T, Taki AC, Karnaneedi S, Nie S, Kalic T, Dai D, et al. Expanding the allergen repertoire of salmon and catfish. *Allergy* (2021) 76(5):1443–53. doi: 10.1111/all.14574

22. Kuehn A, Swoboda I, Arumugam K, Hilger C, Hentges F. Fish allergens at a glance: variable allergenicity of parvalbumins, the major fish allergens. *Front Immunol* (2014) 5:179. doi: 10.3389/fimmu.2014.00179

23. Hoshino A, Kanegane H, Nishi M, Tsuge I, Tokuda K, Kobayashi I, et al. Identification of autoantibodies using human proteome microarrays in patients with IPEX syndrome. *Clin Immunol* (2019) 203:9–13. doi: 10.1016/j.clim.2019.03.011

24. Uchida S, Izawa K, Ando T, Yamada H, Uchida K, Negishi N, et al. CD300f is a potential therapeutic target for the treatment of food allergy. *Allergy* (2019) 75(2):471–4. doi: 10.1111/all.14034

25. Yamada H, Kaitani A, Izawa K, Ando T, Kamei A, Uchida S, et al. Staphylococcus aureus δ-toxin present on skin promotes the development of food allergy in a murine model. *Front Immunol* (2023) 14:1173069. doi: 10.3389/fimmu.2023.1173069

26. Izawa K, Yamanishi Y, Maehara A, Takahashi M, Isobe M, Ito S, et al. The receptor LMIR3 negatively regulates mast cell activation and allergic responses by binding to extracellular ceramide. *Immunity* (2012) 37(5):827–39. doi: 10.1016/j.immuni.2012.08.018

27. Takamori A, Izawa K, Kaitani A, Ando T, Okamoto Y, Maehara A, et al. Identification of inhibitory mechanisms in pseudo-allergy involving Mrgprb2/MRGPRX2-mediated mast cell activation. *J Allergy Clin Immunol* (2019) 143(3):1231–5. doi: 10.1016/j.jaci.2018.10.034

28. Izawa K, Kaitani A, Ando T, Maehara A, Nagamine M, Yamada H, et al. Differential lipid recognition by mouse versus human CD300f, inhibiting passive cutaneous anaphylaxis, depends on a single amino acid substitution in its immunoglobulin-like domain. *J Invest Dermatol* (2020) 140(3):710–3. doi: 10.1016/j.jid.2019.08.439

29. Shiba E, Izawa K, Kaitani A, Isobe M, Maehara A, Uchida K, et al. Ceramide-CD300f binding inhibits lipopolysaccharide-induced skin inflammation. *J Biol Chem* (2017) 292(7):2924–32. doi: 10.1074/jbc.M116.768366

30. Kato Y, Akasaki S, Muto-Haenuki Y, Fujieda S, Matsushita K, Yoshimoto T. Nasal sensitization with ragweed pollen induces local-allergic-rhinitis-like symptoms in mice. *PloS One* (2014) 9(8):e103540. doi: 10.1371/journal.pone.0103540

31. Akasaki S, Matsushita K, Kato Y, Fukuoka A, Iwasaki N, Nakahira M, et al. Murine allergic rhinitis and nasal Th2 activation are mediated via TSLP- and IL-33-signaling pathways. *Int Immunol* (2016) 28(2):65–76. doi: 10.1093/intimm/dxv055

32. Galand C, Leyva-Castillo JM, Yoon J, Han A, Lee MS, McKenzie ANJ, et al. IL-33 promotes food anaphylaxis in epicutaneously sensitized mice by targeting mast cells. *J Allergy Clin Immunol* (2016) 138(5):1356–66. doi: 10.1016/j.jaci.2016.03.056

33. Tordesillas L, Goswami R, Benedé S, Grishina G, Dunkin D, Järvinen KM, et al. Skin exposure promotes a Th2-dependent sensitization to peanut allergens. *J Clin Invest* (2014) 124(11):4965–75. doi: 10.1172/JCI75660

34. Bartnikas LM, Gurish MF, Burton OT, Leisten S, Janssen E, Oettgen HC, et al. Epicutaneous sensitization results in IgE-dependent intestinal mast cell expansion and food-induced anaphylaxis. *J Allergy Clin Immunol* (2013) 131(2):451–60. doi: 10.1016/j.jaci.2012.11.032

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Positive effects of dietary *Clostridium butyricum* supplementation on growth performance, antioxidant capacity, immunity and viability against hypoxic stress in largemouth bass

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The effects of dietary supplementation of *Clostridium butyricum* (CB) on growth performance, serum biochemistry, antioxidant activity, mRNA levels of immune-related genes and resistance to hypoxia stress were studied in largemouth bass. Feed with CB0 (control, 0 CFU/kg), CB1 (4.3×10^8 CFU/kg), CB2 (7.5×10^8 CFU/kg), CB3 (1.5×10^9 CFU/kg) and CB4 (3.2×10^9 CFU/kg) CB for 56 days, and then a 3 h hypoxic stress experiment was performed. The results showed that dietary CB significantly increased the WGR (weight gain rate), SGR (specific growth rate), PDR (protein deposition rate) and ISI (Intestosomatic index) of largemouth bass ($P<0.05$). Hepatic *GH* (growth hormone)/*IGF-1* (insulin-like growth factor-1) gene expression was significantly upregulated in the CB3 and CB4 groups compared with the CB0 group ($P<0.05$), while the FC (feed conversion) was significantly decreased ($P<0.05$). Serum TP (total protein) and GLU (glucose) levels were significantly higher in the CB4 group than in the CB0 group ($P<0.05$), while the contents of serum AST (aspartate transaminase), ALT (alanine transaminase), AKP (alkline phosphatase) and UN (urea nitrogen) in CB4 were significantly lower than those in CB0 ($P<0.05$). T-AOC (total antioxidant capacity), SOD (superoxide dismutase), CAT (catalase), POD (peroxidase) and GSH-Px (glutathione peroxidase) activities were significantly higher in CB3 and CB4 groups than in CB0 group ($P<0.05$). The liver MDA (malondialdehyde) content of CB1, CB2, CB3 and CB4 groups was significantly higher than that of CB0 group ($P<0.05$). The relative expressions of *IL-1β* (interleukin 1β), *TNF-α* (tumor necrosis factor α) and *TLR22* (toll-like receptor-22) genes in CB2, CB3 and CB4 groups were significantly lower than those in CB0 group ($P<0.05$). The relative expression of *IL-8* (malondialdehyde) and *MyD88* (Myeloid differentiation factor 88) genes in the CB4 group was significantly lower than that in the CB0 group ($P<0.05$). The liver LZM (lysozyme) content of CB2, CB3 and CB4 groups was significantly higher than that of CB0 group ($P<0.05$). The relative expression of *IL-10* (interleukin 10) and *TGF-β* (transforming growth factor β) genes in the

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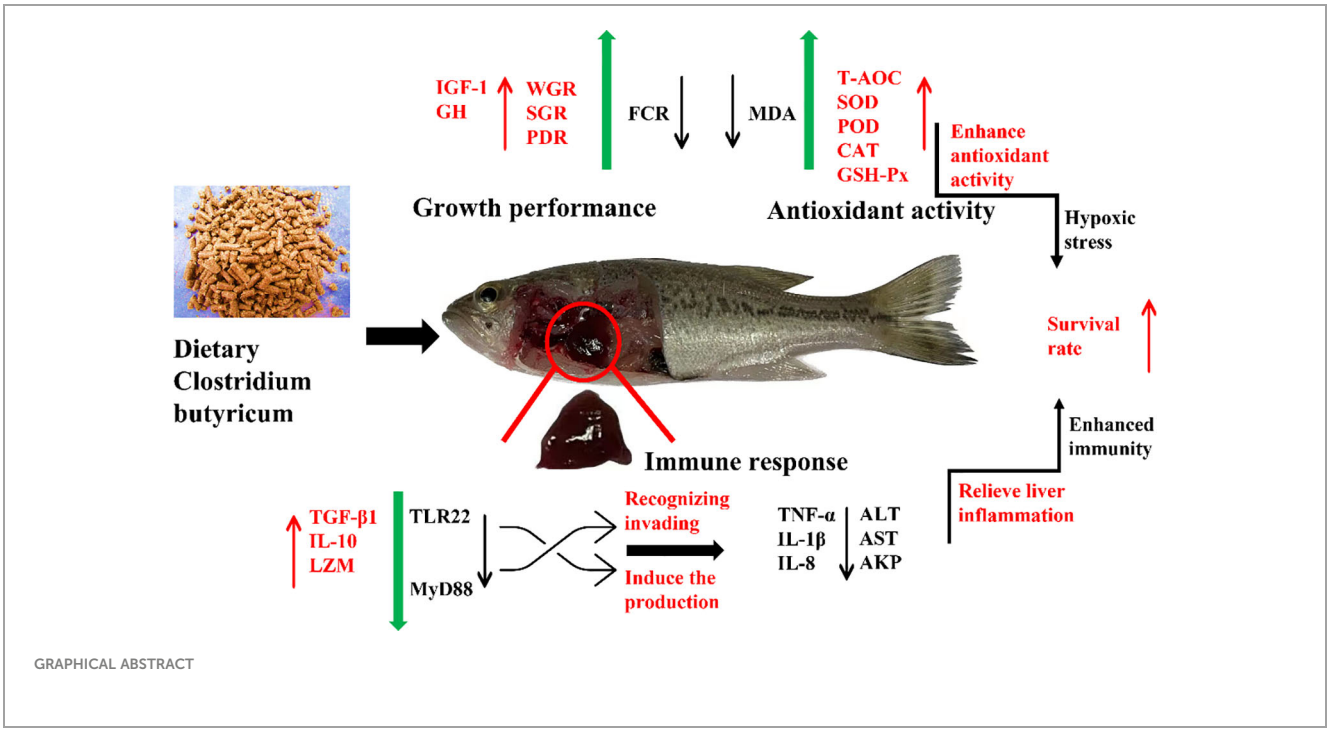
CB4 group was significantly higher than that in the CB0 group ($P<0.05$). Under hypoxic stress for 3 h, the CMR of CB0 group was significantly higher than that of CB1, CB2, CB3 and CB4 groups ($P<0.05$). Dietary CB can improve the growth performance and resistance to hypoxic stress of largemouth bass by regulating the expression of *GH/IGF-1* gene and inflammatory factors and inhibiting *TLR22/MyD88* signaling pathway.

KEYWORDS

Clostridium butyricum, Largemouth bass, growth performance, antioxidant status, immune response



GRAPHICAL ABSTRACT

1 Introduction

The extensive use of antibiotics in intensive farming to control bacterial diseases exacerbates the development and spread of antibiotic resistance (1). The incidence of infectious diseases caused by antibiotic-resistant bacteria is currently increasing, resulting in a higher mortality rate than cancer (2). The misuse of antibiotics results in microbial dysbiosis and the emergence of antimicrobial resistance, thereby posing significant public health challenges (3, 4). Consequently, the utilization of antibiotics as feed additives was prohibited in the European Union, United States, and China in 2006, 2014, and 2020 correspondingly (5). Therefore, it is imperative to explore alternatives that can substitute antibiotics. Probiotics are defined as live microorganisms that optimize the microbial balance and promote health (6). Probiotics have been successfully used in farmed freshwater or marine fish, including Nile tilapia (*Oreochromis niloticus*), Javanese carp (*Puntius gonionotus*), and rainbow trout (*Oncorhynchus mykiss*), among others (7–9). Probiotics are the preferred therapeutic agents for managing inflammatory disorders, such as diarrheal disease and inflammatory bowel disease. A plethora of studies have demonstrated that dietary supplementation with probiotics can significantly augment fish health (5, 10–12).

Clostridium butyricum (CB) is a Gram-positive bacterium that exerts beneficial effects on growth promotion, inflammation suppression, and pathogenic bacteria reduction (13, 14). In recent years, CB has gained widespread application in the livestock and aquatic animal industries, primarily for enhancing growth performance and bolstering disease resistance (15, 16). Additionally, due to its ability to withstand low pH and high temperatures, CB is frequently utilized in fish as a preventative measure against fish pathogens or antibiotic resistance (17). Studies have shown that the addition of CB in the range of 10^7 - 10^{11} CFU/kg significantly improved the growth performance of silver pomfret

(*Pampus argenteus*) (18), large yellow croaker (*Larimichthys crocea*) (19), and Nile tilapia (20), enhanced the immune response of giant freshwater prawn (Macrobrachium rosenbergii) (21), and increased the antioxidant activity of kuruma shrimp (*Marsupenaeus japonicas*) (22). However, the impact of dietary supplementation with CB on growth, antioxidant activity, immune response and hypoxic stress resistance in largemouth bass remains uncertain.

Largemouth bass (*Micropterus salmoides*), a freshwater farmed fish, commands high market value owing to its rapid growth and delectable flesh (23). Meanwhile, due to its immense popularity among Chinese consumers, it constitutes a significant portion of China's breeding industry (24, 25). However, nutritional deficiencies, high densities, and environmental changes often lead to metabolic disorders in fish, which can result in reduced disease resistance and increased susceptibility to disease outbreaks (26). Dietary nutrients and supplementation significantly boost growth of Largemouth bass (27–29). Studies have demonstrated that probiotics have been efficaciously employed in numerous aquaculture species to stimulate growth, optimize feed utilization, and augment organismal resistance against diseases (30). Revised sentence: However, there is a lack of research on the utilization of CB as a feed additive for largemouth bass, and the mechanisms underlying CB's promotion of growth and enhancement of disease resistance remain unclear. The objective of this investigation was to examine the impact of CB on growth performance, antioxidant capacity, immune response, and hypoxic stress in juvenile largemouth bass.

2 Materials and methods

2.1 Experimental diets

The CB with a count of 8×10^8 colony-forming units (colony-forming units, CFU)/ml was obtained from China Organic Biotechnology Co., Ltd., China. Juvenile largemouth bass were fed with five isonitrogenous (50%) and isolipidic (9%) diets, which were supplemented with CB at 0 (CB0), 2.5 (CB1), 5 (CB2), 10 (CB3), and 20 (CB4) ml/kg of diet, respectively. The final CB concentrations in the five diets were 0, 4.3×10^8, 7.5×10^8, 1.5×10^9 and 3.2×10^9 CFU/kg, which were determined by the plate count method (31). The raw materials were weighed in accordance with the feed formulation, crushed, and sieved. Prior to mixing into the raw materials, the CB solution was mixed with water. All diets were prepared and pelleted into 1.5 mm diameter by twin screw extruder (SLX-80, South China University of Technology, China). Dry at 55° C for 6 h and store the feed at -20°C after packaging in groups. The ingredients and proximate composition of experimental diets are presented in Table 1.

2.2 Feeding and management

This feeding experiment was carried out in an indoor recirculating aquaculture system in the aquatic laboratory of the Institute of Animal Science, Guangdong Academy of Agricultural

TABLE 1 Composition and approximate composition of the experimental rations (in dry matter, %).

Item	Group				
	CB0	CB1	CB2	CB3	CB4
Ingredients					
Fish meal (Peru, crude protein 67.7%) [a]	35.00	35.00	35.00	35.00	35.00
Soy protein concentrate (crude protein 64.6%) [a]	9.00	9.00	9.00	9.00	9.00
Blood meal [a]	3.00	3.00	3.00	3.00	3.00
Shrimp shell meal [a]	5.00	5.00	5.00	5.00	5.00
Soybean meal[a]	20.00	20.00	20.00	20.00	20.00
Cottonseed protein meal[a]	9	9	9	9	9
Tapioca meal[a]	9	9	9	9	9
Fish oil[a]	3.00	3.00	3.00	3.00	3.00
Soybean oil[a]	3.00	3.00	3.00	3.00	3.00
Vitamin premix[b]	1.00	1.00	1.00	1.00	1.00
Mineral premix[c]	1.00	1.00	1.00	1.00	1.00
$Ca(H_2PO4)_2$[a]	1.00	1.00	1.00	1.00	1.00
Choline chloride[a]	0.20	0.20	0.20	0.20	0.20
Sodium alginate[a]	0.80	0.80	0.80	0.80	0.80

(Continued)

TABLE 1 Continued

Item	Group				
	CB0	CB1	CB2	CB3	CB4
Clostridium butyricum	0.00	0.25	0.50	1.00	2.00
Total	100.00	100.00	100.00	100.00	100.00
Nutrients compositions of experimental diets (in dry matter, %)					
Clostridium butyricum	0	4.3×10^5	7.5×10^5	1.5×10^6	3.2×10^6
Crude protein	50.55	50.63	50.96	50.21	50.35
Crude lipid	9.63	9.40	9.45	9.03	9.49
Ash	12.44	12.45	12.53	12.47	12.52
Moisture	3.73	4.20	3.46	4.14	3.64

[a]Fishtech Fisheries Science & Technology Company, LTD, Institute of Animal Science, Guangdong Academy of Agricultural Sciences (Guangzhou, China).
[b]Vitamin premixes: VA 4,000,000 IU, VD_3 2,000,000 IU, VE 30 g, VK_3 10 g, VB_1 5 g, VB_2 15 g, VB_6 8 g, VB_{12} 0.02 g, nicotinic acid 40 g, calcium pantothenate 25 g, folic acid 2.5 g, inositol 150 g, biotin 0.08 g. Moisture ≤ 10%.
[c]Mineral premixes: $MgSO_4{\cdot}H_2O$ 12 g, KCl 90 g, Met-Cu 3 g, $FeSO_4{\cdot}H_2O$ 1 g, Ca $(IO_3)_2$ 0.06 g, Met-Co 0.16 g, $ZnSO_4{\cdot}H_2O$ 10 g, $NaSeO_3$ 0.003 6 g. Moisture ≤ 10%.

Sciences (Guangzhou, China) and lasted for 56 d. The fish were purchased from Guangzhou, China, and were acclimated with the control diet for 1 week in an indoor recirculating aquaculture system. Six hundred fish (5.02 ± 0.01 g) were stocked into fifteen cylindrical fiberglass tanks (water volume 300 L) at 40 fish per tank to conduct the experiment. Five experimental diets were randomly allocated to triplicate groups of fish. During the 56-day feeding trial, the water temperature ranged from 25 to 32°C, pH 7.8-8.0, and dissolved oxygen > 8.0 mg/L. Fish were reared under 12 h light: 12 h dark dialcycle photoperiod. All the fish were manually fed to satiation with the experimental diets two times daily (8:30 and 18:30) (initially 5% to 6% of body weight per day and then gradually increased).

2.3 Sample collection and analysis

After the end of the 8-week feeding experiment, the fish were fasted for 24 h, and the final body weight of each cage was weighed, and the survival rate (SR) was calculated. Eighteen fish were randomly selected from each replicate and anesthetized in 120 mg/L tricaine methanesulfonate (MS-222) solution, and 3 fish were stored at -20 °C for the determination of routine nutrient composition of whole fish. The body weight, body length, intestines weight and viscera weight of six fish were measured to calculate weight gain rate (WGR), specific growth rate (SGR), protein deposition rate (PDR), feed conversion (FC), intestosomatic index (ISI) and condition factor (CF). Blood samples were collected from the tail vein of 9 fish, left at room temperature for 4 h, centrifuged at 3500 r/min for 10 min, and the supernatant was taken to prepare serum and stored at -80° C until use. The blood of 9 fish was collected and placed on ice for rapid dissection, and the liver of 3 fish was determined for immune antioxidant indexes. Livers from three fish were fixed in 10% formalin solution and stored at room temperature to produce liver paraffin sections. The routine nutrient composition of experimental diets and whole fish was determined as follows: Water content was determined by oven drying to constant weight at 105 °C (GB/T 6435-2014), crude protein content (N×6.25) was determined by semi-automatic Kjeldner nitrogen determination method (GB/T 6432-2018), crude fat content was determined by ether extraction method (GB/T 6433-2006), crude ash content was determined by the method of burning to constant weight at 550 °C (GB/T 6433-2006).

2.4 Serum biochemical analysis

After the fish were anesthetized, blood was collected from the tail vein, and the blood samples were centrifuged (3500 × g, 10 min, 4°C) to separate the serum. Serum total protein (TP) content was determined by biuret method. alanine transaminase (ALT) activity was measured by spectrophotometry. glucose (GLU) content was measured by glucose oxidase method. UN), triglyceride (TG) and aspartate transaminase (AST) activity were measured by enzyme coupling rate method, enzymatic method and spectrophotometric method, respectively. High-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), The contents of total cholesterol (TCHO) and alkaline phosphatase (AKP) were determined by enzymatic method. 1. The Hitachi 7600 fully automated analyzer was utilized to measure all parameters at Guangzhou Jinyu Medical Testing Center (Guangzhou, China).

2.5 Enzyme activity analysis

The liver samples were added with normal saline according to the ratio of weight liver: volume of normal saline (1:9), and the liver samples were fully broken under the condition of ice water bath. After the broken liver samples were centrifuged for 10 min (2500 r/min, 4 °C), the supernatant was taken after centrifugation for enzyme activity detection. The activity of peroxidase (POD) was determined by A084-2 colorimetric method, the activity of Lysozyme (LZM) was determined by A050 turbidimetric method,

and the activity of superoxide dismutase was determined by superoxide dismutase. SOD activity was determined by A001-1 hydroxylamine method, catalase (CAT) activity was determined by A007-1 visible light method, total antioxidant capacity, T-AOC capacity was determined by A015 colorimetric method. Content of malondialdehyde (MDA) and activity of glutathione peroxidase (GSH-Px) were determined by A003-1 TBA method. The enzyme activity kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and the measurement procedure, principle and calculation formula were referred to the kit manual.

2.6 qRT-PCR analysis

Total RNA was extracted from the whole liver of largemouth bass using RNA Isolation Kit (Vazyme, Nanjing, China), following the protocol of the manufacturer, and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA reverse was then transcribed to cDNA using HiScript®III RT SuperMix for qPCR kit (Vazyme, Nanjing, China). The qPCR assay was carried out using ChamQ Universal SYBQ qPCR Master Mix kit (Vazyme, Nanjing, China). The amplification was carried out in a 20 μL reaction volume containing 10 μL SYBQ Green Master Mix, 0.4 μL of each respective primer (10 μmol/L), 2 μL cDNA product, and 7.2 μL RNA-free water. The specific primers and primer sequences of the housekeeping gene (*β-actin*) are shown in Table 2, and all primers were synthesized by Shanghai Sangon Biotechnology Co., LTD. (Shanghai, China). *β-actin* was used as a nonregulated reference gene to normalize target gene transcript levels in largemouth bass studies, furthermore, *β-actin* gene expression of the intestine was also stable and was not significantly affected by dietary CB in our present research. All reactions were performed in duplicate, and each assay was repeated three times. The gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method (32).

2.7 Hypoxia stress test

At the end of the culture experiment, 16 fish were collected from each experimental group and subjected to hypoxic stress experiments according to the method described by Zeng et al. (33). Drain the water in the tank to the water surface to the bottom 10 cm of the tank (approximately 50 L), cover the tank with transparent plastic film and stop oxygen supply, causing an oxygen deficient environment. Dissolved oxygen (DO) was measured at 30-minute intervals during the experiment (Seven2Go, Mettler Toledo, USA). The DO level decreased from 7.6 mg L^{-1} to 0 mg L^{-1} after 1.5 h of continuous testing and remained at this level. When the cumulative mortality rate (CMR) of the control group (CB0) reached 50%, the experiment was stopped. Observation of the experimental fish was confirmed as a dead state when it did not respond to external stimuli. Fasting was done during the trial, and the number of fish stocks was counted to calculate the CMR after the trial.

2.8 Statistical analysis

SPSS 25.0 software (Chicago, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) and multipole difference test (Tukey's test) were used to determine significant differences ($P< 0.05$). Data are expressed as mean ± standard error (SEM).

2.9 Calculations

The parameters are calculated according to the following formula:

$$\text{Survival rate(SR, \%)} = 100 \times F_{final}/F_{initial}.$$

TABLE 2 Primer sequences.

Genes	Sequence Information		
	Forward primer (5′-3′)	Reverse primer (5′-3′)	GenBank
β-actin	GGACACGGAAAGGATTGACAG	CGGAGTCTCGTTCGTTATCGG	XM_038695351.1
IL-1β	CGTGACTGACAGCAAAAAGAGG	GATGCCCAGAGCCACAGTTC	XM_038696252.1
IL-8	CGTTGAACAGACTGGGAGAGATG	AGTGGGATGGCTTCATTATCTTGT	XM_038704088.1
IL-10	CGGCACAGAAATCCCAGAGC	CAGCAGGCTCACAAAATAAACATCT	XM_038696252.1
TNF-α	CTTCGTCTACAGCCAGGCATCG	TTTGGCACACCGACCTCACC	XM_038710731.1
TGF-β	GCTCAAAGAGAGCGAGGATG	TCCTCTACCATTCGCAATCC	XM_038693206.1
TLR22	TCGCTGTTCACCAATCTG	TAGTTCTCCTCTCCATCTGT	MN807054.1
MyD88	CTCAACCCCAAGAACACA	CGAAGATCCTCCACAATG	XM_038728827.1
IGF-I	CTTCAAGAGTGCGATGTGC	GCCATAGCCTGTTGGTTTACTG	DQ666526
GH	CCCCCAAACTGTCAGAACT	ACATTTCGCTACCGTCAGG	DQ666528

The cDNA sequence of the target gene from NCBI (National Center for Biotechnology Information) was used, and the primer sequence was designed using primer 6, and then synthesized at Shanghai Biotechnology Co.
IL1β, interleukin 1β; IL8, interleukin 8; IL10, interleukin 10; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor β1; TLR22, toll-like receptor 2; MyD88, myeloid differentiation primary response gene 88; IGF-I, insulin-like growth factor-I; GH, growth hormone.

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$$\text{Weight gain rate(WGR, \%)} = 100 \times (W_{final} - W_{initial})/W_{initial}.$$

$$\text{Feed conversion(FC)} = D_{total}/(W_{final} + W_{dead} - W_{initial}).$$

$$\text{Protein deposition rate(PDR, \%)} = 100 \times (W_{final} \times CP_{final} - W_{initial} \times CP_{initial})/(D \times CP_{feed}).$$

$$\text{Specific growth rate(SGR, \%)} = 100 \times (\text{Ln}W_{final} - \text{Ln}W_{initial})/T.$$

$$\text{Condition factor(CF, g/cm}^3\text{)} = 100 \times W/L^3.$$

$$\text{Intestosomatic index(ISI, \%)} = 100 \times W_{intestinal}/W.$$

In the formula: $F_{initial}$ is the initial mantissa; F_{final} is the final mantissa; W is the body weight; L is the body length; $W_{initial}$ is the initial fish weight; W_{final} is the final fish body weight; W_{dead} is the dead fish weight; $CP_{initial}$ is the protein content of the initial fish body; CP_{final} is the protein content of the final fish; CP_{feed} is the protein content of the feed; D_{total} is the total weight of the feed; D is the feed intake; T is the breeding time; $W_{intestinal}$ is the intestinal weight.

3 Results

3.1 Growth performance

The growth performance is shown in Figure 1. WGR and PDR were significantly higher in CB2, CB3 and CB4 groups than in CB0 group ($P< 0.05$). The SGR of CB3 and CB4 groups was significantly higher than that of CB0 group ($P< 0.05$). ISI was significantly higher in CB1, CB2, CB3, and CB4 groups than in CB0 group ($P< 0.05$). FC was significantly lower in CB3 and CB4 groups than in CB0 group ($P< 0.05$). SR was 100% in all groups, and the difference between groups was not statistically significant ($P >0.05$). No significant change in CF between groups ($P >0.05$).

3.2 Liver GH-IGF system

GH-IGF signaling pathway mRNA expression is shown in Figure 2. The IGF-I mRNA expression levels in CB3 and CB4 groups were significantly higher than those in CB0 group ($P< 0.05$). The expression of GH mRNA in CB2, CB3, and CB4 was significantly higher than that in CB0 ($P< 0.05$).

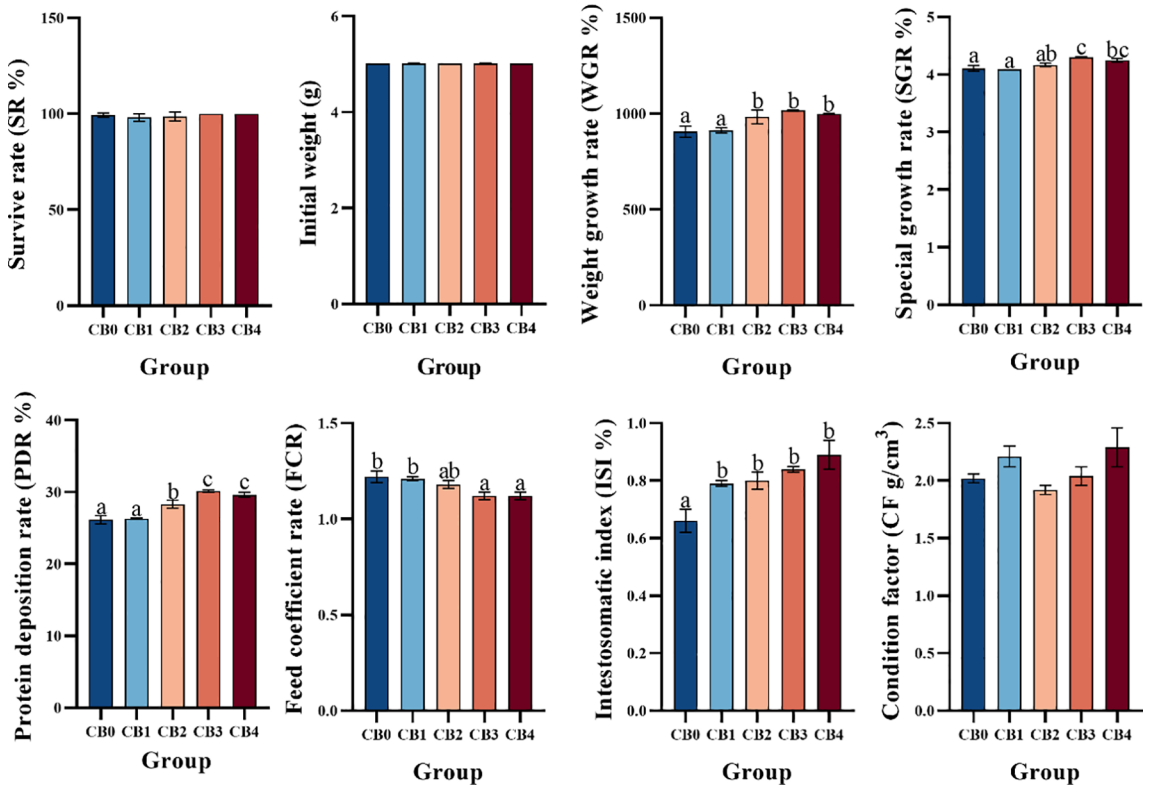

FIGURE 1
Effects of dietary CB growth performance, feed efficiency, and intestinal growth of juvenile largemouth bass *Micropterus salmoides*. [a,b,c] Bars with different superscripts represent significant difference ($P< 0.05$). Data presented are means ± SEM of 3 replicates. The same picture below.

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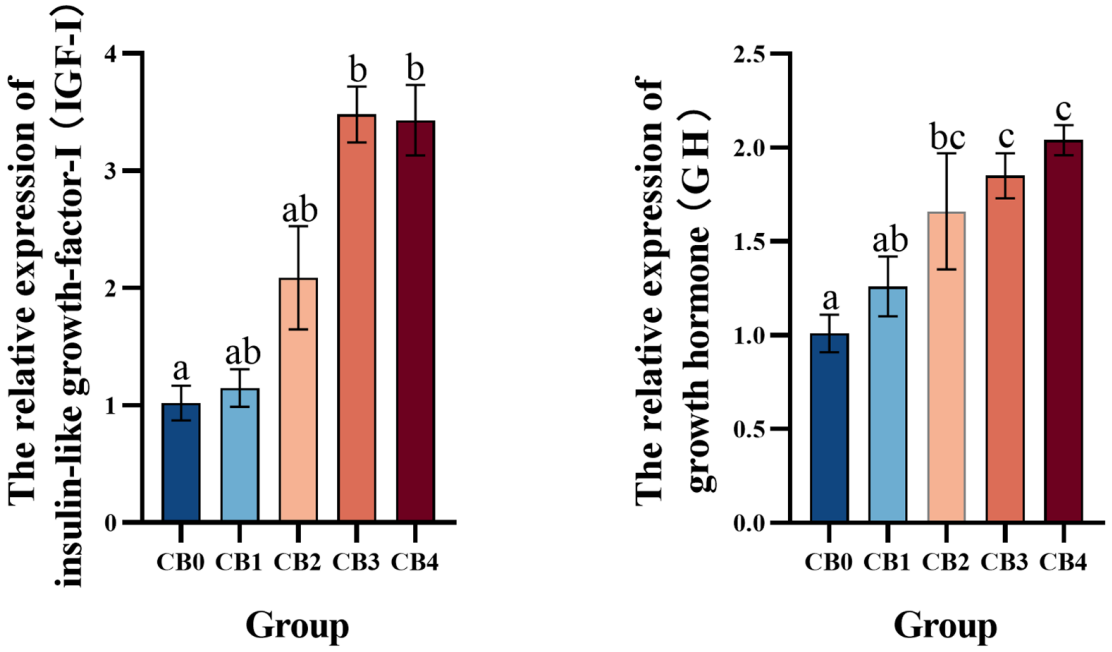

FIGURE 2
Effect of dietary CB levels on the relative expression of GH and IGF-I in the liver of largemouth bass.

3.3 Serum biochemistry

The serum biochemical indices are shown in Figure 3. AST and AKP were significantly lower in CB3 and CB4 groups than in CB0 group ($P< 0.05$). ALT, UN and TG were significantly lower in the CB4 group than in the CB0 group ($P< 0.05$). CB2, CB3 and CB4 serum TP levels were significantly higher than CB0 ($P< 0.05$). No significant changes between groups in TC, HDL, LDL ($P >0.05$).

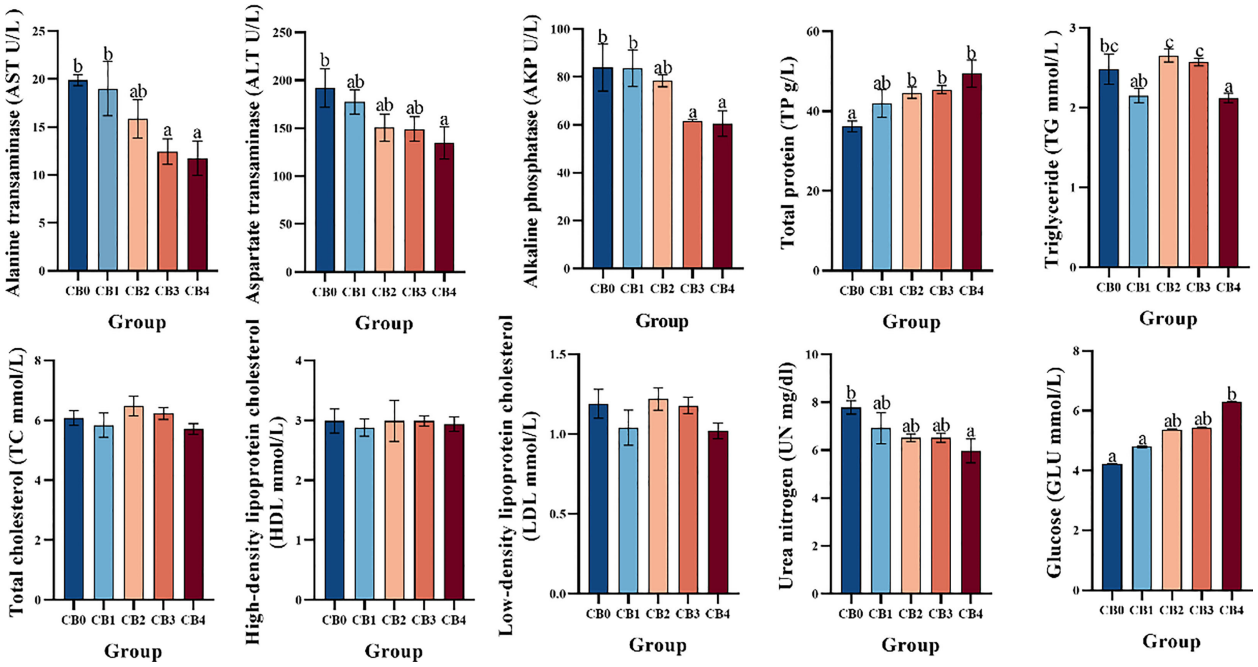

FIGURE 3
Effect of dietary CB on serum biochemistry of largemouth bass.

3.4 Antioxidative parameters

Antioxidant activity is shown in Figure 4. TP and T-AOC were significantly higher in CB1, CB2, CB3 and CB4 groups than in CB0 group ($P< 0.05$). CAT and SOD were significantly higher in CB2, CB3 and CB4 groups than in CB0 group ($P< 0.05$). POD and GSH-Px were significantly higher in CB3 and CB4 than in CB0 ($P< 0.05$). The MDA of CB3 and CB4 groups was significantly lower than that of CB0 group ($P< 0.05$).

3.5 Immune parameters

Immune-related gene expression is shown in Figure 5. *TLR22*, *TNF-α* and *IL-1β* were significantly lower in CB2, CB3 and CB4 groups than in CB0 group ($P< 0.05$). *IL-8* was significantly lower in CB4 than in CB0 ($P< 0.05$). *MyD88* was significantly lower in CB3 and CB4 groups than in CB0 group ($P< 0.05$). *IL-10* and *TGF-β* were significantly higher in the CB4 group than in the CB0 group ($P< 0.05$). LZM was significantly higher in CB2, CB3 and CB4 groups than in CB0 group ($P< 0.05$).

3.6 Hypoxic stress test

Hypoxic stress mortality is shown in Figure 6. CMR of largemouth bass significantly increased 3 hours into the experiment. The CMRs of fish were 68.75% (CB0 DO 0), 54.17% (CB1 DO 0), 52.08% (CB2 DO 0), 31.25% (CB3 DO 0) and 39.58% (CB4 DO 0) after 3 h.

4 Discussion

Dietary supplementation with CB significantly enhanced the growth performance of largemouth bass, as evidenced by increased WGR, SGR, ISI, and PDR and decreased FC. Consistent with our findings, CB has been observed to have beneficial effects on the growth of various livestock and fish species, including broilers (16), weaned piglets (34), Pacific white shrimp (*Penaeus vannamei*) (35), Yellow Catfish(*Pelteobagrus fulvidraco*) (36), large yellow croaker (19), tilapia (*Oreochromis niloticus*) (4), and silver pomfret (*silver pomfret*) (18). The *GH-IGF-I* signaling pathway is an endocrine signaling pathway that regulates growth and responds to the body's nutrient metabolism (37–39). The liver is also highly responsive to the nutritional status of the host and exerts significant regulatory effects on host nutrient metabolism and immune function (40–42). The findings of this investigation indicate that the inclusion of CB in the diet significantly upregulated *GH* and *IGF-I* mRNA expression, with *GH* and *IGF-I* being particularly responsive to varying levels of CB. This promotion of *GH* and *IGF-I* mRNA expression by dietary CB may account for its growth-promoting effects on largemouth bass.

For largemouth bass, it is equally important to ensure their health and optimize growth performance. Blood biochemical indicators can provide valuable insights into the fish's overall

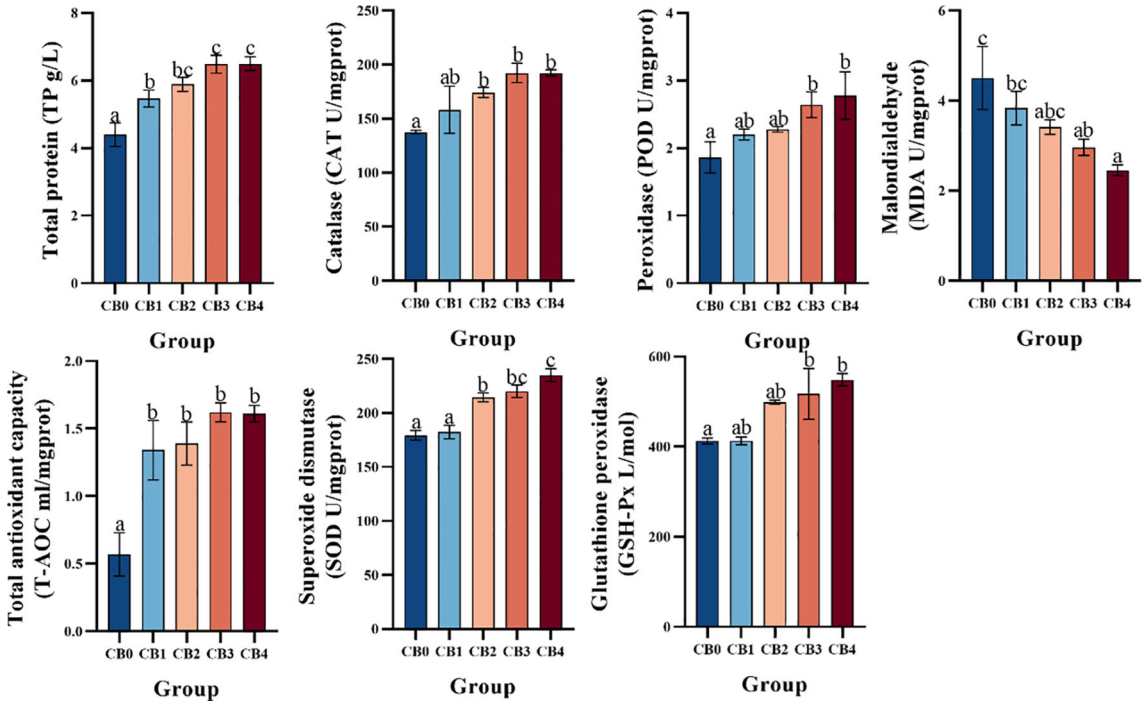

FIGURE 4
Effect of dietary CB on antioxidant activity in the liver of largemouth bass.

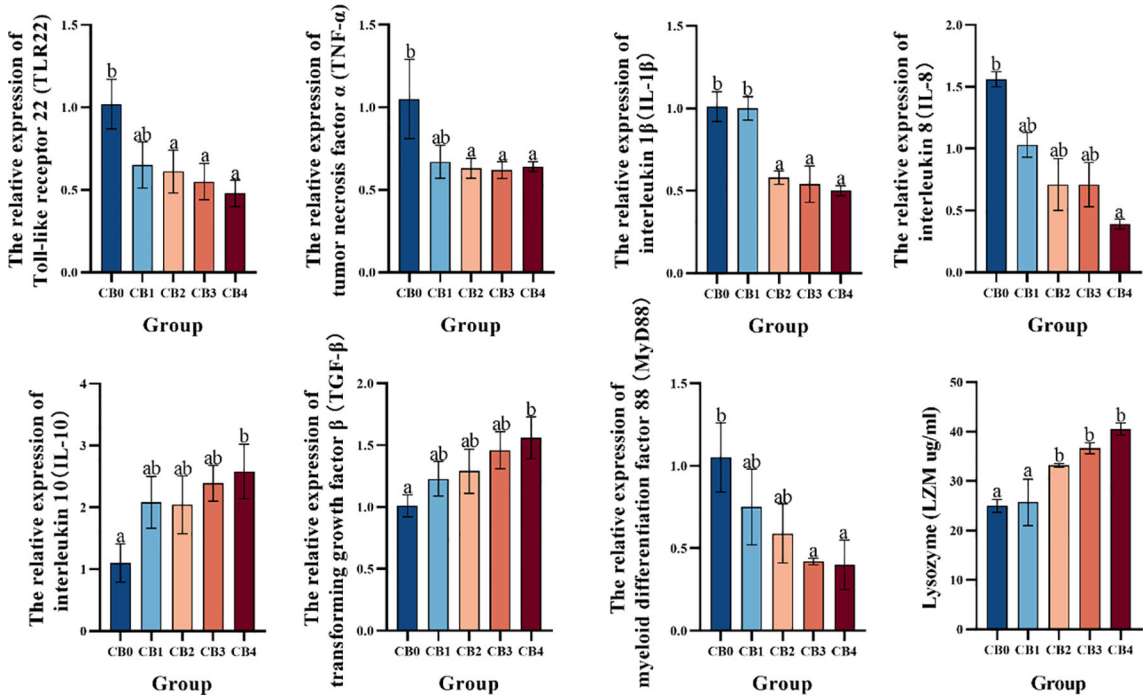

FIGURE 5
Effect of dietary CB on hepatic immune genes in the liver of largemouth bass.

health status, nutritional condition, and adaptability to its environment (43–46). Therefore, the effect of dietary additives on blood biochemical indicators must be verified before application. The content of TP in blood plays a crucial role in maintaining normal osmotic pressure and pH of blood vessels, serving as the basis for protein metabolism intake by the body (47). The findings of this study demonstrate that dietary CB can significantly elevate the serum total protein (TP) and glucose (GLU) levels in largemouth bass, which is consistent with the growth trend of protein deposition efficiency in fish. This suggests that CB supplementation can effectively enhance nutrient metabolism and promote protein deposition in largemouth bass. This indirectly indicates an enhancement in the growth performance of largemouth bass, which may be correlated. Serum ALT, AST and AKP serve as crucial serum markers for identifying animal nutrition and health status (48, 49). The changes in the activities of enzymes, including ALT, AST, and AKP, are directly correlated with the extent of organ-specific cellular damage. As an extracellular

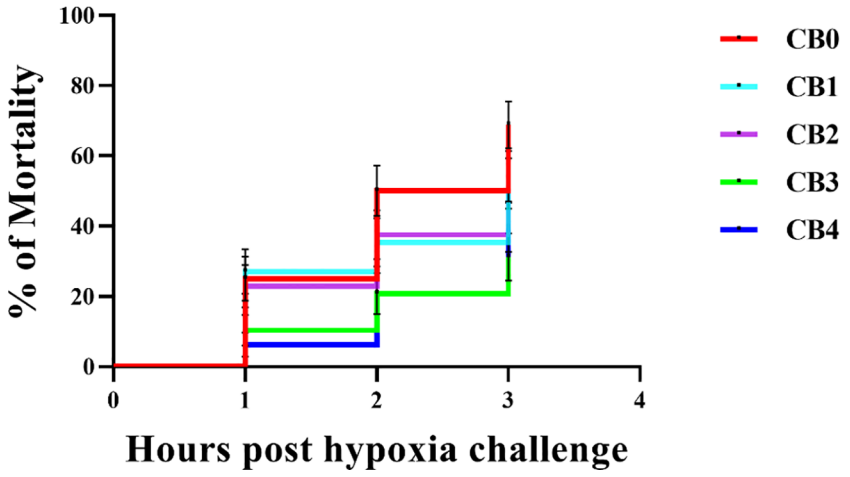

FIGURE 6
Mortality of largemouth bass *Micropterus salmoides* after 3 hours of hypoxic stress.

enzyme, AKP plays a crucial role in various biological processes including growth, cell differentiation, protein synthesis and immune metabolism (50–52). Elevated serum AKP activity indicates compromised cell membrane permeability and integrity, resulting in cellular damage and reflecting the occurrence of host hepatobiliary inflammation (53, 54). The findings of this investigation demonstrate that dietary supplementation with CB can significantly modulate the serum AKP activity in largemouth bass, both increasing and decreasing it. Moreover, when liver inflammation occurs, CB has the potential to enhance phagocytic activity of immune cells against foreign bodies and thus protect the liver from toxic invasion. The results also indicates that dietary CB significantly enhances nutrient digestion, a finding corroborated in yellow catfish. ALT and AST are important transaminases in the human body, and their content can reflect the health status of the liver. Normally, the activity of serum aminotransferases is low. However, elevated levels of ALT and AST indicate liver damage (44). UN, or blood urea nitrogen, is not a protein; however, it serves as an indicator of the body's protein metabolism status (54–57). Under the experimental conditions, it was observed that the control group exhibited elevated levels of serum AST, ALT, and UN, indicating potential hepatic inflammation or other pathological states. Notably, dietary supplementation of CB resulted in a significant reduction in serum AST, ALT and UN levels, indicating the hepatoprotective effect of CB on largemouth bass. However, further investigation is required to elucidate the underlying mechanism. Therefore, our objective was to identify immune and antioxidant genes in the liver of largemouth bass and comprehensively analyze the protective mechanism of CB against liver inflammation and disease states in this species.

The liver plays a crucial role in the response to oxidative stress and immune challenges (58). Oxygen free radical and lipid peroxidation reactions are integral components of the body's metabolic processes (50, 52, 59, 60). Under normal circumstances, the two systems work in concert to maintain a multitude of physiological, biochemical, and immunological responses within the body (48, 51). SOD, POD, GSH-Px and CAT are crucial enzymes in the biological antioxidant defense system that play a vital role in scavenging reactive oxygen species within the body to safeguard cell membranes and nucleic acids (49, 61). T-AOC reflects the body's compensatory ability to external stimuli and the status of free radical metabolism (62, 63). The MDA content serves as an indicator of the extent of cellular damage (63–66). The findings of this experiment indicate that dietary CB significantly enhances the hepatic activities of T-AOC, SOD, CAT, POD and GSH-Px in largemouth bass, which is consistent with the results obtained from a previous study on hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂ *E. lanceolatu*) (60). It was also observed that dietary CB significantly reduced the MDA content in the liver of freshwater shrimp, which is consistent with the results of the study on yellow catfish (36), freshwater prawn (21), and Pacific white shrimp (67). The findings indicated that dietary supplementation of CB could significantly enhance the antioxidant enzyme activity in largemouth bass, decrease the level of MDA, suppress hepatic lipid peroxidation and safeguard against oxidative stress-induced damage. The incorporation of CB significantly augmented the antioxidant potential of largemouth bass, potentially ascribed to its pivotal role in scavenging highly reactive oxygen free radicals and producing antioxidant enzymes, thereby mitigating lipid peroxidation and effectively quenching free radicals, ultimately conferring cellular protection against oxidative damage (21).

Oxidative stress and inflammatory response are interrelated processes that significantly contribute to the body's response to various stressors (68). The inflammatory response is initiated and modulated by pro-inflammatory cytokines (69, 70). Interleukin-8 (*IL-8*), a pro-inflammatory cytokine, stimulates the activation of macrophages and neutrophils, thereby promoting tissue regeneration in response to damage (71–73). Interleukin-1β (*IL-1β*) and tumor necrosis factor-α (*TNF-α*) primarily elicit inflammatory responses by modulating the expression of other cytokines (74). Interleukin-10 (*IL-10*) and transforming growth factor β (*TGF-β*) exert inhibitory effects on the proliferation, activation, and migration of T and B lymphocytes, thereby limiting the inflammatory response (75–78). Lysozyme (LZM) is a crucial antibacterial agent that can disrupt bacterial cell walls, stimulate the alternative complement pathway and phagocytic activity, and contribute to the body's non-specific immune defense (74, 79–81). Toll-like receptors (TLRs) recognize invading microbial pathogens and initiate cellular signaling pathways. Toll-like receptor 22 (*TLR22*) and myeloid differentiation factor 88 (*MyD88*) are implicated in the regulation of inflammatory processes (82–84). Upregulation of *IL-6* and *TNF-α* expression may be attributed to tissue damage caused by infection or oxidative stress (85, 86). Under the current experimental conditions, dietary CB significantly suppressed the expression of hepatic *IL-8*, *IL-1β*, *TNF-α*, *MyD88* and *TLR22* genes in largemouth bass. Notably, the expression of *IL-10* and *TGF-β* genes was significantly upregulated in the liver of largemouth bass, while the activity of LZM was also significantly elevated. The findings indicated that dietary supplementation of CB could activate Toll receptors in largemouth bass, mediate *MyD88*-dependent signaling pathway, ultimately inhibit the release of proinflammatory cytokines *IL-8*, *IL-1β* and *TNF-α*, activate the acquired immune response, ultimately regulate immune function, enhance body resistance and mitigate liver inflammatory response. Similar studies have demonstrated that dietary CB significantly reduces serum levels of tumor necrosis factor-alpha (*TNF-α*) and increases interleukin-10 (*IL-10*) expression in children (87), markedly inhibits the expression of *IL-6* and *TNF-α* in chicken intestines (88), substantially elevates serum *IL-10* content and decreases the content of *IL-1β* in weanling piglets (89), and significantly reduces the expression of *IL-1β* and *IL-8* in yellow catfish (36). In summary, dietary CB can protect the liver from oxidative damage by regulating antioxidant enzyme activity and alleviate the hepatic inflammatory response by upregulating the expression of anti-inflammatory factors and inhibiting the expression of pro-inflammatory factors. Interestingly, the inclusion of CB in the diet resulted in a significant upregulation of *TLR22* expression in piglet ileum and promoted *MyD88* gene expression, which contrasts with the findings of this experiment. The expression of *MyD88* is modulated by its associated signaling cascade. In general, Toll-like receptors are the only ones stimulated by pathogens. *MyD88* is

transcriptionally activated by anti-inflammatory factors through *NF-kB* family genes. Under the experimental conditions, when largemouth bass was exposed to oxidative stress, the liver underwent pathological changes and the body was stimulated by bacteria or toxins. Toll-like receptors (TLRs) mediated immune responses through the *MyD88*-dependent pathway. The inclusion of CB in the diet resulted in a significant increase in hepatic LZM activity and stimulated phagocytic function. The expression of pro-inflammatory cytokines *IL-8*, *IL-1β* and *TNF-α* was significantly reduced, while the genes for anti-inflammatory cytokines *IL-10 and TGF-β* were up-regulated, leading to suppression of the inflammatory response. The comprehensive analysis revealed that CB exerted significant effects on the expression of *TLR22* pathway-associated genes in largemouth bass. CB has the potential to modulate *TLR22* pathway-related factors, thereby mitigating inflammatory response and enhancing immune function.

Hypoxia is a common stressor experienced by aquatic animals in aquaculture, which can result in inhibited behavior and physiological metabolism of fish, ultimately leading to oxidative damage across various organs. Fish can enhance their antioxidant activities by increasing the activity of antioxidant enzymes. The dietary addition of CB significantly boosts the antioxidant capacity of tilapia, *Macrobrachium robertsoni*, *Penaeus vannamei*, and *Penaeus prefixus*. Reaffirming the findings of previous studies, the results of our current experiment demonstrate that dietary CB significantly enhances the activities of antioxidant enzymes in a similar manner. Interestingly, the results of the 3-hour hypoxic stress experiment on largemouth bass revealed that CB1, CB2, CB3 and CB4 had significantly lower CMRs than CB0 by 21.22%, 24.25%, 54.55% and 42.43% respectively, indicating a significant improvement in hypoxia stress survival rate due to dietary CB. The enhanced anti-hypoxic activity of CB in promoting survival may be attributed to the upregulated hepatic antioxidant enzyme activities (SOD, GSH-Px, CAT, POD) and decreased hepatic MDA concentration observed in this study, which ultimately improved the antioxidative status of largemouth bass by elevating hepatic T-AOC levels.

5 Conclusions

Dietary CB (1.5×10^9 CFU/kg, CB3) can enhance the growth, antioxidant activity and hypoxic stress resistance of largemouth bass. By regulating GH/IGF-1 gene expression and inflammatory factors, as well as inhibiting TLR22/MyD88 signaling pathway, dietary CB can improve the growth performance and hypoxic stress resistance of largemouth bass.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was reviewed and approved by Collaborative Innovation Center of Aquatic Sciences, Guangdong Key Laboratory of Animal Breeding and Nutrition, Institute of Animal Science, Guangdong Academy of Agricultural Sciences.

Author contributions

PL wrote the paper; XC and BC performed the experiments; DH performed the experiments; KP performed the experiments; WH analyzed the data; JC designed the experiments; HZ conceived the experiments.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Defoirdt T, Sorgeloos P, Bossier P. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Curr Opin Microbiol* (2011) 14:251–8. doi: 10.1016/j.mib.2011.03.004

2. McAllister TA, Wang Y, Diarra MS, Alexander T, Stanford K. Challenges of a one-health approach to the development of alternatives to antibiotics. *Anim Front* (2018) 8:10–20. doi: 10.1093/af/vfy002

3. Wang Y, Zhou J, Wang G, Cai S, Zeng X, Qiao S. Advances in low-protein diets for swine. *J Anim Sci Biotechnol* (2018) 9:1–14. doi: 10.1186/s40104-018-0276-7

4. Li H, Zhou Y, Ling H, Luo L, Qi D, Feng L. The effect of dietary supplementation with Clostridium butyricum on the growth performance, immunity, intestinal

microbiota and disease resistance of tilapia (*Oreochromis niloticus*). *PloS One* (2019) 14: e0223428. doi: 10.1371/journal.pone.0223428

5. Fu J, Wang T, Xiao X, Cheng Y, Wang F, Jin M, et al. Clostridium butyricum ZJU-F1 benefits the intestinal barrier function and immune response associated with its modulation of gut microbiota in weaned piglets. *Cells* (2021) 10:527. doi: 10.3390/cells10030527

6. Hasan KN, Banerjee G. Recent studies on probiotics as beneficial mediator in aquaculture: a review. *J Basic Appl Zool* (2020) 81:1–16. doi: 10.1186/s41936-020-00190-y

7. Vendrell D, Balcázar JL, de Blas I, Ruiz-Zarzuela I, Gironés O, Muzquiz JL. Protection of rainbow trout (*Oncorhynchus mykiss*) from lactococcosis by probiotic bacteria. *Comp Immunol Microbiol Infect Dis* (2008) 31:337–45. doi: 10.1016/j.cimid.2007.04.002

8. Allameh SK, Yusoff FM, Ringø E, Daud HM, Saad CR, Ideris A. Effects of dietary mono-and multiprobiotic strains on growth performance, gut bacteria and body composition of J avanese carp (*Puntius gonionotus*, B leeker 1850). *Aquac Nutr* (2016) 22:367–73. doi: 10.1111/anu.12265

9. Van Doan H, Lumsangkul C, Jaturasitha S, Meidong R, Hoseinifar SH, Dawood MAO. Modulation of growth, skin mucus and serum immunities, and disease resistance of Nile tilapia fed host-associated probiotic (*Lactobacillus paracasei* l61-27b). *Aquac Nutr* (2021) 27:3–12. doi: 10.1111/anu.13314

10. Nayak SK. Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol* (2010) 29:2–14. doi: 10.1016/j.fsi.2010.02.017

11. Miao R, Zhu X, Wan C, Wang Z, Wen Y, Li Y. Effect of Clostridium butyricum supplementation on the development of intestinal flora and the immune system of neonatal mice. *Exp Ther Med* (2018) 15:1081–6. doi: 10.3892/etm.2017.5461

12. Mohammadian T, Nasirpour M, Tabandeh MR, Heidary AA, Ghanei-Motlagh R, Hosseini SS. Administrations of autochthonous probiotics altered juvenile rainbow trout Oncorhynchus mykiss health status, growth performance and resistance to *Lactococcus garvieae*, an experimental infection. *Fish Shellfish Immunol* (2019) 86:269–79. doi: 10.1016/j.fsi.2018.11.052

13. Liu L, Zeng D, Yang M, Wen B, Lai J, Zhou Y, et al. Probiotic Clostridium butyricum improves the growth performance, immune function, and gut microbiota of weaning rex rabbits. *Probiot Antimicrob Proteins* (2019) 11:1278–92. doi: 10.1007/s12602-018-9476-x

14. Tran NT, Li Z, Ma H, Zhang Y, Zheng H, Gong Y, et al. Clostridium butyricum: a promising probiotic confers positive health benefits in aquatic animals. *Rev Aquac* (2020) 12:2573–89. doi: 10.1111/raq.12459

15. Hsiao Y-P, Chen H-L, Tsai J-N, Lin M-Y, Liao J-W, Wei M-S, et al. Administration of Lactobacillus reuteri combined with Clostridium butyricum attenuates cisplatin-induced renal damage by gut microbiota reconstitution, increasing butyric acid production, and suppressing renal inflammation. *Nutrients* (2021) 13:2792. doi: 10.3390/nu13082792

16. Xu L, Sun X, Wan X, Li K, Jian F, Li W, et al. Dietary supplementation with Clostridium butyricum improves growth performance of broilers by regulating intestinal microbiota and mucosal epithelial cells. *Anim Nutr* (2021) 7:1105–14. doi: 10.1016/j.aninu.2021.01.009

17. Zhang L, Cao GT, Zeng XF, Zhou L, Ferket PR, Xiao YP, et al. Effects of Clostridium butyricum on growth performance, immune function, and cecal microflora in broiler chickens challenged with Escherichia coli K88. *Poult Sci* (2014) 93:46–53. doi: 10.3382/ps.2013-03412

18. Gao Q, Xiao C, Min M, Zhang C, Peng S, Shi Z. Effects of probiotics dietary supplementation on growth performance, innate immunity and digestive enzymes of silver pomfret, *Pampus argenteus*. *Indian J Anim Res* (2016) 50:936–41. doi: 10.18805/ijar.9640

19. Yin Z, Liu Q, Liu Y, Gao S, He Y, Yao C, et al. Early life intervention using probiotic clostridium butyricum improves intestinal development, immune response, and gut microbiota in large yellow croaker (*Larimichthys crocea*) larvae. *Front Immunol* (2021) 12:640767. doi: 10.3389/fimmu.2021.640767

20. Poolsawat L, Li X, He M, Ji D, Leng X. Clostridium butyricum as probiotic for promoting growth performance, feed utilization, gut health and microbiota community of tilapia (Oreochromis niloticus× O. aureus). *Aquac Nutr* (2020) 26:657–70. doi: 10.1111/anu.13025

21. Wangari MR, Gao Q, Sun C, Liu B, Song C, Tadese DA, et al. Effect of dietary Clostridium butyricum and different feeding patterns on growth performance, antioxidant and immune capacity in freshwater prawn (*Macrobrachium rosenbergii*). *Aquac Res* (2021) 52:12–22. doi: 10.1111/are.14865

22. Duan Y, Dong H, Wang Y, Zhang Y, Zhang J. Effects of the dietary probiotic Clostridium butyricum on intestine digestive and metabolic capacities, SCFA content and body composition in *Marsupenaeus japonicus*. *J Ocean Univ China* (2018) 17:690–6. doi: 10.1007/s11802-018-3464-3

23. Li S, Sang C, Turchini GM, Wang A, Zhang J, Chen N. Starch in aquafeeds: the benefits of a high amylose to amylopectin ratio and resistant starch content in diets for the carnivorous fish, largemouth bass (*Micropterus salmoides*). *Br J Nutr* (2020) 124:1145–55. doi: 10.1017/S0007114520002214

24. Gong Y, Yang F, Hu J, Liu C, Liu H, Han D, et al. Effects of dietary yeast hydrolysate on the growth, antioxidant response, immune response and disease resistance of largemouth bass (*Micropterus salmoides*). *Fish Shellfish Immunol* (2019) 94:548–57. doi: 10.1016/j.fsi.2019.09.044

25. Yusuf A, Huang X, Chen N, Apraku A, Wang W, Cornel A, et al. Impact of dietary vitamin c on plasma metabolites, antioxidant capacity and innate immunocompetence in juvenile largemouth bass, *Micropterus salmoides*. *Aquac Rep* (2020) 17:100383. doi: 10.1016/j.aqrep.2020.100383

26. Adeoye AA, Yomla R, Jaramillo-Torres A, Rodiles A, Merrifield DL, Davies SJ. Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis niloticus*) growth, intestinal morphology and microbiome. *Aquaculture* (2016) 463:61–70. doi: 10.1016/j.aquaculture.2016.05.028

27. Dawood MAO, Koshio S, Esteban MÁ. Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Rev Aquac* (2018) 10:950–74. doi: 10.1111/raq.12209

28. Bharathi S, Antony C, Cbt R, Arumugam U, Ahilan B, Aanand S. Functional feed additives used in fish feeds. *Int J Fish Aquat Stud* (2019) 7:44–52.

29. Ogunkalu O. Effects of feed additives in fish feed for improvement of aquaculture. *Eurasian J Food Sci Technol* (2019) 3:49–57.

30. Elsabagh M, Mohamed R, Moustafa EM, Hamza A, Farrag F, Decamp O, et al. Assessing the impact of Bacillus strains mixture probiotic on water quality, growth performance, blood profile and intestinal morphology of Nile tilapia, *Oreochromis niloticus*. *Aquac Nutr* (2018) 24:1613–22. doi: 10.1111/anu.12797

31. Wei CL, Chao SH, Tsai WB, et al. Analysis of bacterial diversity during the fermentation of inyu, a high- temperature fermented soy sauce, using nested PCR-denaturing gradient gel electrophoresis and the plate count method. *Food Microbiol* (2013) 33(2):252–61.

32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *methods* (2001) 25:402–8. doi: 10.1006/meth.2001.1262

33. Zeng L, Wang Y-H, Ai C-X, Zheng J-L, Wu C-W, Cai R. Effects of β-glucan on ROS production and energy metabolism in yellow croaker (*Pseudosciaena crocea*) under acute hypoxic stress. *Fish Physiol Biochem* (2016) 42:1395–405. doi: 10.1007/s10695-016-0227-1

34. Liang J, Kou S, Chen C, Raza SHA, Wang S, Ma X, et al. Effects of Clostridium butyricum on growth performance, metabonomics and intestinal microbial differences of weaned piglets. *BMC Microbiol* (2021) 21:1–16. doi: 10.1186/s12866-021-02143-z

35. Luo K, Tian X, Wang B, Wei C, Wang L, Zhang S, et al. Evaluation of paraprobiotic applicability of Clostridium butyricum CBG01 in improving the growth performance, immune responses and disease resistance in Pacific white shrimp, *Penaeus vannamei*. *Aquaculture* (2021) 544:737041. doi: 10.1016/j.aquaculture.2021.737041

36. Li P, Hou D, Zhao H, Wang H, Peng K, Cao J. Dietary Clostridium butyricum Improves Growth Performance and Resistance to Ammonia Stress in Yellow Catfish (*Pelteobagrus fulvidraco*). *Aquac Nutr* (2022) 2022:6965174. doi: 10.1155/2022/6965174

37. Yi C-C, Liu C-H, Chuang K-P, Chang Y-T, Hu S-Y. A potential probiotic Chromobacterium aquaticum with bacteriocin-like activity enhances the expression of indicator genes associated with nutrient metabolism, growth performance and innate immunity against pathogen infections in zebrafish (*Danio rerio*). *Fish Shellfish Immunol* (2019) 93:124–34. doi: 10.1016/j.fsi.2019.07.042

38. Yang P, Wang W, Chi S, Mai K, Song F, Wang L. Effects of dietary lysine on regulating GH-IGF system, intermediate metabolism and immune response in largemouth bass (*Micropterus salmoides*). *Aquac Rep* (2020) 17:100323. doi: 10.1016/j.aqrep.2020.100323

39. Al-Samerria S, Radovick S. The role of insulin-like growth factor-1 (IGF-1) in the control of neuroendocrine regulation of growth. *Cells* (2021) 10:2664. doi: 10.3390/cells10102664

40. Sheridan MA, Hagemeister AL. Somatostatin and somatostatin receptors in fish growth. *Gen Comp Endocrinol* (2010) 167:360–5. doi: 10.1016/j.ygcen.2009.09.002

41. Norbeck LA, Sheridan MA. An in *vitro* model for evaluating peripheral regulation of growth in fish: effects of 17β-estradiol and testosterone on the expression of growth hormone receptors, insulin-like growth factors, and insulin-like growth factor type 1 receptors in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* (2011) 173:270–80. doi: 10.1016/j.ygcen.2011.06.009

42. Mohammed-Geba K, Martos-Sitcha JA, Galal-Khallaf A, Mancera JM, Martínez-Rodríguez G. Insulin-like growth factor 1 (IGF-1) regulates prolactin, growth hormone, and IGF-1 receptor expression in the pituitary gland of the gilthead sea bream *Sparus aurata*. *Fish Physiol Biochem* (2016) 42:365–77. doi: 10.1007/s10695-015-0144-8

43. Bonvini E, Bonaldo A, Parma L, Mandrioli L, Sirri R, Grandi M, et al. Feeding European sea bass with increasing dietary fibre levels: Impact on growth, blood biochemistry, gut histology, gut evacuation. *Aquaculture* (2018) 494:1–9. doi: 10.1016/j.aquaculture.2018.05.017

44. Guardiola FA, Saraiva-Fraga M, Cuesta A, Esteban MA. Changes in natural haemolytic complement activity induced by stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* (2018) 78:317–21. doi: 10.1016/j.fsi.2018.04.056

45. Parma L, Pelusio NF, Gisbert E, Esteban MA, D'Amico F, Soverini M, et al. Effects of rearing density on growth, digestive conditions, welfare indicators and gut bacterial community of gilthead sea bream (*Sparus aurata*, L. 1758) fed different fishmeal and fish oil dietary levels. *Aquaculture* (2020) 518:734854. doi: 10.1016/j.aquaculture.2019.734854

46. Pelusio NF, Bonaldo A, Gisbert E, Andree KB, Esteban MA, Dondi F, et al. Different fish meal and fish oil dietary levels in European sea bass: welfare implications after acute confinement stress. *Front Mar Sci* (2022) 8:2003. doi: 10.3389/fmars.2021.779053

47. Saurav K, Raman RP, Kundan K, Pandey PK, Neeraj K, Mallesh B, et al. Effect of azadirachtin on haematological and biochemical parameters of Argulus-infested goldfish *Carassius auratus* (Linn. 1758). *Fish Physiol Biochem* (2013) 39:733–47. doi: 10.1007/s10695-012-9736-8

48. Akbary P, Sartipi Yarahmadi S, Jahanbakhshi A. Hematological, hepatic enzymes' activity and oxidative stress responses of gray mullet (*Mugil cephalus*) after sub-acute exposure to copper oxide. *Environ Sci Pollut Res* (2018) 25:1800–8. doi: 10.1007/s11356-017-0582-1

49. Ao H, Jiang J, Liu L, Liu Y, Hu B, Chen Y. Effects of dietary fermentation products of kitchen waste on growth, apparent digestibility, digestive enzyme activities and serum biochemistry in juvenile allogynogenetic gibel carp (*Carassius auratus gibelio*) var. CAS III. *J World Aquac Soc* (2021) 52:895–912. doi: 10.1111/jwas.12826

50. Dufour DR, Lott JA, Nolte FS, Gretch DR, Koff RS, Seeff LB. Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. *Clin Chem* (2000) 46:2027–49. doi: 10.1093/clinchem/46.12.2027

51. Zhang C, Zhang Q, Pang Y, Song X, Zhou N, Wang J, et al. The protective effects of melatonin on oxidative damage and the immune system of the Chinese mitten crab (*Eriocheir sinensis*) exposed to deltamethrin. *Sci Total Environ* (2019) 653:1426–34. doi: 10.1016/j.scitotenv.2018.11.063

52. Wu YC, Li RM, Shen G, Huang F, Yang Q, Tan B, et al. Effects of dietary small peptides on growth, antioxidant capacity, nonspecific immunity and ingut microflora structure of *Litopenaeus vannamei*. *J Guangdong Ocean Univ* (2021) 41:1–9.

53. Lin H, Tan X, Zhou C, Niu J, Xia D, Huang Z, et al. Effect of dietary arginine levels on the growth performance, feed utilization, non-specific immune response and disease resistance of juvenile golden pompano *Trachinotus ovatus*. *Aquaculture* (2015) 437:382–9. doi: 10.1016/j.aquaculture.2014.12.025

54. Tan X, Sun Z, Chen S, Chen S, Huang Z, Zhou C, et al. Effects of dietary dandelion extracts on growth performance, body composition, plasma biochemical parameters, immune responses and disease resistance of juvenile golden pompano *Trachinotus ovatus*. *Fish Shellfish Immunol* (2017) 66:198–206. doi: 10.1016/j.fsi.2017.05.028

55. Lin S-M, Shi C-M, Mu M-M, Chen Y-J, Luo L. Effect of high dietary starch levels on growth, hepatic glucose metabolism, oxidative status and immune response of juvenile largemouth bass, *Micropterus salmoides*. *Fish Shellfish Immunol* (2018) 78:121–6. doi: 10.1016/j.fsi.2018.04.046

56. Yi Y, Zhang Z, Zhao F, Liu H, Yu L, Zha J, et al. Probiotic potential of Bacillus velezensis JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*. *Fish Shellfish Immunol* (2018) 78:322–30. doi: 10.1016/j.fsi.2018.04.055

57. Chen X-Q, Zhao W, Xie S-W, Xie J-J, Zhang Z-H, Tian L-X, et al. Effects of dietary hydrolyzed yeast (*Rhodotorula mucilaginosa*) on growth performance, immune response, antioxidant capacity and histomorphology of juvenile Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol* (2019) 90:30–9. doi: 10.1016/j.fsi.2019.03.068

58. Jin H, Yan C, Xiao T, Yan N, Xu J, Zhou L, et al. High fish oil diet promotes liver inflammation and activates the complement system. *Mol Med Rep* (2018) 17:6852–8. doi: 10.3892/mmr.2018.8687

59. Zhao J, Liu Y, Jiang J, Wu P, Jiang W, Li S, et al. Effects of dietary isoleucine on the immune response, antioxidant status and gene expression in the head kidney of juvenile Jian carp (*Cyprinus carpio* var. *Jian). Fish Shellfish Immunol* (2013) 35:572–80. doi: 10.1016/j.fsi.2013.05.027

60. Ni P-J, Jiang W-D, Wu P, Liu Y, Kuang S-Y, Tang L, et al. Dietary low or excess levels of lipids reduced growth performance, and impaired immune function and structure of head kidney, spleen and skin in young grass carp (*Ctenopharyngodon idella*) under the infection of Aeromonas hydrophila. *Fish Shellfish Immunol* (2016) 55:28–47. doi: 10.1016/j.fsi.2016.03.163

61. Li Y, Wu J, Chen X, Chu Z, Jin J, Tang D. Dietary myo-inositol requirements of juvenile hybrid sturgeon (Acipenser baerii♀× A. schrenckii♂). *Aquac Res* (2020) 51:1143–51. doi: 10.1111/are.14461

62. Tan X, Lin H, Huang Z, Zhou C, Wang A, Qi C, et al. Effects of dietary leucine on growth performance, feed utilization, non-specific immune responses and gut morphology of juvenile golden pompano *Trachinotus ovatus*. *Aquaculture* (2016) 465:100–7. doi: 10.1016/j.aquaculture.2016.08.034

63. Kong Y, Li M, Chu G, Liu H, Shan X, Wang G, et al. The positive effects of single or conjoint administration of lactic acid bacteria on *Channa argus*: Digestive enzyme activity, antioxidant capacity, intestinal microbiota and morphology. *Aquaculture* (2021) 531:735852. doi: 10.1016/j.aquaculture.2020.735852

64. Cheng C-H, Yang F-F, Ling R-Z, Liao S-A, Miao Y-T, Ye C-X, et al. Effects of ammonia exposure on apoptosis, oxidative stress and immune response in pufferfish (*Takifugu obscurus*). *Aquat Toxicol* (2015) 164:61–71. doi: 10.1016/j.aquatox.2015.04.004

65. Lan Y, Ye T, Xue Y, Liu H, Zhang H, Cheng D, et al. Physiological and immunological responses to mass mortality in noble scallop Chlamys nobilis cultured in Nan'ao waters of Shantou, China. *Fish Shellfish Immunol* (2018) 82:453–9. doi: 10.1016/j.fsi.2018.08.049

66. Yu Z, Wu X-Q, Zheng L-J, Dai Z-Y, Wu L-F. Effect of acute exposure to ammonia and BFT alterations on Rhynchocypris lagowski: Digestive enzyme, inflammation response, oxidative stress and immunological parameters. *Environ Toxicol Pharmacol* (2020) 78:103380. doi: 10.1016/j.etap.2020.103380

67. Duan Y, Zhang Y, Dong H, Wang Y, Zheng X, Zhang J. Effect of dietary Clostridium butyricum on growth, intestine health status and resistance to ammonia stress in Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol* (2017) 65:25–33. doi: 10.1016/j.fsi.2017.03.048

68. Zhang J-X, Guo L-Y, Feng L, Jiang W-D, Kuang S-Y, Liu Y, et al. Soybean β-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. *PloS One* (2013) 8:e58115. doi: 10.1371/journal.pone.0058115

69. Zhao J, Feng L, Liu Y, Jiang W, Wu P, Jiang J, et al. Effect of dietary isoleucine on the immunity, antioxidant status, tight junctions and microflora in the intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immunol* (2014) 41:663–73. doi: 10.1016/j.fsi.2014.10.002

70. Chen L, Feng L, Jiang W-D, Jiang J, Wu P, Zhao J, et al. Intestinal immune function, antioxidant status and tight junction proteins mRNA expression in young grass carp (*Ctenopharyngodon idella*) fed riboflavin deficient diet. *Fish Shellfish Immunol* (2015) 47:470–84. doi: 10.1016/j.fsi.2015.09.037

71. Stalnikowitz DK, Weissbrod AB. Liver fibrosis and inflammation. A review. *Ann Hepatol* (2003) 2:159–63. doi: 10.1016/S1665-2681(19)32127-1

72. Yin G, Li W, Lin Q, Lin XI, Lin J, Zhu Q, et al. Dietary administration of laminarin improves the growth performance and immune responses in *Epinephelus coioides*. *Fish Shellfish Immunol* (2014) 41:402–6. doi: 10.1016/j.fsi.2014.09.027

73. El-Leithy AAA, Hemeda SA, El Naby WSHA, El Nahas AF, Hassan SAH, Awad ST, et al. Optimum salinity for Nile tilapia (*Oreochromis niloticus*) growth and mRNA transcripts of ion-regulation, inflammatory, stress-and immune-related genes. *Fish Physiol Biochem* (2019) 45:1217–32. doi: 10.1007/s10695-019-00640-7

74. Zhang C, Zhang J, Ren H, Zhou B, Wu Q, Sun P. Effect of tributyltin on antioxidant ability and immune responses of zebrafish (*Danio rerio*). *Ecotoxicol Environ Saf* (2017) 138:1–8. doi: 10.1016/j.ecoenv.2016.12.016

75. Li MO, Flavell RA. Contextual regulation of inflammation: a duet by transforming growth factor-β and interleukin-10. *Immunity* (2008) 28:468–76. doi: 10.1016/j.immuni.2008.03.003

76. Giri SS, Sen SS, Chi C, Kim HJ, Yun S, Park SC, et al. Effect of guava leaves on the growth performance and cytokine gene expression of *Labeo rohita* and its susceptibility to Aeromonas hydrophila infection. *Fish Shellfish Immunol* (2015) 46:217–24. doi: 10.1016/j.fsi.2015.05.051

77. Fabregat I, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G, et al. TGF-β signalling and liver disease. *FEBS J* (2016) 283:2219–32. doi: 10.1111/febs.13665

78. Zhang D, Tang J, Zhang J, Hu CX. Responses of pro-and anti-inflammatory cytokines in zebrafish liver exposed to sublethal doses of *Aphanizomenon flosaquae* DC-1 aphantoxins. *Aquat Toxicol* (2019) 215:105269. doi: 10.1016/j.aquatox.2019.105269

79. Chiu C-H, Cheng C-H, Gua W-R, Guu Y-K, Cheng W. Dietary administration of the probiotic, Saccharomyces cerevisiae P13, enhanced the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. *Fish Shellfish Immunol* (2010) 29:1053–9. doi: 10.1016/j.fsi.2010.08.019

80. Li Z-H, Zlabek V, Grabic R, Li P, Machova J, Velisek J, et al. Effects of exposure to sublethal propiconazole on the antioxidant defense system and Na+–K+-ATPase activity in brain of rainbow trout, *Oncorhynchus mykiss*. *Aquat Toxicol* (2010) 98:297–303. doi: 10.1016/j.aquatox.2010.02.017

81. Liu H, Wang S, Cai Y, Guo X, Cao Z, Zhang Y, et al. Dietary administration of Bacillus subtilis HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol* (2017) 60:326–33. doi: 10.1016/j.fsi.2016.12.003

82. Ding X, Lu D, Hou Q, Li S, Liu X, Zhang Y, et al. Orange-spotted grouper (*Epinephelus coioides*) toll-like receptor 22: molecular characterization, expression pattern and pertinent signaling pathways. *Fish Shellfish Immunol* (2012) 33:494–503. doi: 10.1016/j.fsi.2012.05.034

83. Zhang J, Kong X, Zhou C, Li L, Nie G, Li X. Toll-like receptor recognition of bacteria in fish: ligand specificity and signal pathways. *Fish Shellfish Immunol* (2014) 41:380–8. doi: 10.1016/j.fsi.2014.09.022

84. Zhao S, Chen Z, Zheng J, Dai J, Ou W, Xu W, et al. Citric acid mitigates soybean meal induced inflammatory response and tight junction disruption by altering TLR signal transduction in the intestine of turbot, *Scophthalmus maximus* L. *Fish Shellfish Immunol* (2019) 92:181–7. doi: 10.1016/j.fsi.2019.06.004

85. Takano T, Kondo H, Hirono I, Saito-Taki T, Endo M, Aoki T. Identification and characterization of a myeloid differentiation factor 88 (MyD88) cDNA and gene in Japanese flounder, *Paralichthys olivaceus*. *Dev Comp Immunol* (2006) 30:807–16. doi: 10.1016/j.dci.2005.11.003

86. Heng J, Su J, Huang T, Dong J, Chen L. The polymorphism and haplotype of TLR3 gene in grass carp (*Ctenopharyngodon idella*) and their associations with susceptibility/resistance to grass carp reovirus. *Fish Shellfish Immunol* (2011) 30:45–50. doi: 10.1016/j.fsi.2010.09.004

87. Chen C-C, Kong M-S, Lai M-W, Chao H-C, Chang K-W, Chen S-Y, et al. Probiotics have clinical, microbiologic, and immunologic efficacy in acute infectious diarrhea. *Pediatr Infect Dis J* (2010) 29:135–8. doi: 10.1097/INF.0b013e3181b530bf

88. Yang CM, Cao GT, Ferket PR, Liu TT, Zhou L, Zhang L, et al. Effects of probiotic, Clostridium butyricum, on growth performance, immune function, and cecal microflora in broiler chickens. *Poult Sci* (2012) 91:2121–9. doi: 10.3382/ps.2011-02131

89. Chen L, Li S, Zheng J, Li W, Jiang X, Zhao X, et al. Effects of dietary Clostridium butyricum supplementation on growth performance, intestinal development, and immune response of weaned piglets challenged with lipopolysaccharide. *J Anim Sci Biotechnol* (2018) 9:1–14. doi: 10.1186/s40104-018-0275-8

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Enterosorbents in complex therapy of food allergies: a focus on digestive disorders and systemic toxicity in children

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The review analyzes mechanisms and concomitant factors in developing IgE-associated allergic diseases provoked by food allergens and discusses clinical symptoms and current approaches for the treatment of food allergies. The expediency of using enterosorbents in complex therapy of food allergies and skin and respiratory manifestations associated with gastroenterological disorders is substantiated. The review summarizes the experience of using enterosorbents in post-Soviet countries to detoxify the human body. In this regard, special attention is paid to the enterosorbent White Coal (Carbowhite) based on silicon dioxide produced by the Ukrainian company OmniFarma.

KEYWORDS

food allergy, pseudoallergy, enterosorbents, detoxification, silicon dioxide, White Coal (Carbowhite), immunotoxicity

1 Introduction

According to the World Health Organization (WHO), allergic diseases are the third most common after cardiovascular and oncological pathologies. Approximately 40% of the world population is allergic to drugs, household and industrial allergens, insect bites, food ingredients, etc. According to WHO forecasts, by the end of the 21st century, every second person will suffer from some form of allergy (1).

Allergic diseases are one of the most common somatic diseases of childhood. In recent years, the prevalence of allergic diseases in schoolchildren has increased, reaching 20% (2,

Abbreviations: DCs, Dendritic Cells; IL, Interleukin; ILC, Innate Lymphoid Cell; MHC-II, Major Histocompatibility Complex Type II; TFH, T Follicular Helper Cell; Th2, T Helper Cell Type 2; TSLP, thymic stromal lymphopoietin.

3). The formation of allergies in children is characterized by the stages of development of sensitization and the transformation of clinical manifestations, depending on the child's age. In children with atopy, the allergy generally manifests in food allergies and atopic dermatitis already in early childhood. Later, 10-15% of children develop allergic rhinitis, and 40-43% develop bronchial asthma. The severe course of atopic dermatitis and atopic rhinitis is a risk factor for the subsequent development of bronchial asthma (2–5). Since this process is often triggered by early childhood food allergies, therapeutic and preventive measures aimed at timely detection and compensation of allergic manifestations in children caused by food components are relevant.

The increasing prevalence of allergic diseases among schoolchildren and the high prevalence of food allergies in early childhood are complex issues influenced by a combination of genetic, environmental, and lifestyle factors. While the exact causes are not fully understood, several potential contributing factors are discussed (2–5): 1) Allergies often have a genetic component. Children with a family history of allergies are at a higher risk of developing allergic diseases. 2) The hygiene hypothesis suggests that reduced exposure to infections and certain microbes in early childhood due to increased hygiene and reduced family size may lead to an improperly developed immune system resulting in allergies. 3) Changes in diet, such as increased consumption of processed foods and a decrease in the consumption of fresh fruits and vegetables, may influence the development of allergies. 4) Exposure to environmental factors like pollution and a lack of contact with animals and rural environments may impact immune system development and contribute to the rise in allergies. 5) Early life events, including the mode of birth (cesarean section vs. vaginal birth) and breastfeeding practices, can influence a child's susceptibility to allergens. 6) The composition of the gut microbiota in early life can play a crucial role in immune system development. Changes in the gut microbiota due to factors like antibiotic use, diet, and hygiene practices may impact the risk of allergies. 7) Psychological stress and lifestyle factors, such as lack of physical activity or excessive screen time, may also influence immune system function and contribute to the development of allergies. 9) Vaccination practices and antibiotic use in early childhood may influence allergy development.

It's important to note that these factors likely interact with each other, and the specific combination of influences can vary from one individual to another.

Food allergy is one of the types of allergic reactions that develops when eating certain foods that contain an allergen, which in turn causes an aggressive pathological response of the immune system, which manifests, in most cases in an immediate type I reaction, due to hypersynthesis and critical participation of IgE (4–6). Food allergy occupies a special place among allergic manifestations in terms of prevalence and peculiarities of its etiology, prevention, and treatment. It is one of the main reasons for developing allergic diseases in children (5, 7–10). Medical observations suggest that food allergy occurs in 6-8% of children under two years of age (60-94% of those cases occur in the first year of life), with a subsequent decrease in its prevalence to 2% among the adult population (4, 5, 10).

Food allergy symptoms are often presented through itching in the mouth and larynx, laryngeal edema, coughing for no reason, wheezing and shortness of breath, and pain during a conversation. Nausea, vomiting, and diarrhea may meanwhile occur in more acute cases. With food allergies, hives may appear due to a sharp influx of blood to the face. Urticaria, in turn, can provoke bronchial spasms, laryngeal edema, and arterial hypertension, leading to severe consequences, even death. Most patients have a combination of several symptom complexes (7–10). Prevalence of food allergies varies widely and ranges from 0.9 to 13% of all allergic reactions (8, 10).

One of the causes of allergic diseases is considered to be a sizable antigenic load on the body due to combined effects of natural and anthropogenic factors, in particular artificial pollutants, which also include bio-pollutants. In addition to exogenous factors, there are also endogenous factors, primarily burdened heredity. It has been established that 40% of people have a hereditary tendency to atopy and that this population is susceptible to adverse environmental conditions (1, 8, 10). Over time, these individuals develop immediate-type allergic hypersensitivity with elevated levels of IgE, i.e., atopic allergic diseases type I (1, 3, 11). Often there is a combined effect of several factors: an unfavorable environmental situation, occupational hazards, social conditions, etc., that primarily affect patients with a hereditary predisposition (1–3, 12).

Of the endogenous factors, concomitant diseases of liver, kidneys, alimentary canal, respiratory system, and skin also play an essential role in allergy formation. Analysis of physical status of allergic patients showed a high percentage (67.5%) of concomitant pathology, especially liver pathology (chronic hepatocholecystitis, cholelithiasis, biliary dyskinesia), the alimentary canal (intestinal dysbiosis, enterocolitis, helminthic invasion, etc.), kidneys, etc. (3–6, 10). These diseases play an essential role in pathogenesis of allergic disease relapses.

Impairment of barrier function of internal organs in chronic diseases facilitates entry of various exoallergens (drugs, dust, food components, etc.) and xenobiotics of industrial origin into the body and also deteriorates detoxification and elimination of these foreign substances from the body. As a result of significant antigenic stimulation of immunocompetent cells, hyperproduction of IgE occurs (primarily in individuals with hereditary atopy), increased synthesis of immune complexes with damage to the membrane of mast cells (blood basophils), and release of biologically active substances from them into bloodstream - histamine, serotonin, acetylcholine, etc. Accumulation of these substances in the body leads to development of endotoxicosis and aggravates the patient's condition (3, 10–15).

Endotoxicosis is understood to be a multifactorial pathological process based on systemic tissue hypoxia with all its complex metabolic consequences. With endogenous intoxication, catabolism processes intensify, tissue alteration, liver and kidney failure, microcirculation disorders, and metabolic disorders occur. Exposure of cells to xenobiotics leads to changes in the properties of their membranes and disruption of intracellular homeostasis and metabolism. As a result of these pathological processes, toxins penetrate intercellular space and enter bloodstream. Regardless of how exoallergens enter the body, biologically active substances

enter bloodstream and are distributed throughout organs and tissues affecting them either at the site of penetration or at the level of organs and systems. Toxic products of allergic reactions and various ecopathogens (in a native or transformed form) enter through liver, pancreas, and intestinal mucous secretions, into the digestive canal's lumen, from where they can be reabsorbed into the blood. Having passed phases of biotransformation, xenobiotics as endogenous toxic substances are thus distributed between the blood, tissues, and enteric system (15–22).

Given the mechanisms of pathogenesis and toxic manifestations of allergic reactions, it is advisable to use detoxification methods in the complex treatment of patients with allergic diseases. From this point of view, enterosorbents, and in particular, enterosorbents based on silicon dioxide, which have been available in the Ukrainian and other post-Soviet markets for over 30 years, have proven themselves as safe and effective. These are affordable nutritional supplements and medicines that have universal sorption and detoxifying properties to address a variety of pathogenic intestinal microorganisms, food, helminthic, household and industrial toxins and allergens, circulating immune complexes, and inflammatory mediators (23–35) – all factors that play a critical role in sensitization and development of allergic reactions of various origins. These nonspecific properties of enterosorbents make it possible to be considered reasonable components in complex therapy of allergic diseases, which can significantly alleviate the course of these diseases and improve the life quality of sensitized patients.

2 Immunological mechanisms of food allergy

2.1 Role of gut microbiota

Dgestion and absorption of food products depend on the state of the neuroendocrine system, structure and function of the digestive and hepatobiliary systems, composition and volume of digestive juices, composition of the intestinal microflora, state of local immunity of the intestinal mucosa (lymphoid tissue, secretory immunoglobulins, etc.) and other factors. Usually, food products break down into compounds that do not have sensitizing properties (amino acids and other non-antigenic structures), and the intestinal wall is impermeable to non-digested products that have or may have, under certain conditions, sensitizing activity or able to cause pseudoallergic reactions (6, 8–10). Food allergens are digested and absorbed mainly in the small intestine, which is enriched by symbiotic microbiota. Thus, the intestinal cavity presents a homeostatic environment in which immune cells respond favorably to food allergens. However, the type and number of symbiotic microbiota in the gut may change and be affected by many external factors particularly, changes in dietary patterns, antibiotics use, breastfeeding, vaccines, pathogen exposure, etc. Experimental and epidemiological studies suggest that the gut microbiota composition is related to the clinical manifestation of food allergies and that early life (0–6 months) is a critical period for gut microbial colonization. Gut microbiota has been shown to affect the growth of immune tolerance to food antigens by modifying regulatory T cell (Tregs) differentiation, regulating basophil populations, and enhancing intestinal barrier function. Several gut microbial metabolites, such as short-chain fatty acids, secondary bile acids, and amphoteric polysaccharide A may directly or indirectly regulate Tregs differentiation, most of which express the retinoic acid receptor (RAR)-associated orphan receptor γt (RORγt) (36).

RORγt is a master regulator of interleukin (IL)-17-producing intestinal CD4+ T helper (Th17) cells (37, 38). This transcriptional factor is highly expressed also by IL-22-producing CD4+ Th cells and IL-17/IL-22-producing innate lymphoid cells (ILCs), in particular, subset group 3 (ILCs3) located in the intestine (39–43). Aryl Hydrocarbon Receptor (AhR) is another transcription regulator acting synergistically with RORγt (44, 45). The AhR can either directly regulate IL-22 gene expression and cytokine production or regulate the production and development of ILC3 and Th17 cells (42, 44–46). AhR ligands derived from gut microbiota are an essential component in initiating the transcription of IL-22 (46–48). Through IL-17- and IL-22-mediated signaling, RORγt and AhR can enhance the intestinal epithelial barrier, defend against pathogenic microflora, and regulate the balance of symbiotic gut microbiota which play a crucial role in the formation of intestinal immunity (38, 42–45).

Intestinal immune cells are mostly reserved in the gut-associated lymphoid tissues. However, a diverse array of immune cells (both, the innate and the adaptive immune system) are also present in the lamina propria and the layer of epithelial cells forming mucosa which is colonized by symbiotic gut microbiota (49–51). This provides easy communication signaling between gut microbiota and immune cells supporting the healthy microbiota balance and correct immunoreactivity and preventing dysbiosis and allergic reactions to food components which may damage the gut epithelial barrier and promote the expansion of pathogenic microflora. These highlights support a strong relationship between gut microbiota quality and food allergies or tolerance development, but the exact regulatory mechanism remains unclear.

Various factors, including dietary patterns, antibiotic usage, environmental factors, and early-life factors like breastfeeding, can impact the gut microbiota composition and development of food allergies. Antibiotic use in infancy can selectively deplete specific microbial species, potentially favoring the overgrowth of undesirable bacteria, affecting immune system maturation, and increasing the risk of allergies. A diverse microbiota is generally associated with a healthier immune system and reduced allergy risk. There are several therapeutic strategies proposed to prevent dysbiosis and gut microbiota restoring. Diets rich in a variety of fibers and nutrients can promote a diverse gut microbiota. Prebiotics (nondigestible fibers that feed beneficial gut bacteria) and probiotics (live beneficial bacteria) can help maintain a balanced gut microbiota. These supplements have been explored as potential interventions to reduce the risk of food allergies, although more research is needed to determine their effectiveness (49, 50). Breast milk contains beneficial microbes and prebiotic compounds that support the growth of beneficial gut bacteria in infants. Breastfeeding is associated with a reduced risk of food

allergies, likely due to its positive impact on the infant's gut microbiota (49). Certain microbial metabolites, such as short-chain fatty acids, have immunomodulatory effects. Bacterial metabolites can promote the development of Tregs, which help maintain immune tolerance. Therapies aimed at boosting the production of these metabolites, such as dietary interventions or microbial supplementation, are being explored (51). As each individual's gut microbiota composition is unique, personalized approaches to food allergy prevention and treatment may become more common. These approaches might involve gut microbiota profiling and tailoring interventions based on an individual's specific microbial makeup. Strategies to reduce environmental factors that disrupt the gut microbiota, such as reducing unnecessary antibiotic use and promoting a cleaner but microbiota-friendly living environment, may be explored. Finally, we consider enterosorbent use as a beneficial approach to support the normalization of symbiotic gut microbiota and prevent the growth of pathogenic and opportunistic microflora and thus reduce risks of food allergy development. While specific mechanisms through which enterosorbents improve the balance of the gut microbiota are absent, the symbiotic microflora colonizing the mucosa of the intestine is less accessible for nonspecific adhesion in contrast to pathogenic transitory bacteria entering the intestine from the external environment and directly contacting with an enterosorbent. As a result, pathogenic bacteria are fixed by enterosorbents and naturally excreted from the intestine. A similar mechanism apparently also operates in the endogenous opportunistic microflora, if it persists in the intestine and has not yet colonized unspecific places for it.

It's important to emphasize that while these therapeutic avenues hold promise, they are still areas of active research, and more clinical trials and studies are needed to establish their safety and effectiveness fully. More details about the correlation between the gut microbiota and immunological mechanisms of food allergies and tolerance and gut microbiota normalizing strategies are provided in recent reviews (49–51).

2.2 Role of IgE

Food allergies most often develop according to general mechanisms of IgE-mediated type I hypersensitivity, pathogenesis of which is associated with hereditary hyperproduction of IgE or due to allergic sensitization to various allergens, including some food components (1, 6). In this case, a patient synthesizes IgE antibodies to specific epitopes of food allergens (4). In genetically predisposed individuals, exposure to allergens leads to an increase in specific IgE that can bind to effector cells through a high-affinity receptor known as FcεRI, expressed by blood basophils and mast cells in various organs, including skin, digestive and respiratory tracts. As a result of complex formation and receptor activation, basophils and mast cells secrete proinflammatory mediators and cytokines, which develop inflammation and allergy symptoms (4, 6, 11, 12, 19). IgE is very short-lived (about one day) when free in plasma, but IgE bound to the FcεRI receptor forms pathogenic immune complexes and can remain attached to tissue cells for weeks or even months. Moreover, binding to the FcεRI receptor, IgE increases cell survival and receptor activation. Contact with a specific allergen causes the release of pharmacologically active mediators that are stored in mast cell granules and blood basophils, which leads to clinical manifestations of type I hypersensitivity (6, 13, 14).

2.3 Role of T helper type 2 cells

Allergic diseases result from a distorted immune response of Th2 cells to environmental antigens or allergens. In the case of food allergy, these allergens are usually food proteins causing predominantly IgE-mediated allergy (4–6, 8–10). The first step in allergy development is allergic sensitization, during which allergen-specific T and B cells activate, form clones, and differentiate (52). Allergic sensitization can occur through various routes of exposure to allergens. In type I hypersensitivity, allergens are initially presented to antigen-specific CD4+Th2 cells (53), which stimulate B cells to produce IgE antibodies, which are also antigen-specific (6). During IgE sensitization, antibodies bind to FcεRI on the surface of mast cells localized in tissues and blood basophils (12–14). Repeated exposure to the same allergen leads to binding of IgE on sensitized cells, resulting in degranulation and secretion of preformed pharmacologically active mediators, first of all, histamine (6, 14). All this happens as an immediate reaction that develops within a few seconds. The late response caused by induced synthesis and release of leukotrienes, chemokines, and cytokines by activated mast cells allows the recruitment of other leukocytes, eosinophils, basophils, and Th2-type lymphocytes to the site of allergic inflammation (Figure 1).

2.4 Role of dendritic cells

Most of the knowledge about mechanisms of food allergy and tolerance to food allergens comes from mouse models. Oral exposure usually induces tolerance but may also lead to sensitization, especially when adjuvants are present that disrupt the natural barriers of the alimentary canal, such as staphylococcal enterotoxin B (54). However, exposure to food allergens through the skin and respiratory tract can also lead to body sensitization (20–22). The association between food allergy and skin barrier impairment is also illustrated by high prevalence of food allergies among patients with atopic dermatitis, especially in its severe forms (20, 22).

Dietary proteins can pass through the intestinal epithelial barrier via transcytosis, paracellular diffusion, or endocytosis with help of microfold (M) cells (55, 56). In addition, intestinal epithelial cells can express MHC-II molecules and thus can directly present allergenic peptides to CD4+ T cells in the intestine (57, 58). Dietary proteins can also be taken up via transluminal processes by CX3CR1+ antigen-presenting DCs, which can select intestinal antigens by spreading transepithelial dendrites into the intestinal lumen. It is hypothesized that these CX3CR1+ DCs do not appear to migrate and fail to activate naive T cells, remaining in the intestinal

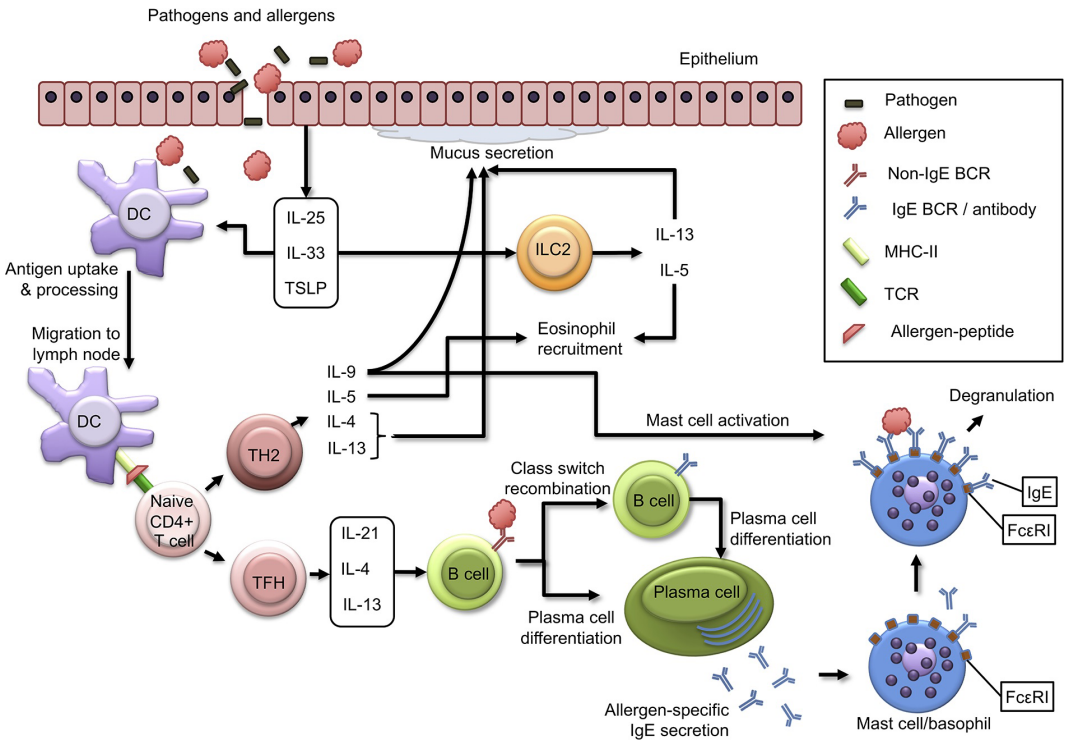

FIGURE 1
Immunological mechanisms of allergic sensitization. Description in the text. BCR, B Cell Receptor; DC, Dendritic Cell; IL, Interleukin; ILC2, Innate Lymphoid Cell Type 2; MHC-II, Major Histocompatibility Complex Type II; TCR, T Cell Receptor; TFH, T Follicular Helper Cell; TH2, T Helper Cell Type 2. Adopted from Schoos et al., 2020 (6); this is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY).

epithelium. However, these cells can carry antigens to migrating CD103+ DCs in the intestinal mucosa (59, 60). A healthy response to food antigens is characterized by immune tolerance, mediated by antigen presentation in the gut by DCs. Peyer's patches of the intestine are enriched in CD11c+ CD103+ DCs. After antigen uptake, these DCs can migrate to local lymph nodes, where they perform classical functions of DCs and control adaptive immune responses to food antigens (57–60).

Skin CD11b+ DCs and Langerhans cells are central players in induction of tolerance to allergens in the skin (61–63). Because of tissue damage and inflammation, epithelial cells produce Th2-inducing cytokines, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). These cytokines are primary regulators of type 2 immunity as they act on DCs to induce a Th2 cell response and activate innate lymphoid cells type 2 (ILC2), producing IL-13 and IL-5 (64). IL-33 is critical for activating the OX40 ligand (OX40L) on DCs in a mouse model of oral sensitization using cholera toxin, resulting in differentiation of naive Th2 cells (65). Allergens and pathogens that pass through cutaneous or epithelial mucosal barriers are captured and processed by DCs. When DCs exposed to epithelial-derived cytokines, they take up the allergen and migrate to the draining lymph nodes, where they present allergen peptides on MHC class II molecules to naive T cells leading to their clonal expansion, which, in turn (depending on costimulatory and cytokine signals) can differentiate into Th2 or TFH (T follicular helper) cells. Th2 cells become polarized towards producing type 2 cytokines, IL-4, IL-13, IL-5, and IL-9, and act as effector cells that control many aspects of allergic inflammatory responses.

2.5 Role of THF cells

While Th2 cells have long been considered critical for induction of IgE production by B cells, it has recently become apparent that IgE production requires interaction of B cells with TFH cells rather than with Th2 (66, 67). A recent study indicated that the production of high-affinity anaphylactogenic IgE antibodies depends on a subpopulation of TFH cells called TFH13 cells that secrete IL-13 in addition to IL-4 and IL-21 (66). Through the secretion of these mediators, TFH cells stimulate B cells to differentiate into allergen-specific IgE-producing plasma cells that can bind to surface FcεRIs receptors on basophils in blood and mast cells localized in tissues. Upon repeated exposure to the allergen, cross-linking of these FcεRIs receptors with allergen-specific IgE leads to degranulation of mast cells and basophils involved in Th2 response and a type I hypersensitivity reaction, culminating in clinical phase of the manifestation of food allergy (5, 6, 15, 17, 36, 52, 68, 69).

2.6 Role of cytokines

IL-4 and IL-13 are structurally and functionally similar cytokines playing a central role in allergic inflammation - they induce a switch in synthesis of immunoglobulin classes to IgE recombination, smooth muscle cell contraction, goblet cell hyperplasia, and mucus production (15). IL-5 also significantly contributes to allergic inflammation by recruiting eosinophils (68, 69). While IL-5-mediated eosinophilic inflammation has been convincingly demonstrated in some forms of asthma, it appears to be much less so in IgE-mediated food allergy (70). Interestingly, allergen-specific IL-5+ Th2 cells were found only in allergic patients with eosinophilic gastroenteritis. At the same time, peanut allergy is associated with a predominant IL-5+ Th2 cell response, indicating that heterogeneity in Th2 responses may contribute to IgE-mediated inflammation of the digestive system with predominance of eosinophilia (68). IL-9 contributes to occurrence of allergic diseases through stimulation of mucus secretion and release of chemokines by epithelial cells, as well as activation of mast cell proliferation (17, 71) (Figure 1). Food allergy can sometimes occur through a cell-mediated mechanism, leading to delayed symptoms or a chronic process. An example of such allergy is food-induced enterocolitis syndrome, which develops as a result of production of cytokines by T cells (68, 70, 72). Sometimes food allergies can develop in response to some food additives, especially azo dyes, in which case the latter act as haptens and, forming complexes with a protein, such as serum albumin, become fully-fledged allergens (6, 8, 10, 16). Mechanisms of formation of true food allergies have however yet to be thoroughly studied.

3 Associated factors of food allergy

Many external agents, including allergens, enter the digestive system. Due to unique properties of the mucosa of the digestive canal, a barrier exists in the intestine that serves to prevent penetration of allergens into the blood, including dangerous microorganisms, viruses, and toxins. Low acidity (pH) of gastric juices and presence of proteolytic enzymes both contribute to destruction of protein allergens, which in turn leads to loss of their allergenic properties (7–9, 19, 50, 73). Immune cells meanwhile protect the body from foreign agents and increase barrier properties of the intestine. Failure of the immunological defense program in the intestine's immune system leads to increased permeability of the digestive canal mucosa for allergens and food allergies. This failure often manifests itself in overproduction of IgE antibodies and inability of the body to defend against invasion of antigens (4, 8, 10, 74). The antigen thus causes an alternating sequence of reactions: burning in the mouth, vomiting, abdominal pain, diarrhea, etc. When it enters the bloodstream, the allergen can cause drop in pressure, rash or eczema on skin, and bronchospasm in lungs (7–10). Almost any food product can become an allergen and cause development of food allergies. Protein products containing animal and vegetable proteins have more pronounced sensitizing properties, although there is no direct relationship between protein content and allergenicity of products (10, 75).

Of the endogenous factors, a significant role in formation of allergies is concomitant diseases of the liver (chronic hepatocholecystitis, cholelithiasis, biliary dyskinesia), the digestive canal (intestinal dysbiosis, enterocolitis, helminthic infestations, etc.), kidneys, respiratory system and skin. These diseases play an essential role in pathogenesis of relapses of hypersensitivity, both with and without an immunological mechanism of development (9, 16, 51). Presence of intestinal dysbiosis, even in subcompensated forms, often results in presence of products of incomplete digestion in the intestine. These, in turn, support clinical manifestations of food allergies since they can be allergens and, at the same time, enhance inflammatory changes in the digestive canal mucosa due to direct irritating effects (9, 54, 58, 72, 74).

Functional immaturity of the immune and digestive systems, insufficient production of digestive enzymes, deficiency of beneficial microflora, and intestinal infections, all play an essential role in formation of food allergies in children. The risk of a food allergy developing in a child may increase in presence of toxicosis, allergy, infection (helminthic, bacterial, fungal), and associated diseases, as well as being the result of an unbalanced diet during the mother's pregnancy and during breastfeeding. Allergic sensitization of a child may be influenced by the nature of feeding (natural or artificial), types and timing of introduction of complementary foods, drug therapy, and allergens that enter the body from the environment. In addition, genetic predisposition to food allergies is of great importance. If neither parent has an allergy, then allergies are likely to occur in 4-10% of children; if one of the parents has allergies, then this probability increases to 25-50%; if both parents have allergies, then the probability of the child having an allergy increases to 40-80% (6, 8–10). A special low-allergenic diet currently is however not recommended for healthy people and pregnant and lactating women with a hereditary predisposition to atopy. On the contrary, it is believed that presence of allergens in the blood and milk of the mother contributes to development of tolerance to these food allergens in the child (76–78). However, exposure to epigenetic factors during fetal development and the first thousand days of life after birth remains a high-risk factor for child sensitization (79).

4 Non-allergic hypersensitivity (pseudoallergy)

With regard to allergic reactions to food components, it is important to distinguish between two main types of food allergy: true food allergy and pseudoallergy (non-allergic hypersensitivity). Non-allergic food hypersensitivity is clinically similar to an immediate type allergic reaction but it develops without participation of basic immunological mechanisms, such as IgE sensitization and Th2 response. Pseudoallergy differs from other reactions associated with food intolerance (defects or absence of digestive enzymes, psychoemotional intolerance) in that the same mediators are involved in its manifestation as in true food allergies (histamine, leukotrienes, prostaglandins, cytokines) albeit they are released from allergy target cells in a nonspecific way, that is, without the participation of IgE or other allergic antibodies. In this way, pseudoallergy is a result of the direct

impact of food substrate antigens on target cells, in particular, mast cells, and, indirectly, with the activation of several biological systems by the antigen, such as the kinin system, the complement system, etc. in the absence of the immunological link of allergic sensitization (4, 8–10, 70).

Among the mediators of pseudoallergy, histamine plays a unique role. Many factors provoke development of food pseudoallergy: excessive intake of histamine into the body with foods rich in histamine, tyramine, and histamine liberators; excessive formation of histamine from food substrates; increased absorption of histamine with functional insufficiency of the digestive system mucosa; increased release of histamine from target cells; violation of the synthesis of prostaglandins, and leukotrienes (8, 9, 80).

Diagnosis of non-allergic hypersensitivity is based on the clinical pattern, dynamics of the course, and response to the elimination of pseudoallergy causes (80–83). True allergic reactions to food allergens are detected in approximately 35% of cases and pseudoallergic in 65% (9, 10, 12, 70).

Development of food allergies or pseudoallergies provokes disorders common to adults and children. It results in an increase in the permeability of the intestinal mucosa, pancreatic insufficiency, enzymopathy, inflammation of the biliary tract, intestinal dyskinesia, etc. Disorderly eating and rare or frequent meals meanwhile lead to impaired stomach secretion, gastritis, mucus hypersecretion, and other disorders contributing to development of food allergies or pseudoallergies (9, 10, 81, 84, 85). When digestive and hepatobiliary systems function correctly, food products supplied through the enteral route do not result in sensitization. Often the reason for pseudoallergies in food is not the foods themselves but various chemical additives introduced to improve taste, smell, and color and ensure shelf life (83).

Polymorphism of pseudoallergic skin rashes to food varies from urticaria (10–20% of the examined persons), papular eruption (20–30%), erythematous, and macular (15–30%) to hemorrhagic and bullous rashes (18, 19, 80, 82, 83). However, dividing food allergies into true and pseudoallergy is somewhat arbitrary. The same patient may develop hypersensitivity reactions to foods due to involvement of specific immune mechanisms and non-immune reactions. In addition, with a true food allergy, the patient may develop pseudo-allergic reactions to food over time, which aggravates the severity of the disease, making diagnosis difficult, and reducing the effectiveness of treatment.

5 Clinical manifestations of food allergy

5.1 Diversity of manifestations

Clinical manifestations of food allergies are varied from gastrointestinal disorders to respiratory and skin manifestations, such as urticaria, angioedema (Quincke's edema), and dermatitis. There are systemic and local allergic reactions that occur after exposure to food allergens, and their severity ranges from mild to extremely severe, such as in anaphylactic shock. A variety of conditions associated with food allergies have been described in children, among which the most common are atopic dermatitis, neurodermatitis, urticaria, angioedema, itchy morbilliform rashes, as well as lesions of the digestive system, which are manifested by nausea, vomiting, dysmotility, diarrhea, and abdominal pain, severe colic, constipation, weight loss, dystrophy. Less common are bronchial obstruction, laryngeal edema, rhinoconjunctivitis, and vulvovaginitis. Most patients have a combination of several symptom complexes (4, 5, 7–10, 19–22). Figure 2 presents the main stages of food allergies development and its manifestations.

The earliest and most typical manifestation of a true food allergy is the development of oral allergy syndrome. After ingesting a particular food allergen, these patients develop swelling and itching in the lips, tongue, pharynx, and hard or soft palate (72). This syndrome is caused by cross-reactivity between certain types of food and pollen allergens. For example, a patient allergic to tree pollen may develop oral syndrome after eating apples, fresh carrots, peaches, cherries, and hazelnuts (9, 86, 87).

The most severe manifestation of food allergies is anaphylactic shock. Anaphylactic shock in food allergies differs in the speed of its development (from a few seconds to 4 hours), and the severity of the course impacting the likelihood of mortality, which ranges from 20 to 70%. Among the symptoms observed in anaphylactic shock, skin manifestations (84%), cardiovascular (72%) and respiratory symptoms (68%), are notably present. Anaphylaxis can also develop in the absence of skin manifestations. Respiratory and cardiovascular symptoms are however potentially life-threatening. Respiratory symptoms are more common in children, and cardiovascular symptoms are dominant among adults. Digestive symptoms such as nausea and vomiting may also occur. Biphasic anaphylactic reactions can occur in 20% of all cases. They usually appear 4 to 12 hours after the first symptoms and may be more severe. Delayed or insufficient administration of adrenaline (epinephrine) or erroneous administration of glucocorticosteroids may increase the risk of biphasic anaphylactic reactions (4, 8, 9, 88, 89).

Unlike true food allergies manifested by anaphylaxis, pseudoallergies can manifest as non-immunological anaphylaxis, known as anaphylactoid shock. Food-induced non-immunological anaphylaxis may resemble anaphylactic shock in clinical symptoms but differs in that it may lack polysyndromicity and a favorable prognosis. In non-immunological anaphylaxis, symptoms are observed mainly from one of the body systems, for example, drop in blood pressure or loss of consciousness. Still, all other parameters (skin, mucous membranes, breathing, etc.) are unchanged. The prognosis for non-immunological anaphylaxis is therefore favorable. With the timely appointment of adequate symptomatic therapy, the clinical response to treatment is observed quickly, normally in first hours of treatment (8–10, 70).

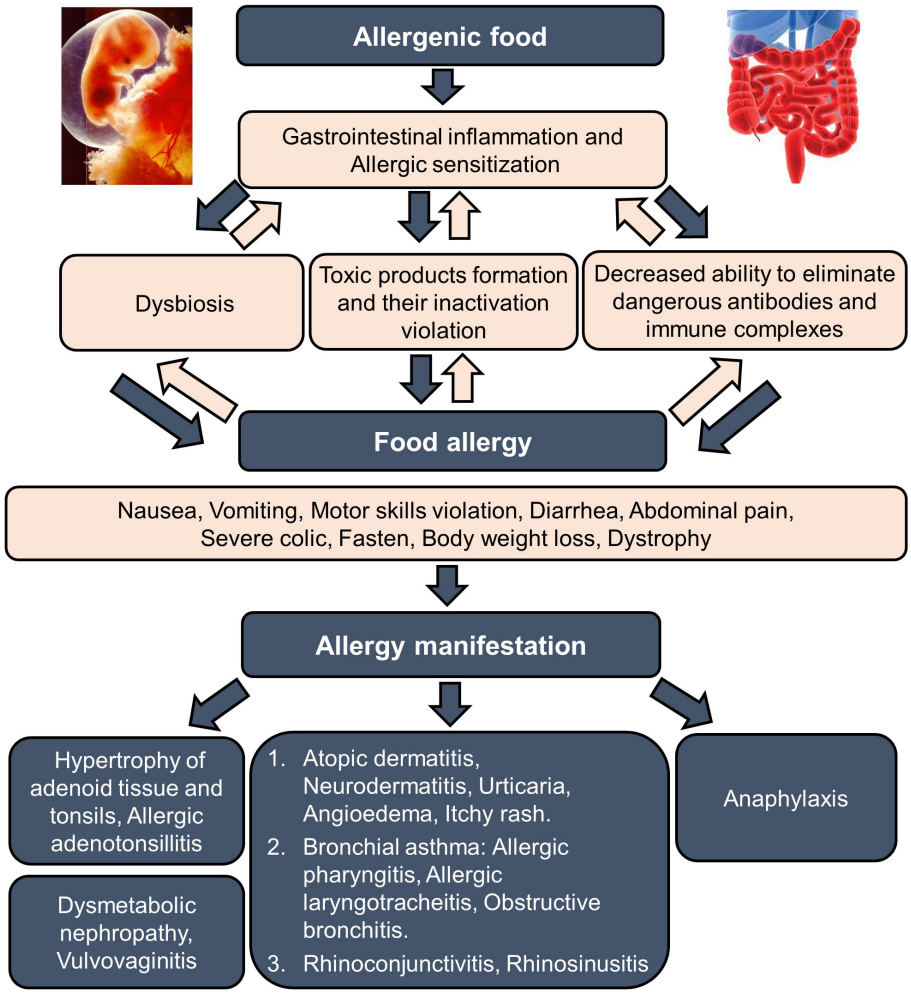

FIGURE 2
Food allergy formation and manifestation. The formation of food allergy in children to food components begins with a hereditary predisposition of parents to allergic diseases and intrauterine sensitization of the fetus (stage 1). These prerequisites are manifested in the first six months after birth as a food allergy with primary gastrointestinal and skin manifestations (stage 2). Damage to the alimentary canal provokes further sensitization to other allergens, including food. As a result, the food allergy progresses further, encouraging the development of atopic dermatitis and neurodermatitis, the maximal manifestations observed in the child age before 1-year-old (stage 3). After that, a progressing decrease in the allergic reaction to food allergens can be observed; however, the phenomena of bronchial obstruction (beginning of bronchial asthma) and allergic rhinitis may increase, which reach their pics to the ages 7-8 and 10-12 years, respectively (stage 4).

5.2 Gastrointestinal manifestations

Clinical manifestations of IgE-mediated diseases of the digestive system usually occur in combination with skin lesions and manifest in a variety of symptoms (vomiting, nausea, pain, diarrhea). In most children in first years of life, atopic dermatitis is a consequence of food allergies. 89-94% of children with atopic dermatitis caused by food allergens are diagnosed with the gastrointestinal allergy. In these children, especially in the first year of life, gastrointestinal allergies have the character of allergic enteropathy or allergic colitis. It most often has such manifestations as abdominal pain (colic), flatulence, regurgitation, and vomiting. Repeated exposure to food allergens leads to chronic inflammation, itching, and scratching, followed by clinically significant skin lesions (4, 5, 9, 10, 84). Any part of the digestive canal can be involved in the pathological process - from the oral cavity to the rectum. The most common gastrointestinal clinical manifestations of food allergies include vomiting, colic, anorexia, constipation, diarrhea, eosinophilic esophagitis, and allergic enterocolitis (9, 72, 74, 84, 90). Clinical manifestations are diverse and often nonspecific- infantile intestinal colic, regurgitation, vomiting, gastroesophageal reflux, eosinophilic esophagitis, gastritis, gastroenteritis, and proctitis. Chronic constipation resistant to conventional therapy, as well as malabsorption syndrome or protein-losing enteropathy, may also occur. Vomiting can occur from a few minutes to 4–6 hours after food ingestion and is often persistent. Vomiting is mainly associated with the spastic reaction of the pylorus when a food allergen enters the stomach. Eosinophilic esophagitis during endoscopy is detected in 15% of all patients who complained of dysphagia (10, 90). In 14–75% of cases, eosinophilic esophagitis is accompanied by other manifestations of allergy (bronchial asthma, atopic rhinitis, atopic dermatitis), and some patients have a history of IgE-mediated food

anaphylaxis (8–10, 68, 69, 90). A combined lesion of several parts of the digestive system is possible (7–10, 18, 19, 72, 74, 75, 81, 84, 85). Symptoms of damage to the digestive system in patients with atopic dermatitis and allergic rhinitis may also indicate the role of food allergies in the etiopathogenesis of these diseases.

Allergic colicky abdominal pain can occur immediately after a meal or several hours later and is caused by smooth muscle spasms of the intestine associated with specific (true allergy) or nonspecific (pseudoallergy) liberation of allergy mediators. Abdominal pains are usually intense, sometimes displaying themselves as "acute abdomen," accompanied by decreased appetite, mucus in the stool, and other dyspeptic disorders. Stool disorders, such as constipation and diarrhea, are also common manifestations of food allergies. Frequent, loose stools after ingesting a causally significant food allergen are among the most common clinical symptoms of food allergy in adults and children, especially in milk allergy (7, 9, 19, 72, 84, 85).

Allergic enterocolitis with food allergies is characterized by severe pain in the abdomen, flatulence, and loose stools with the discharge of vitreous mucus, which contains many eosinophils. Patients with allergic enterocolitis complain of extreme weakness, loss of appetite, headache, and dizziness (9, 12, 13, 73). Histological examination of patients with allergic enterocolitis reveals hemorrhagic changes, pronounced tissue eosinophilia, local edema, and mucus hypersecretion (7, 9, 68, 72, 81, 84, 85).

With food allergies, the intestinal microflora changes qualitatively and quantitatively due to the existing allergic inflammation and disturbances in the processes of digestion and absorption - frequency of dysbiotic disorders in children with food allergies reaching 94% (18, 19, 81, 84). Dysbiotic states create the conditions for implementing the virulent action of opportunistic pathogens. Putrefactive or fermentative flora and fungi, mainly of the genus Candida, develop abundantly, and microorganisms not typical for normal microflora are present (58, 84). Inflammation in various parts of the digestive system leads to impaired enzymatic activity, parietal and membrane absorption, and changes in microbiota. These changes in turn lead to increased formation of toxic products and their impaired inactivation, including a decrease in the ability to eliminate antibodies, and antigen-antibody complexes, which worsen the condition of patients with allergies and stimulate transition of the disease to more severe forms (4, 5, 8–10, 84, 88, 89).

5.3 Skin manifestations

Skin manifestations are the most common symptoms of food allergies in adults and children. In children under one year old, one of the first signs of food allergies can be persistent diaper rash, the appearance of perianal dermatitis, and perianal itching that occurs after feeding. Localization of skin changes in food allergies is different, but more often, they appear on the face and around the mouth, and can spread across the entire skin's surface. At the onset of the disease, there is a clear connection between skin exacerbations and intake of a causally significant food allergen. Over time, allergic changes in the skin become persistent and constantly relapse, which makes it difficult to determine etiological factors. For true food allergies, most typical skin manifestations are urticaria, angioedema, and atopic dermatitis (5, 9, 10, 20, 21, 53, 91).

5.4 Respiratory manifestations

Allergic rhinitis in food allergies is characterized by presence of profuse mucus-watery discharge from nose, sometimes nasal congestion, and difficulty in nasal breathing. Rhinoscopy reveals swelling of the nasal concha mucosa, which has a pale cyanotic color. Often, along with rhinorrhea or swelling of the mucosa, patients sneeze and itch skin around or inside nose. The most frequent cause of allergic rhinitis in patients with food allergies is fish and fish products, crabs, milk, eggs, honey, etc. (4, 5, 9, 10).

5.5 Bronchial asthma

According to most researchers, the role of food allergens in developing bronchial asthma is small. Clinical manifestations of food allergies in the form of asthma attacks are observed in approximately 3% of cases (8–10, 19, 22, 92).

6 Treatment of food allergy

Main principles of treating food allergies are an integrated approach with phased-in therapy aimed at elimination of allergy symptoms and prevention of exacerbations. To reach optimal results, the treatment should include drug therapy, elimination therapy, environmental control, allergen-specific immunotherapy (3, 6, 8–10, 93–95), and nonspecific approaches, such as enterosorption (24, 27, 32, 33). The most promising treatment method being studied is allergen-specific immunotherapy (AIT), which primarily includes oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and epicutaneous immunotherapy (EPIT) approaches (96–103). Additionally, there are currently actively studied adjunct and alternative treatment methods, such as use of IgE inhibitors, probiotics, and early introduction of allergenic foods in infancy (93–95, 97, 104–106). However, typical treatments still include food allergen elimination and AIT (96, 97, 107).

6.1 Allergen-specific immunotherapy

AIT is a specific desensitizing immunological method for treatment of allergic conditions, which consists of administering gradually increasing doses of a food allergen (food extracts or purified and sometimes modified food allergens) to the patient until desensitization is reached. This means switching the synthesis of allergen-specific IgE to immune IgG (3, 52, 96, 97). AIT is carried out only when the disease is based on the IgE-mediated mechanism, and a food product is vital (for example, milk allergy in children). AIT represents the only therapy capable of inducing a state of

immune tolerance and provides potential to affect a sustained clinical benefit with long-lasting clinical remission of the allergic condition. In addition, AIT offers the possibility of preventing development of new food allergen sensitivities, inhibiting progression of food allergies toward allergic rhinitis and asthma, and improving a patient's quality of life and lowering medication requirements. Currently, various routes of administration of food allergens are under evaluation, including oral, sublingual, and epicutaneous (6, 52, 62, 63, 91, 96–103). Research and clinical trials over the past few decades have elucidated the mechanisms underlying AIT-induced tolerance, involving reduction of allergen-specific TH2 cells, induction of regulatory T and B cells, and production of IgG and IgA blocking antibodies. To better harness these mechanisms, novel strategies are being explored to achieve safer, more effective, more convenient regimens and more durable long-term tolerance. These include alternative routes for current AIT approaches, novel adjuvants, use of recombinant allergens (including hypoallergenic variants), and combination of allergens with immune modifiers or monoclonal antibodies targeting the TH2 cell pathway (96–103). Although there have been many experimental and clinical trials, there is no consensus on criteria for assessing clinical and immunological outcomes of research, which is a severe obstacle to widespread adoption of these new treatments, and their effectiveness requires further study.

6.2 Oral immunotherapy

OIT is an emerging treatment for food allergies. Food allergy patients ingest increasing doses of allergens to desensitize their immune system and train it to not react to the problem food. OIT typically starts with very small doses of food allergens consumed under medical supervision. These doses are increased every one to several weeks until a small tolerated dose is reached that can be taken each day for months or years. Typical OIT doses of food proteins are measured in milligrams or grams. The food allergies treated with OIT in clinical trials included allergies to milk, egg, peanut, tree nut, wheat, soy, and sesame, as well as baked milk and baked eggs (97, 98, 103). Results of these researches show that OIT can be effective in inducing desensitization and, in some people with IgE-mediated food allergies, can induce sustained unresponsiveness (remission). In January 2020, an OIT treatment for peanut allergies received approval from the U.S. Food and Drug Administration (FDA) (97). However, this method is not routinely recommended for patients with food allergies due to uncertainty about outcomes including safety, long-term effectiveness, and overall impact on quality of life.

6.3 Sublingual immunotherapy

In order to undergo SLIT, patients must have a documented IgE-mediated food allergy, such as food allergies to peanuts, eggs, or milk. In the SLIT method, food allergens are dissolved in a small amount of liquid (usually this is a glycerinated allergen) and held under the tongue for several minutes before being spat out or swallowed. The procedure is repeated every day. SLIT doses are increased during an escalation phase until a consistent daily maintenance dose is reached. Compared to OIT, SLIT uses smaller doses because the amount of liquid that fits under the tongue is limited. SLIT doses are usually measured in micrograms or milligrams of protein. SLIT has been studied in treatment of hazelnut, peach, apple, milk, and peanut allergies with a substantial focus on treatment of peanut allergies. Phase II studies have shown that SLIT for treatment of peanut allergies increases the tolerated dose of peanut by a substantial margin with fewer and less severe side effects than other modalities. Long-term SLIT has been shown to induce sustained unresponsiveness, and there is evidence that high-dose SLIT protocols can achieve tolerance that approximates that of OIT. However, the cost of allergenic extract may make long-term, high-dose SLIT prohibitive. Therefore, some allergists have used food allergy SLIT as a temporary bridge to OIT (99, 100). Because long-term maintenance dosing regimens for food allergy SLIT have not been standardized, studies are needed to determine the minimum effective doses and duration of food allergy SLIT for various foods.

6.4 Epicutaneous immunotherapy

Epicutaneous (on the skin) immunotherapy, or EPIT, exposes tolerance-promoting immune cells in the skin to an adhesive dermal patch containing a small (micrograms) dose of food protein. Patches are being developed to treat peanut, milk, and egg allergies. EPIT starts with a small initial dose that is increased over time by wearing the patch for longer periods of day until a maintenance dose is reached in which each patch is worn 24 hours and replaced daily. EPIT has been tested in clinical trials for children with peanut allergies for its safety and efficacy in inducing desensitization (101–103).

6.5 Early introduction of allergenic foods in infancy

Studies support the existence of a critical time early in infancy during which the genetically predisposed atopic infant is at higher risk for developing allergic sensitization to food allergens. Therefore, dietary interventions in the first years of life have been analyzed for their effects on prevalence of allergic diseases including food allergies. Several observational and interventional studies demonstrated potential effectiveness of the early allergic food introduction strategy in prevention of food allergies, although strong evidence from randomized controlled trials is lacking and, sometimes, contrasting. Nevertheless, for children considered at high risk of developing food allergies the evidence for early introduction of allergenic foods, and in particular peanut and egg, is robust. The consensus exists that not only should such foods not be delayed, but that they should be introduced at approximately 4 to 6 months of age in order to minimize the risk of food allergies. Thus, currently, prevention strategies for food allergies have shifted from avoidance of foods to the early introduction of high-allergenic

foods. American and European allergy expert committee guidelines as well as some other organizations recommend that solid foods should be introduced between four to six months of age in all infants (104–106). However, further studies are required to provide patients with evidence-based best practices.

6.6 Elimination diet

The elimination diet is considered the most effective modern concept for treatment and prevention of food allergies. An elimination diet is characterized by eliminating significant food allergens and replacing highly allergenic foods with low-allergenic or non-allergenic foods. An elimination diet requires exclusion from the diet not only of a specific food product responsible for the development of sensitization but also of any other foods in which it is included, even in trace amounts. For most patients, strict avoidance of confirmed food allergens remains the recommended standard of care. At this, of great importance is the appointment of adequate rational nutrition, corresponding in volume and ratio of food ingredients to the patient's age and weight, concomitant somatic diseases, and other factors (6, 8–10, 19, 107).

6.7 Pharmacotherapy

Pharmacotherapy of food allergies aims to eliminate the symptoms of the existing disease and prevent exacerbations. Histamine is one of the most critical mediators responsible for developing clinical signs of food intolerance in true food allergies and pseudoallergies. Histamine has a wide range of pharmacological actions. It can influence pathophysiological reactions from various organs and systems: the respiratory tract (mucosal edema, bronchospasm, and mucus hypersecretion), the skin (itching, blistering-hyperemic reaction), the alimentary canal (intestinal colic, stimulation of gastric secretion), the cardiovascular system (expansion of capillary vessels, increased permeability, arterial hypotension, and cardiac arrhythmias), and smooth muscles (spasm). The critical role of histamine in pathogenesis of most allergic diseases determines the widespread use of histamine H1 receptor antagonists (6, 10, 108–110). These drugs have a pronounced antipruritic effect, ability to almost instantly alleviate the symptoms of allergic and pseudoallergic reactions, and have different clinical forms that provide flexible dosing for different age categories of patients, starting from infancy (8, 108–110).

6.8 Anti-IgE treatment

Understanding pathways that underlie development of various forms of allergic reactions has opened up new possibilities for their treatment with new immunobiological preparations aimed at binding antigen-specific IgE or its receptors (93–95). For example, omalizumab (OmAb), a recombinant anti-IgE antibody, has already been approved for treating severe allergic asthma and chronic urticaria not controlled by conventional therapies (36, 93). In recent years, several other drugs specific against IgE have also appeared but it remains unclear whether they will also be used in clinical practice.

A favorable factor in food allergies is that with age, the tolerance of the body to most food allergens increases. It is believed that if the food allergy has not stopped to 5-year age then age-related tolerance should not be expected to develop further (9, 10).

7 Clinical application of enterosorbents

An effective measure to detoxify the body is an efferent (sorption) therapy aimed at accelerated removal from the body of xenobiotics, harmful metabolites, circulating immune complexes, mediators of allergic reactions, inflammatory cytokines, food and industrial toxins, decay products of helminths, and other toxic substances. Experience with this type of treatment, mainly in post-Soviet countries, has shown the feasibility of its use in the acute period of allergic diseases and to prevent recurrence of the disease. Today, enterosorption is a component of complex therapy for various types of intoxication, including allergic, with a scientifically based and clinically proven efficacy (23–35, 111–125).

Sorbents are used to fix and remove food allergens from the digestive canal, in addition to products of incomplete enzymatic cleavage (medium molecular weight toxic metabolites) released as a result of allergic or eosinophilic inflammation, biologically active substances (serotonin, histamine, bradykinin, neuropeptides, prostaglandins, leukotrienes, etc.), pathogenic, opportunistic microorganisms and viruses, bacterial endotoxins associated with impaired intestinal microbiota. Binding of these substances in the intestinal lumen prevents their absorption, promotes their rapid and safe excretion, and improves conditions of the digestive system (23, 25–30, 34, 35). Indirect effects of sorbents are elimination or weakening of toxic-allergic reactions, prevention of endotoxicosis, reduction of the metabolic load on organs of excretion and detoxification, restoration of the integrity and permeability of the intestinal mucosa, stimulation of intestinal motility, and improvement of blood supply (24, 27–33, 120, 121, 124, 125). Figure 3 summarizes clinically significant effects of enterosorbents application. The main advantage of enterosorption is its non-invasiveness, a small number of contraindications, the absence of complications, and changes in the biochemical composition of the blood through a sustained course of treatment (26, 30, 32, 116–118, 120, 121, 123). Enterosorbents are successfully used not only as a pathogenetic but also as an etiotropic mono- and combined therapy for intestinal infections and other infectious diseases (23, 26, 29, 30, 35). The clinical efficacy of some enterosorbents in mild and moderate forms of intestinal infections is not inferior to antibiotics (32). Effectiveness of enterosorption is comparable to the use of probiotics (120, 121).

There are several sorption methods using different physiological mechanisms. In practice of allergists, the most acceptable method is enterosorption, based on binding and removal of toxic substances

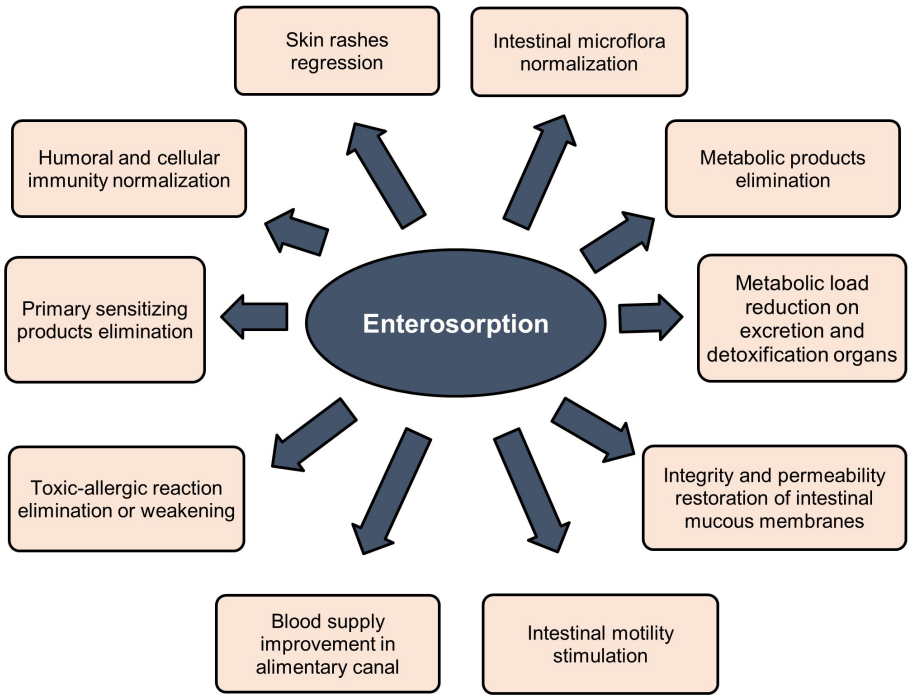

FIGURE 3
Clinical effects of enterosorption.

and metabolites from the digestive canal (33, 111, 112). The gastroenterosorption detoxification involves the possibility of reverse passage of toxic substances from the blood into the intestine with their further binding by enterosorbents (9, 24, 30) The use of enterosorbents is applicable both in acute periods of allergic diseases and in inter-recurrent periods. Since the barrier function of the gastrointestinal canal mucosa is impaired, many toxic substances can be absorbed and lead to endotoxicosis, which is often found in patients with urticaria, atopic dermatitis, and other allergic reactions (24, 32, 33).

Enterosorption, as an effective detoxification method, is used for treatment of urticaria, psoriasis, atopic dermatitis, eczema, lupus erythematosus, dermorespiratory and dermointestinal syndromes, bronchial asthma, and other diseases (23–28, 32, 33, 114, 115, 119). With food and drug allergies and atopic dermatitis, using sorbents in complex therapy leads to more rapid regression of skin rashes and subjective sensations, as well as normalization of the gastrointestinal canal function. There is a positive effect in terms of laboratory parameters such as the decreasing number of eosinophils and level of total IgE in blood (28, 32, 119), increasing in cellular and humoral immunity, in particular, the number of T lymphocytes increases, eosinophilia decreases, level of circulating immune complexes decreases, while content of immunoglobulins class M and E stabilizes, itching reduces, and edema phenomena and hives decrease (24, 28, 33, 34, 113–119, 122). It is significant that at the same time, enterosorbents increase sensitivity to hormones, making it possible to reduce the amount of glucocorticosteroid therapy by 50 percent on average and, in some patients, complete withdrawal of hormonal therapy (32).

In patients with bronchial asthma, enterosorption has demonstrated positive results when combined with unloading and dietary therapy: signs of intoxication, intensity of concomitant skin-allergic manifestations, hormone dependence, drug intolerance, frequency and severity of bronchospasm attacks decrease (24, 33). The use of enterosorbents is effective in metabolic disorders in allergic patients and in detoxification of organs. Function of internal organs is improved with concomitant pathology of the hepatobiliary system, kidneys, cardiovascular vascular diseases, and diabetes. The ability of enterosorbents to reduce antigenic load on the body makes them suitable as a preventative measure for persons who come into contact with a large number of harmful substances in manufacturing and everyday life, as well as those living in industrial regions (23–35, 113, 115).

Development of intestinal dysbiosis plays an important role in formation of allergic reactions, in particular in food allergies. Dysbiosis contributes to increasing the permeability of the intestinal wall for products with sensitizing activity. Also, metabolic products of microorganisms themselves can be allergens. Therefore, normalizing intestinal microbiota, including the use of enterosorbents, is the fundamental principle of food allergies treatment (9, 29, 33). When the intestinal barrier function is intact, with typical microflora composition, endotoxins penetrate bloodstream in small amounts and are detoxified in liver hepatocytes. Conversely, with dysbiosis development, endogenous intoxication is maintained, resulting in increased permeability of the intestinal wall and weakening of adaptive and protective mechanisms. At the same time, some pathological processes in the body can cause intestinal dysbiosis and formation of

endogenous intoxication by themselves, thus creating a vicious circle. These are diseases of the gastrointestinal canal with impaired motor evacuation and secretory functions (chronic pancreatitis, cholelithiasis, chronic gastritis, liver steatosis, hepatitis, etc.), resection of the stomach, small or large intestine, diverticular disease, past intestinal infections, helminthic infestations, giardiasis, or taking certain medications, including antibiotics, as well as chronic diseases of other organs and systems. In all these cases it is also advisable to prescribe enterosorption therapy (23–29, 35, 84, 100–105, 113–115).

Enterosorbents should be included in complex therapy in the first days or hours of exacerbation of an allergic disease or an acute allergic reaction. They are introduced into the gastrointestinal cavity orally. Usually, sorbents are prescribed 1.5-2 hours before meals. This period is needed for the enterosorbent to react with the stomach content and to be partially evacuated to the intestine, where its interaction with components of the intestinal contentcontinues. Sorbents and other drugs should not be applied simultaneously; there should be 2-3 hours between respective intakes. For most sorbents, the daily therapeutic dose is 0.2-1 g/kg of body weight, and maximal doses - are up to 2 g/kg of body weight. At the same time, the daily dose of enterosorbents is evenly distributed into 3-4 doses between breakfast, lunch, and dinner. The course of treatment is 6-8 days (no more than 14 days) with a gradual dose reduction over the last 2-3 days (23–30, 113, 115, 119, 120, 123).

Preventive (anti-relapse) dose of enterosorbents is 0.2-0.5 g/kg of body weight. Sorbents should be used for a 7-10-day period, during which it is possible to take them both in the morning and in the evening, 1.5-2 hours after dinner. A prophylactic course for allergic patients should be carried out monthly (in the first three months), then once a quarter during a year. Frequency of prophylactic enterosorption treatment is decided individually for each patient, taking into account the severity of both underlying and concomitant pathologies (24–27, 29).

Clinical effect of enterosorbents depends on timelines of their prescription - the earlier the drug is administered, the higher the sorption coefficient and clinical effect. A negative factor of several sorbents is, however, the sorption of vitamins, mineral salts, and other valuable substances, as well as the nonspecific sorption of enzymes (pepsin, trypsin, amylase), which in some cases requires correction by enzyme replacement therapy (26, 27, 32, 123).

General contraindications for the use of enterosorbents are erosive gastritis, peptic ulcer of the stomach and duodenum, ulcerative lesions of the intestine, and its atony. Cautious use of enterosorbents is recommended for patients with reduced intestinal peristaltic activity. A relative contraindication is a tendency to constipation. Excretion during the process of enterosorption should be daily; if necessary, laxatives are prescribed. While taking enterosorbents, increasing the drinking regimen and consuming foods with a high fiber content are recommended. Vitamin preparations are also recommended (24, 26–29, 32, 33, 35, 113–115, 119–121).

Sorption therapy of persons with allergic diseases should be individualized for each patient, considering comorbidity and tolerance to the sorption preparation. Mandatory indications for including enterosorbents in the complex of rehabilitation measures are acute allergic reactions of any etiology. Enterosorbents are indicated for prophylactic purposes for people with hereditary atopy and for patients receiving long-term corticosteroid therapy with concomitant liver, kidneys, and intestines pathology that slows down the detoxification of xenobiotics. Sorption methods for treating allergic diseases and concomitant pathological conditions are highly effective and practice safe methods of endogenous detoxification. They are recommended for widespread introduction into clinical practice in inpatient and outpatient settings for different age groups of patients (9, 24, 33, 113–119, 123). In post-Soviet countries, enterosorbents are included in the protocols for treatment of various manifestations of food allergy in children, along with an elimination diet and systemic treatment, depending on clinical symptoms of the disease (24–26, 29, 32, 33).

For enterosorbents, the main considerations that should be taken into account, especially for enterosorbents used in pediatric practice are the following:

- lack of toxicity;
- good evacuation from the stomach;
- no damaging effect on the gastrointestinal canal;
- high sorption capacity with minimal loss of valuable ingredients;
- as they pass through the intestine, the bound components should not be desorbed and should not change the medium pH;
- convenient form and ease of dosing;
- good organoleptic properties;
- the sorbent should favorably influence the secretion processes and the intestinal microbiota (23, 32, 111, 112, 126, 127).

Many enterosorbents that meet the above requirements already exist in the arsenal of therapies available to pediatricians. These methods have been used in clinical practice for over 30 years and are used globally (23–33, 111, 112, 128–132).

All of them have certain advantages and can be prescribed to allergic patients depending on individual indications, including such characteristics as tolerability, efficacy, features of the course of the underlying disease, and presence of concomitant diseases. This review is limited to detailed consideration of enterosorbents based on silicon dioxide, particularly a drug known under the trade name “White Coal” (Carbowhite) produced by the Ukrainian company OminiFarma (Figure 4).

8 Silicon dioxide and silicon dioxide-based enterosorbents

Silicon (Si) is one of the chemical elements found in the Earth’s crust, and it ranks second in abundance after oxygen (125, 133). The oxide forms of silicon are silicate (SiO_4) and silica (silicon dioxide, SiO_2), which are also present in various plants, organs, and tissues of humans and animals, and enter the human body with food, being

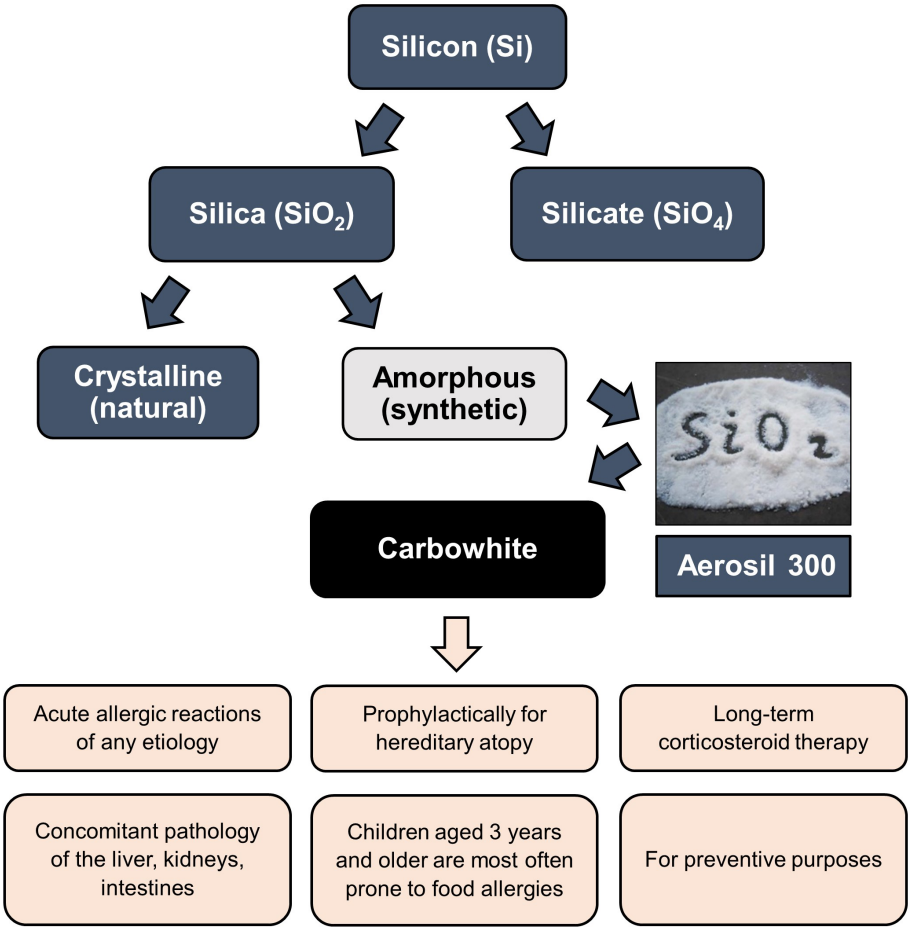

FIGURE 4
The place of White Coal (Carbowhite) in the structure of silicon derivatives and indications for its use. The active substance of White Coal is Aerosil 300, produced by the company Evonik (Germany) - patented the highly dispersed non-porous anhydrous amorphous colloidal silicon dioxide raw material (silica, SiO2), having a powdery or granular structure of white color.

physiological components of the body (125, 133). Silicon is widely used in industry, and its oxide forms are often used in biomedical applications. Silica nanoparticles have several rare properties, such as ease of synthesis, modifiable surface, strong mechanical properties, and relatively inert chemical composition, which ensure the durability of biomaterials based on them (134–137).

There are two primary states of silica - crystalline and amorphous (Figure 4). Both conditions have the same molecular formula but differ in structural arrangement (133, 134). The lattices of crystalline silica are arranged regularly, while the lattices of amorphous silica are arranged irregularly (121–139).

Crystalline silica has several forms. A well-known form is α-quartz, which can be converted to β-quartz, tridymite, and cristobalite when heated. There is also porous crystalline silica called porosil; all porosils are synthetic. Terms nanoporous, mesoporous, and microporous refer to the pore diameter. “Nanoporous” refers to materials with pore diameters less than 2 nm, “microporous” refers to materials with pore diameters greater than 100 nm, and “mesoporous” refers to materials with pore diameters between 2 and 100 nm (133, 134, 136, 140). Mesoporous silicon and silica particles are ideal candidates for controlled drug release due to their rare properties, such as high surface area, large pore volumes, controlled pore sizes, and good chemical and thermal stability (135, 136, 141).

Amorphous silica can be divided into three groups: natural form (rare), a by-product of power plants and metallurgical production, and synthetically created silica (133, 134). Amorphous synthetic silica is a promising candidate for gene carrier and molecular imaging, mainly due to its highly tunable biocompatibility and stability (126, 127). In addition, it is also used in dietary supplements (142), catheters, implants, and dental fillers (143).

Nanoparticles are one of the most general terms for designating isolated ultradispersed objects, duplicating previously known terms (colloidal particles, ultradispersed particles) but differing from them in clearly defined dimensional boundaries. Solid particles less than 1 nm in size are usually referred to as clusters and more than 100 nm - to submicron particles. A nanoparticle is an isolated solid-phase object, the dimensions of which in all three sizes range from 1 to 100 nm. At the same time, in some fields of knowledge, in particular, in biomedical nanotechnologies, nanoparticles are often conventionally referred to as objects with a diameter of up to several hundred nanometers, the small size of which also plays a significant role in

their properties and application, in particular, providing increased mucosal absorption during oral administration. At present, it is generally accepted by the world scientific community that nanoparticles of synthetic amorphous silica and, especially, submicron particles do not have toxic side effects when used in sufficiently high doses and are almost eliminated from the gastrointestinal canal physiologically without entering into biological fluids (23–30, 32, 33, 91, 92, 114, 133–136, 138, 144–151). Moreover, the use of amorphous silica nanoparticles (Aerosil 380 and Aerosil 200) has been approved in the European Union as a safe food additive E551, and Aerosil 300 is widely used in pharmaceutical and cosmetic industries (135, 144, 145, 151–153).

The Manufacturers Association of amorphous silica and independent studies have verified the safety of synthetic amorphous silica. In particular, in several works, based on a comparison of numerous clinical and laboratory studies, it was shown that none of the forms of silica, including colloidal nanoparticles, bioaccumulates. All of them are eliminated from living organisms within a short time using physiological mechanisms (150, 151). Research centers and individual experts in the European Union and the USA have evaluated the daily dosage limits of synthetic amorphous silica, which would lead to undesirable toxicological effects. A brief review of animal studies on the oral safety of amorphous silica has been published by the Organization for Economic Co-operation and Development (OECD) (152) and the European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) (153).

Silicon dioxide-based enterosorbents are produced from high-quality patented raw materials from Evonik (Germany), obtained using a unique technology that allows the creation of soluble porous and non-porous materials from the lightest fraction of silicon dioxide (nanoparticles 7-40 nm in size), which include Aerosil Ox50, Aerosil 130, Aerosil 150, Aerosil 200, Aerosil 300, Aerosil 380 (135–137, 144, 152, 153). They have a high degree of dispersion and a large sorption surface area. Therefore, the daily dose of silica preparations is only 2-4 g, almost ten times less than that of activated carbon (29). Silica preparations are prescribed for adults and children over three years old, 3-4 tablets up to 4 times daily. With prolonged administration in therapeutic doses, silicon dioxide preparations do not cause noticeable changes in the activity of enzymes of the intestinal mucosa; they differ in lower (compared to other sorbents) excretion of vitamins and microelements and their rapid recovery to normal levels without additional drug load (30, 32, 34, 35, 116, 118), which is typical, in particular, for the enterosorbent White Coal (Carbowhite).

9 White Coal (Carbowhite)

The active substance of the enterosorbent White Coal is a highly dispersed, non-porous colloidal anhydrous silicon dioxide (silica, SiO2). The drug is manufactured using modern, highly dispersed technology from patented raw materials, silicon dioxide Aerosil 300 from Evonik (Figure 4). The safe dosage range of Aerosil 300, used in the drug White Coal varies from 100 to 300 mg/kg of body weight/day, with short-term use - up to 1000 mg/kg per day (23).

The production technology guarantees that the White Coal tablet will disintegrate in the body in a few minutes, turning into a highly dispersed suspension, while 1 g covers 380-400 m^2. The product was introduced to the Ukrainian market in 2008. To date, the clinical evaluation of the efficacy and safety of the product has been shown by multiple studies, which also showed the absence of any undesirable side effects of treatment (23, 35, 115–118, 154–160). White Coal is registered as a drug in Ukraine, Georgia, and Uzbekistan.

According to the manufacturing specification, the particles of silicon dioxide in feedstock used for production of the White Coal have a size of 7 nm, which during the production process under the influence of high temperature, form submicron agglomerates with a size of more than 100 nm (137, 160). It is essential that during the production of the White Coal, silicon dioxide Aerosil 300 is subjected to the process of further additional coarsening of agglomerated particles according to the patented wet granulation technology of the Omnifarma company, that is, the White Coal is not a nanoproduct and does not carry the risks associated with possible systemic toxicity or toxicity to the immune system when taking therapeutic doses of silicon dioxide nanoproducts (146–153, 161). No evidence exists of natural mechanisms for the degradation of Aerosil 300 agglomerates to their original nanoparticles in the human body. Such deagglomeration can only be achieved by ultrasonic irradiation (138) or by passage through the gastrointestinal canal with pH > 12 (151), which is not possible in the human body (162). Therefore, amorphous silicon dioxide used in White Coal is a structurally stable submicron agglomerate of nanoparticles that remain intact in the gastrointestinal canal. Moreover, these agglomerates cannot pass through the intestinal epithelial barrier and thus do not circulate in the body fluids with the exception possible presence of a very limited number of free nanoparticles. This unique property of White Coal significantly contributes to its safety profile when used as the enterosorbent and reduces potential negative consequences associated with the possible presence of trace amounts of free amorphous silica nanoparticles in the product. Nevertheless, to clarify the safety profile of the White Coal drug, it is advisable to conduct further studies of its pharmacokinetic and pharmacodynamic properties. White Coal is produced as tablets containing 210 mg of the active substance. It is easily dispersed in the gastrointestinal canal, forming a large sorption surface. It is almost entirely (more than 99%) excreted from the intestine through feces, practically without entering the systemic circulation (less than 1%). From systemic circulation, the drug is excreted through the kidneys with urine. The enterosorbent is widely used as mono and complex therapy for acute intestinal diseases (salmonellosis, food poisoning), acute diarrhea of various etiologies, and exogenous intoxication with household and industrial toxins, drugs, alcohol, and food intoxications. It is also used as an adjuvant for viral hepatitis, allergic diseases, dermatitis, late gestosis of pregnant women, cirrhosis of the liver, chronic toxic hepatitis, chronic non-calculous cholecystitis, non-alcoholic fatty liver disease and other mono and complex pathologies (Figure 4). At the same time, the enterosorbent does not cause constipation and absorbs toxic substances in the intestines selectively, without loss of beneficial

trace elements. In addition, due to the formation of concentration and osmotic gradients, White Coal promotes the removal of various toxic products from the internal environment of the body (blood, lymph, interstitium), including medium molecules, oligopeptides, amines, and other substances, which are formed in a result of systemic inflammatory and allergic reactions (23, 35, 113, 115–118, 154–161). Figure 5 demonstrates the distribution and the mechanism of action and evacuation of the enterosorbent White Coal from the digestive system.

A remarkable feature of enterosorbent White Coal is the combined effect of silicon dioxide (Aerosil 300) and microcrystalline cellulose, which is part of its composition as an additional active substance. Microcrystalline cellulose positively impacts the tablet's properties (163) and functions of the gastrointestinal canal (164). This food additive enhances the buffering effect of food, potentiates the hydrolysis of proteins in the stomach, modifies the secretion of gastrointestinal hormones, affects the transit and hydrolysis of nutrients in the small intestine, inhibits the absorption of monomers and bile acids, reduces intracavitary pressure in the colon, stimulates intestinal motility and growth of microflora, reduces the risk of gallstone formation, and has a hypoglycemic effect (29, 31, 164). Following a request from the European Commission, the EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) delivered a scientific opinion re-evaluating the safety of microcrystalline cellulose (E 460(i)) and modified variations. From the reported studies, the Expert Panel considered a safe application for the microcrystalline, powdered, or modified cellulose to be around 660–900 mg/kg body per day (165).

In 2016, a multicentre, randomized, double-blind, placebo-controlled study of the efficacy and safety of White Coal in acute diarrhea was conducted (level of evidence I (A) according to CEBM (35). Administration of White Coal to patients (children and adults) with acute diarrhea during 3-5 days in doses 1260, 1890, and 4200 mg daily (for different groups) resulted in the termination of diarrhea in an average of 1.7 days without any side effects (35). The clinical trial results were approved by the US FDA - available at the ClinicalTrials.gov web resource (166).

In 2009, the results of a clinical study on the use of the enterosorbent White Coal in the complex treatment of children with manifestations of food allergies were published (113). The study was conducted as an open randomized in the National Children's Specialized Hospital "Okhmatdet" (Kyiv, Ukraine). Under observation were 46 children aged 1 to 15 years with manifestations of food allergies against the background of various allergic pathologies.

The studies were carried out in dynamics: in the acute period of the disease (before treatment), on the 7th and 14th days of complex therapy with the inclusion of the White Coal. The enterosorbent

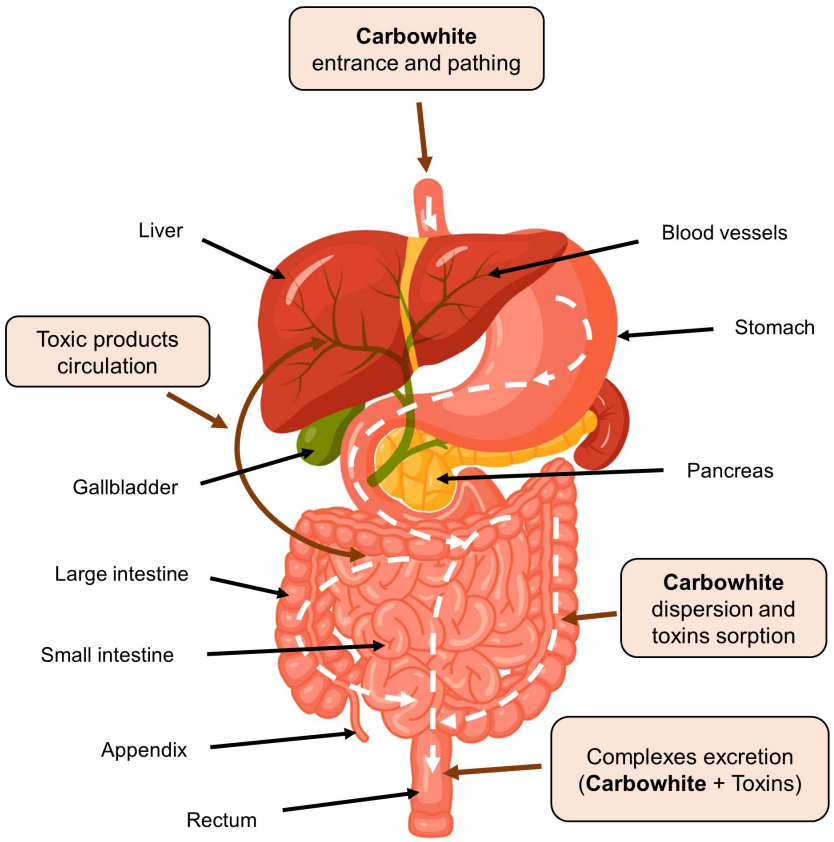

FIGURE 5
The mechanism of sorption and excretion of toxic products from the digestive system by enterosorbent White Coal (Carbowhite).

was administered orally in 3 doses at a total daily dose of 150 mg/kg, 1 - 1.5 hours before meals and between doses of other drugs for 14 days. The effectiveness of treatment was assessed according to the following criteria: SCORAD index, level of total and specific IgE, degree of dysbiosis, level of hematological activity, the full dose of systemic glucocorticosteroids, and duration of the acute period.

The treatment resulted in a reduction in duration of the acute period of atopic dermatitis, urticaria, and angioedema, a decrease in the level of specific IgE to cow's milk proteins in children with atopic dermatitis, and an improvement in the state of the gastrointestinal canal with the decreasing of dysbiosis symptoms. The total dose of systemic glucocorticosteroids was reduced in children with an acute episode of urticaria and angioedema. These data indicate a relatively high clinical efficacy of the 14-day course of the enterosorbent White Coal in children aged 1 to 15 years with manifestations of food allergies against the background of various pathologies (Figure 6).

10 Nanoparticles-associated silica potential risks

The effects of silica exposure on the body, especially crystalline silica, have been studied extensively. Studies have shown that exposure to crystalline silica associated with the use or production of this material causes silicosis (fibrous lung disease) in workers and that this exposure is also associated with other lung diseases such as lung cancer, lung emphysema, pulmonary tuberculosis, autoimmune diseases (149, 166–169). The toxicity and pathogenicity profile of silica, and therefore the risk of silicosis, depends on the inhaled particles' size and physical and chemical properties (167–171). It has long been thought that the crystal structure imparts silica toxicity. Still, recent studies show that the number and distribution of sialon and siloxane groups, rather than crystallinity, act as primary toxic factors (172, 173).

The safety of using amorphous silica and, mainly, silica nanoparticles, their systemic toxicity, and toxicity to the immune system are also discussed (133, 146–153, 161). Amorphous silica used to be considered less harmful than crystalline silica. However, recent studies have shown that amorphous silica nanoparticles may have the same potential toxicity as crystalline ones (146, 173). It is believed that the physicochemical properties of amorphous silica nanoparticles can have various toxic effects *in vitro* and *in vivo*. Several reviews have considered this issue in detail (134, 161, 173, 174).

The studies were mainly focused on the initial stages of the interaction of silica with cells located in the lungs and immunological modifications of the innate immune cellular response. Special attention was paid to the primary interaction of silica with alveolar macrophages. Usually, silica particles enter the alveolar space after inhalation and interact with macrophages, resulting in inhaled particles entering the phagosomes. The macrophage receptor MARCO has been described as the primary molecule responsible for silica recognition and uptake by

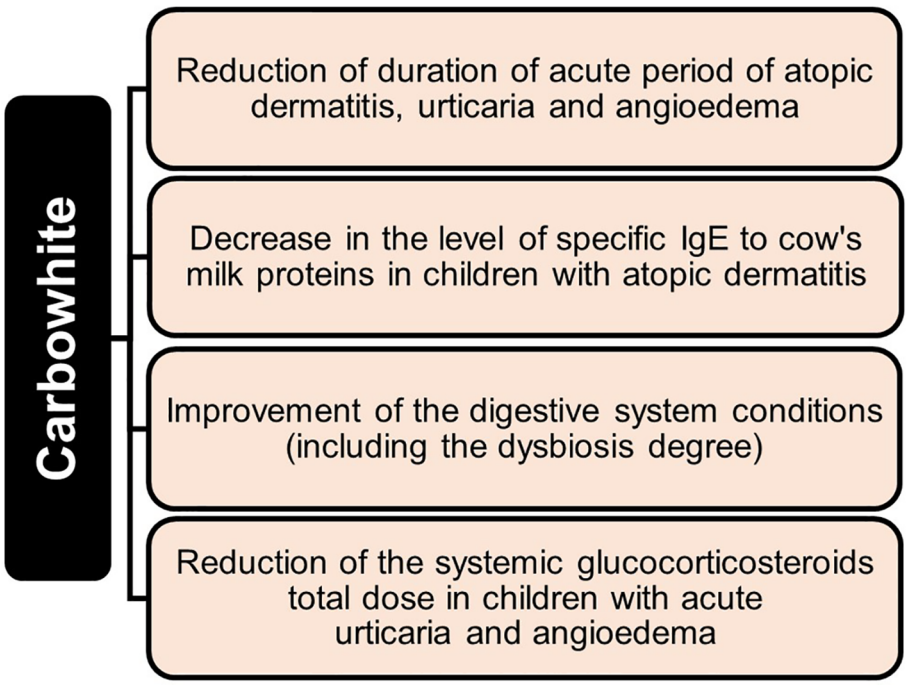

FIGURE 6
Clinical effectiveness of a 14-day course of using enterosorbent White Coal (Carbowhite) in children aged 1 to 15 years with manifestations of food allergies of various etiologies.

macrophages (175). Other receptors from the group of recognition receptors (Pattern receptors) may also be involved in this process. In particular, it has been shown that CD204 can interact with silica and inhibit the activity of macrophages. In addition, significant downregulation of Toll-like receptor 2 (TLR2) after silica exposure may contribute to increased susceptibility to tuberculosis (176). Finally, the inability of macrophages to digest and remove phagocytosed silica particles leads to persistent inflammation and modification of the cellular response (177).

Some authors believe silica nanoparticles can interact with immunocompetent cells and cause immunotoxicity (146, 176, 177). The effect of silica nanoparticles on the immune system depends on the physicochemical properties of the nanoparticles and type of cells they affect and lead to different results (134, 138, 178, 179). The effector cells involved in these reactions are mainly tissue macrophages, peripheral blood monocytes, polymorphonuclear leukocytes, dendritic cells, lymphocytes, mast cells, and other cell types, modifying the immune response (34, 134, 146–149, 161, 178, 179).

Silica exposure can reduce cellular function and DCs' activation, leading to a nonspecific attenuated inflammatory response disrupting antibacterial defense mechanisms and increasing susceptibility to bacterial infections, primarily Mycobacterium tuberculosis and other mycobacteria (176, 180, 181). However, the impact of silica on immune responses is complex and does not always result in increased susceptibility to bacterial infections; some effects promote the elimination of mycobacteria rather than their reproduction (161).

Potential toxicity of nanoparticles to immune cells is related to their ability to induce direct cell damage, such as apoptosis and necrosis. Functions of immune cells may change after interaction with nanoparticles, and this may affect immunospecific signaling pathways. Toxic effects can be assessed by functional activity of cells, development of pro-inflammatory reactions, generation of reactive oxygen species, cellular dysfunction, cytotoxicity, genotoxicity, and their underlying mechanisms (134, 182–191). Assessing the interaction between nanoparticles and cells of the immune system is critical in determining safety of silica nanoparticles, which are widely used in biomedical applications due to their unique chemical and physical properties (192, 193). In general, toxic effects of silica nanoparticles on the immune system *in vivo* are rare and occur only at oral doses significantly higher than the recommended allowable values (134, 170–174, 182–184, 194). Based on risk assessments of amorphous silica toxicity with known sources of silica, such as dietary supplement E551, a safe upper intake level has been estimated at 720 mg/day for a 60 kg adult, equivalent to 12 mg silica/kg body weight/day (133).

Considering the discussion regarding the possible toxicity and immunotoxicity of amorphous silica nanoparticles, the expert committee of the European Commission in 2018 revised the recommendations regarding the food additive E551. It concluded that, at the moment, there are no grounds for conclusions about the toxicity of this product for humans, and the recommendations of the European Commission made for it earlier, in 2009, remain valid (144, 145).

11 Conclusion

Amorphous silicon dioxide food supplements and drugs, particularly White Coal (Carbowhite), are safe at recommended doses because they are structurally stable submicron agglomerates of nanoparticles that do not disaggregate in the gastrointestinal canal and are hardly absorbed into body fluids. Given the composition and properties of White Coal, its usage scope can be extended to a wide range of allergic diseases, primarily food allergies. It is advisable to include White Coal in complex treatment of food allergies in adults and children over three years of age, as it contributes to faster relief of symptoms of damage to the gastrointestinal system, reduces endogenous intoxication, reduces drug load, and improves the patient's quality of life. In addition, the high safety profile of White Coal and its unique sorption properties make it possible to recommend this enterosorbent to pregnant and breastfeeding women, especially during an exacerbation of allergic inflammation, to prevent allergic sensitization in hereditarily predisposed newborns.

Author contributions

VS collected and reviewed the literature, wrote and redacted the manuscript, and designed the illustrations. OK and EO redacted the manuscript and illustrations. VC and DD revised the manuscript and figures, contributed to rewriting Section 6 "Treatment of Food Allergy", approved the final version of the revised manuscript, and agree to be accountable for all aspects of the work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors VS and OK were employed by OmniFarma, and OK is the company's founder.

The remaining author declares that the research was conducted without commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Ansotegui IJ, Melioli G, Canonica GW, Caraballo L, Villa E, Ebisawa M, et al. IgE allergy diagnostics and other relevant tests in allergy, a World Allergy Organization position paper. *World Allergy Organ J* (2020) 13(2):100080. doi: 10.1016/j.waojou.2019.100080

2. Asher I, Montefort S, Björkstén B, Lai CKW, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* (2006) 368:733–43. doi: 10.1016/S0140-6736(06)69283-0

3. Navinés-Ferrer A, Serrano-Candelas E, Molina-Molina G-J, Martín M. IgE-related chronic diseases and anti-IgE-based treatments. *J Immunol Res* (2016) 2016:8163803. doi: 10.1155/2016/8163803

4. Allen JK, Koplin JJ. Epidemiology of Ig E mediated food allergy and anafilaxis. *Immunol Allergy Clin N Am* (2012) 32(1):35–50. doi: 10.1016/j.iac.2011.11.008

5. Longo G, Berti I, Burks AW, Krauss B, Barbi E. IgE-mediated food allergy in children. *Lancet* (2013) 382:1656–64. doi: 10.1016/S0140-6736(13)60309-8

6. Schoos A-MM, Bullens D, Chawes BL, De Vlieger L, DunnGalvin A, Epstein ME, et al. Immunological outcomes of allergen-specific immunotherapy in food allergy. *Front Immunol* (2020) 11:568598. doi: 10.3389/fimmu.2020.568598

7. Koletzko S, Niggemann B, Arato A, Dias JA, Heuschkel R, Husby S, et al. European Society of Pediatric Gastroenterology, Hepatology, and Nutrition. Diagnostic approach and management of cow's-milk protein allergy in infants and children: ESPGHAN GI Committee practical guidelines. *J Pediatr Gastroenterol Nutr* (2012) 55 (2):221–29. doi: 10.1097/MPG.0b013e31825c9482

8. Muraro A, Werfel T, Hoffmann-Sommergruber K, Roberts G, Beyer K, Bindslev-Jensen C, et al. EAACI Food Allergy and Anaphylaxis Guidelines Group. EAACI Food Allergy and Anaphylaxis Guidelines: diagnosis and management of. *Food Allergy Allergy* (2014) 69(8):1008–25. doi: 10.1111/all.12429

9. Sidorovich OI, Luss LV. Pishchevaya allergiya: printsipy diagnostiki i lecheniya [Food allergy: principles of diagnosis and treatment]. *Meditsinskiy Sovet* (2016) 16:141–47. doi: 10.21518/2079-701X-2016-16-141-147

10. Seth D, Poowutikul P, Pansare M, Kamat D. Food allergy: A review. *Pediatr Ann* (2020) 49(1):e50–8. doi: 10.3928/19382359-20191206-01

11. Yamaguchi M, Lantz CS, Oettgen HC, Katona IM, Fleming T, Miyajima I, et al. IgE enhances mouse mast cell Fc(epsilon)RI expression in*vitro* and in *vivo*: evidence for a novel amplification mechanism in IgE-dependent reactions. *J Exp Med* (1997) 185:663–72. doi: 10.1084/jem.185.4.663

12. Oettgen HC. Fifty years later: emerging functions of IgE antibodies in host defense, immune regulation, and allergic diseases. *J Allergy Clin Immunol* (2016) 137 (6):1631–45. doi: 10.1016/j.jaci.2016.04.009

13. Asai K, Kitaura J, Kawakami Y, Yamagata N, Tsai M, Carbone DP, et al. Regulation of mast cell survival by IgE. *Immunity* (2001) 14(6):791–800. doi: 10.1016/s1074-7613(01)00157-1

14. Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, et al. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* (2001) 14(6):801–11. doi: 10.1016/s1074-7613(01)00159-5

15. Akdis M, Aab A, Altunbulakli C, Azkur K, Costa RA, Crameri R, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor b, and TNF-a: Receptors, functions, and roles in diseases. *J Allergy Clin Immunol* (2016) 138:984–10. doi: 10.1016/j.jaci.2016.06.033

16. Dunkin D, Berin MC, Mayer L. Allergic sensitization can be induced via multiple physiologic routes in an adjuvant-dependent manner. *J Allergy Clin Immunol* (2011) 128:1251–58.e2. doi: 10.1016/j.jaci.2011.06.007

17. Sehra S, Yao W, Nguyen ET, Glosson-Byers NL, Akhtar N, Zhou B, et al. TH9 cells are required for tissue mast cell accumulation during allergic inflammation. *J Allergy Clin Immunol* (2015) 136:433–40.e1. doi: 10.1016/j.jaci.2015.01.021

18. Petruláková M, Valík L. Food allergy and intolerance. *Acta Chimica Slovaca* (2015) 8(1):44–51. doi: 10.1515/acs-2015-0009

19. Onyimba F, Crowe SE, Johnson S, Leung J. Food allergies and intolerances: A clinical approach to the diagnosis and management of adverse reactions to food. *Clin Gastroenterol Hepatol* (2021) 19(11):2230–40.e1. doi: 10.1016/j.cgh.2021.01.025

20. Brough HA, Liu AH, Sicherer S, Makinson K, Douiri A, Brown SJ, et al. Atopic dermatitis increases the effect of exposure to peanut antigen in dust on peanut sensitization and likely peanut allergy. *J Allergy Clin Immunol* (2015) 135:164–70. doi: 10.1016/j.jaci.2014.10.007

21. Smeekens JM, Immormino RM, Balogh PA, Randell SH, Kulis MD, Moran TP. Indoor dust acts as an adjuvant to promote sensitization to peanut through the airway. *Clin Exp Allergy* (2019) 49:1500–11. doi: 10.1111/cea.13486

22. Loo EXL, Sim JZT, Goh A, Teoh OH, Chan YH, Saw SM, et al. Predictors of allergen sensitization in Singapore children from birth to 3 years. *Allergy Asthma Clin Immunol* (2016) 12:56. doi: 10.1186/s13223-016-0161-x

23. Chuiko AA. *Meditsinskaya khimiya i klinicheskoye primeneniye dioksida kremniya [Medicinal chemistry and clinical use of silicon dioxide]*. Kyiv: Izdatelstvo Naukova Dumka (2003). p. 416. redaktor.

24. Aleshina RM. Sorbenty v praktike allergologa [Sorbents in the practice of an allergist]. *Klinichna Imunolohiya Alerholohiya Infektolohiya* (2006) 4(5):12–6.

25. Vershinin AS, Polilov AN. Enterosorbtsiya v praktike semeynogo vracha [Enterosorption in the practice of a family doctor]. *Russkiy Meditsinskiy Zhurnal* (2008) 16(4):314–19.

26. Bondarev EV, Shtrygol S, Dyryavy SB. Primeneniye enterosorbentov v meditsinskoy praktike [The use of enterosorbents in medical practice]. *Provizor* (2008) 13:25. http://www.provisor.com.ua/archive/2008/N13/enters_138.php.

27. Panfilova VN, Taranushenko TE. Primeneniye enterosorbentov v klinicheskoy praktike [Application of enterosorbents in clinical practice]. *Pediatricheskaya Farmakologiya* (2012) 9(6):34–9. doi: 10.15690/pf.v9i6.516

28. Alekseeva AA. Primeneniye enterosorbentov v kompleksnoy terapii atopicheskogo dermatita [The use of enterosorbents in the complex therapy of atopic dermatitis]. *Voprosy Sovremennoy Pediatrii* (2012) 11(2):151–54.

29. Shchekina MI, Panchuk MS. Aspekty primeneniya enterosorbentov pri intoksikatsiyakh razlichnogo geneza v ambulatornoy praktike [Aspects of the use of enterosorbents for intoxication of various origins in outpatient practice]. *Meditsinskiy Sovet* (2013) 3:67–71. doi: 10.21518/2079-701X-2013-3-67-71

30. Lototska CB. Obgruntuvannya vykorystannya enterosorbentiv u likuvanni syndrome endohennoyi intoksykatsiyi pry riznomanitnykh zakhvoryuvannyakh (ohlyad literatury) [Justification of the use of enterosorbents in the treatment of endogenous intoxication syndrome in various diseases (literature review)]. *Bukovyns' kyy Medychnyy Visnyk* (2015) 19(1):222–26.

31. Teryoshin VA, Kruglova OV. *Evaluation of the efficiency of enterosorbent White Coal® in patients with the diseases of hepatobiliary system* . Available at: http://www.pancreatology.com.ua/dat/var/journal/2015-3/09.pdf.

32. Menshikova SV, Ketova HG, Popylov MA. *Primeneniye enterosorbenta Polisorb MP (kremniya dioksida kolloidnogo) v kompleksnoy terapii razlichnykh patologicheskikh sostoyaniy u detey [Use of enterosorbent Polysorb MP (colloidal silicon dioxide) in complex therapy of various pathological conditions in children]* (2016). Available at: http://www.science-education.ru/ru/article/view?id=25791.

33. Filatova TA. Vozmozhnosti enterosorbentov pri allergicheskikh zabolevaniyakh u detey [Possibilities of enterosorbents in allergic diseases in children]. *Praktika Pediatra* (2016) 3:14–6.

34. Doroshenko A, Gorchakova N, Zaychenko G. Effect of a nanodispersion silica composite with polyhexamethylene guanidine hydrochloride on immunological indicators and indicators of oxidation and antioxidant homeostasis in rats with thermal burn. *Sci J «ScienceRise: Pharm Science»* (2019) 4(20):45–52. doi: 10.15587/2519-4852.2019.178951

35. Tieroshyn V, Moroz L, Prishliak O, Shostakovich-Koretska L, Kruglova O, Gordienko L. Colloidal silicon dioxide in tablet form (Carbowhite) efficacy in patients with acute diarrhea: results of randomized, double-blind, placebo-controlled, multi-center study. *Sci Rep* (2020) 10(1):6344. doi: 10.1038/s41598-020-62386-0

36. Kreft L, Hoffmann C, Ohnmacht C. Therapeutic potential of the intestinal microbiota for immunomodulation of food allergies. *Front Immunol* (2020) 11:1853. doi: 10.3389/fimmu.2020.01853

37. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* (2006) 126(6):1121–33. doi: 10.1016/j.cell.2006.07.035

38. Mickael ME, Bhaumik S, Basu R. Retinoid-related orphan receptor RORγt in $CD4^+$ T-cell- mediated intestinal homeostasis and inflammation. *Am J Pathol* (2020) 190(10):1984–99. doi: 10.1016/j.ajpath.2020.07.010

39. Sonnenberg GF, Monticelli LA, Elloso MM, Fouser LA, Artis D. CD4(+) lymphoid tissue inducer cells promote innate immunity in the gut. *Immunity* (2011) 34:122–34. doi: 10.1016/j.immuni.2010.12.009

40. Gladiator A, Wangler N, Trautwein-Weidner K, Leibundgut-Landmann S. Cutting edge: IL-17- secreting innate lymphoid cells are essential for host defense against fungal infection. *J Immunol* (2013) 190:521–25. doi: 10.4049/jimmunol.1202924

41. Parks OB, Pociask DA, Hodzic Z, Kolls JK and Good M. Interleukin-22 signaling in the regulation of intestinal health and disease. *Front Cell Dev Biol* (2016) 3:85. doi: 10.3389/fcell.2015.00085

42. Mahapatro M, Erkert L, Becker C. Cytokine-mediated crosstalk between immune cells and epithelial cells in the gut. *Cells* (2021) 10:111. doi: 10.3390/cells10010111

43. Yan J, Yu J, Liu K, Liu Y, Mao C, Gao W. The pathogenic roles of IL-22 in colitis: its transcription regulation by musculin in T helper subsets and innate lymphoid cells. *Front Immunol* (2021) 12:758730. doi: 10.3389/fimmu.2021.758730

44. Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity* (2012) 36:92–04. doi: 10.1016/j.immuni.2011.11.011

45. Helm EY, Zhou L. Transcriptional regulation of innate lymphoid cells and T cells by aryl hydrocarbon receptor. *Front Immunol* (2023) 14:1056267. doi: 10.3389/fimmu.2023.1056267

46. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, Nukaya M, et al. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat Immunol* (2012) 13:144–51. doi: 10.1038/ni.2187

47. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity* (2013) 39:372–85. doi: 10.1016/j.immuni.2013.08.003

48. Lowe MM, Mold JE, Kanwar B, Huang Y, Louie A, Pollastri MP, et al. Identification of cinnabarinic acid as a novel endogenous aryl hydrocarbon receptor ligand that drives IL-22 production. *PloS One* (2014) 9:e87877. doi: 10.1371/journal.pone.0087877

49. Yang H, Qu Y, Gao Y, Sun S, Wu R, Wu J. Research progress on the correlation between the intestinal microbiota and food allergy. *Foods* (2022) 11(18):2913. doi: 10.3390/foods11182913

50. Xiong Y, Xu G, Chen M, Ma H. Intestinal uptake and tolerance to food antigens. *Front Immunol* (2022) 13:906122. doi: 10.3389/fimmu.2022.906122

51. Cheng Y, Liu X, Cheng F, Rolnic BM, Chleilat F, Ling Z, et al. The roles and mechanisms of gut microbiota in food alleregy. *Adv Gut Microbiome Res* (2023) 2023:9575410. doi: 10.1155/2023/9575410

52. van de Veen W, Akdis M. The use of biologics for immune modulation in allergic disease. *J Clin Invest* (2019) 130:1452–62. doi: 10.1172/JCI124607

53. Tordesillas L, Goswami R, Benedé S, Grishina G, Dunkin D, Järvinen KM, et al. Skin exposure promotes a Th2-dependent sensitization to peanut allergens. *J Clin Invest* (2014) 124:4965–75. doi: 10.1172/JCI75660

54. Ganeshan K, Neilsen CV, Hadsaitong A, Schleimer RP, Luo X, Bryce PJ. Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. *J Allergy Clin Immunol* (2009) 123:231–38.e4. doi: 10.1016/j.jaci.2008.10.011

55. Ménard S, Cerf-Bensussan N, Heyman M. Multiple facets of intestinal permeability and epithelial handling of dietary antigens. *Mucosal Immunol* (2010) 3:247–59. doi: 10.1038/mi.2010.5

56. Mabbott NA, Donaldson DS, Ohno H, Williams IR, Mahajan A. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol* (2013) 6:666–77. doi: 10.1038/mi.2013.30

57. Chinthrajah RS, Hernandez JD, Boyd SD, Galli SJ, Nadeau KC. Molecular and cellular mechanisms of food allergy and food tolerance. *J Allergy Clin Immunol* (2016) 137(4):984–97. doi: 10.1016/j.jaci.2016.02.004

58. Ali A, Tan H, Kaiko GE. Role of the intestinal epithelium and its interaction with the microbiota in food allergy. *Front Immunol* (2020) 11:604054. doi: 10.3389/fimmu.2020.604054

59. Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* (2009) 206:3101–14. doi: 10.1084/jem.20091925

60. Mazzini E, Massimiliano L, Penna G, Rescigno M. Oral tolerance can be established via gap junction transfer of fed antigens from $CX3CR1^+$ Macrophages to $CD103^+$ Dendritic cells. *Immunity* (2014) 40(2):248–61. doi: 10.1016/j.immuni.2013.12.012

61. Tordesillas L, Lozano-Ojalvo D, Dunkin D, Mondoulet L, Agudo J, Merad M, et al. PDL2+ CD11b+ dermal dendritic cells capture topical antigen through hair follicles to prime LAP+ Tregs. *Nat Commun* (2018) 9:5238. doi: 10.1038/s41467-018-07716-7

62. Dioszeghy V, Mondoulet L, Laoubi L, Dhelft V, Plaquet C, Bouzereau A, et al. 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. *Front Immunol* (2018) 9:1951. doi: 10.3389/fimmu.2018.01951

63. Luo Y, Wang S, Liu X, Wen H, Li W, Yao X. Langerhans cells mediate the skin-induced tolerance to ovalbumin via Langerin in a murine model. *Allergy* (2019) 74:1738–47. doi: 10.1111/all.13813

64. Hammad H, Lambrecht BN. Barrier epithelial cells and the control of type 2 immunity. *Immunity* (2015) 43:29–40. doi: 10.1016/j.immuni.2015.07.007

65. Chu DK, Llop-Guevara A, Walker TD, Flader K, Goncharova S, Boudreau JE, et al. IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization. *J Allergy Clin Immunol* (2013) 131:187–200. doi: 10.1016/j.jaci.2012.08.002

66. Prussin C, Lee J, Foster B. Eosinophilic gastrointestinal disease and peanut allergy are alternatively associated with IL-5+ and IL-5(-) T(H)2 responses. *J Allergy Clin Immunol* (2009) 124:1326–32.e6. doi: 10.1016/j.jaci.2009.09.048

67. Florence R. Targeting the interleukin-5 pathway for treatment of eosinophilic conditions other than asthma. *Front Med* (2018) 5:49. doi: 10.3389/fmed.2018.00049

68. Brough HA, Cousins DJ, Munteanu A, Wong YF, Sudra A, Makinson K, et al. IL-9 is a key component of memory TH cell peanut-specific responses from children with peanut allergy. *J Allergy Clin Immunol* (2014) 134:1329–38.e10. doi: 10.1016/j.jaci.2014.06.032

69. Simon D, Cianferoni A, Spergel JM, Aceves S, Holbreich M, Venter C, et al. Eosinophilic esophagitis is characterized by a non-IgE-mediated food hypersensitivity. *Allergy* (2016) 71:611–20. doi: 10.1111/all.12846

70. Gowthaman U, Chen JS, Zhang B, Flynn WF, Lu Y, Song W, et al. Identification of a T follicular helper cell subset that drives anaphylactic IgE. *Science* (2019) 365 (6456):eaaw6433. doi: 10.1126/science.aaw6433

71. Crotty S. T follicular helper cell biology: A decade of discovery and diseases. *Immunity* (2019) 50:1132–48. doi: 10.1016/j.immuni.2019.04.011

72. Sicherer SH, Eigenmann PA, Sampson HA. Clinical features of food protein-induced enterocolitis syndrome. *J Pediatr* (1998) 133(2):214–19. doi: 10.1016/s0022-3476(98)70222-7

73. Yang X, Liang R, Xing Q, Ma X. Fighting food allergy by inducing oral tolerance: facts and fiction. *Int Arch Allergy Immunol* (2021) 182:852–62. doi: 10.1159/000515292

74. Samadi N, Klems M, Untersmayr E. The role of gastrointestinal permeability in food allergy. *Ann Allergy Asthma Immunol* (2018) 121:168–73. doi: 10.1016/j.anai.2018.05.010

75. Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, et al. Food processing and allergenicity. *Food Chem Toxicol* (2015) 80:223–40. doi: 10.1016/j.fct.2015.03.005

76. Shumilov PV, Dubrovskaya MI, Yudina OV. Porazheniya zheludochno-kishechnogo trakta u detey s pishchevoy allergiyey [Gastrointestinal disorders in children with food allergies]. *Trudnyy patsiyent* (2007) 10:19–25.

77. Garcia-Larsen V, Ierodiakonou D, Jarrold K, Cunha S, Chivinge J, Robinson Z, et al. Diet during pregnancy and infancy and risk of allergic or autoimmune disease: A systematic review and meta-analysis. *PloS Med* (2018) 15(2):e1002507. doi: 10.1371/journal.pmed.100250

78. Fujimura T, Lum SZC, Nagata Y, Kawamoto S and Oyoshi MK. Influences of maternal factors over offspring allergies and the application for food allergy. *Front Immunol* (2019) 10:1933. doi: 10.3389/fimmu.2019.01933

79. Azad M, Dharma C, Simons E, Tran M, Reyna M, Dai R, et al. Reduced peanut sensitization with maternal peanut consumption and early peanut introduction while breastfeeding. *J Dev Origins Health Dis* (2021) 12(5):811–18. doi: 10.1017/S2040174420001129

80. Di Costanzo M, De Paulis N, Capra ME, Biasucci G. Nutrition during pregnancy and lactation: epigenetic effects on infants' Immune system in food allergy. *Nutrients* (2022) 14:1766. doi: 10.3390/nu14091766

81. Casale TB, Haselkorn T, Ciaccio CE, Sriaroon P, Chipps BE. Harmonization of terminology for tolerated and reactive dose in food allergy immunotherapy. *J Allergy Clin Immunol Pract* (2019) 7:389–92. doi: 10.1016/j.jaip.2018.12.008

82. Gargano D, Appanna R, Santonicola A, De Bartolomeis F, Stellato C, Cianferoni A, et al. Food allergy and intolerance: A narrative review on nutritional concerns. *Nutrients* (2021) 13(5):1638. doi: 10.3390/nu13051638

83. Zhao Y, Zhang X, Jin H, Chen L, Ji J, Zhang Z. Histamine intolerance - A kind of pseudoallergic reaction. *Biomolecules* (2022) 12(3):454. doi: 10.3390/biom12030454

84. Kacik J, Wróblewska B, Lewicki S, Zdanowski R, Kalicki B. Serum diamine oxidase in pseudoallergy in the pediatric population. *Adv Exp Med Biol* (2018) 1039:35–44. doi: 10.1007/5584201781

85. Zuberbier T, Pfrommer C, Specht K, Vieths S, Bastl-Borrmann R, Worm M, et al. Aromatic components of food as novel eliciting factors of pseudoallergic reactions in chronic urticaria. *J Allergy Clin Immunol* (2002) 109(2):343–48. doi: 10.1067/mai.2002.121309

86. Högerle C, Nicolo MS, Gellrich D, Eder K, Gröger M. Clinical relevance of profilin sensitization concerning oral allergy syndrome in birch pollen sensitized patients. *J Asthma Allergy* (2022) 15:249–55. doi: 10.2147/JAA.S348650

87. Carlson G, Coop C. Pollen food allergy syndrome (PFAS): A review of current available literature. *Ann Allergy Asthma Immunol* (2019) 123(4):359–65. doi: 10.1016/j.anai.2019.07.022

88. Cianferoni A, Muraro A. Food-induced anaphylaxis. *Immunol Allergy Clin North Am* (2012) 32(1):165–95. doi: 10.1016/j.iac.2011.10.002

89. Yue D, Ciccolini A, Avilla E, Waserman S. Food allergy and anaphylaxis. *J Asthma Allergy* (2018) 11:111–20. doi: 10.2147/JAA.S162456

90. Dellon ES, Gonsales N, Hirano I, Furuta GT, Liacouras CA, Katzka DA. American College of Gastroenterology. ACG Clinical Guidline: evidence based approach to the diagnosis and managment of esophageal eosinophilia and eosinophilic esophagitis (EoE). *Am J Gastroenterol* (2013) 108:679–92. doi: 10.1038/ajg.2013.71

91. Sicherer SH, Burks AW, Sampson HA. Clinical features of acute allergic reactions to peanut and tree nuts in children. *Pediatrics* (1998) 102:e6. doi: 10.1542/peds.102.1.e6

92. Sampson HA, Gerth van Wijk R, Bindslev-Jensen C, Sicherer S, Teuber SS, Burks AW, et al. Standardizing double-blind, placebo-controlled oral food challenges: American Academy of Allergy, Asthma and Immunology- European Academy of Allergy and Clinical Immunology PRACTALL consensus report. *J Allergy Clin Immunol* (2012) 130:1260–74. doi: 10.1016/j.jaci.2012.10.017

93. Gomez G, Jogie-Brahim S, Shima M, Schwartz LB. Omalizumab reverses the phenotypic and functional effects of IgE-enhanced Fc epsilonRI on human skin mast cells. *J Immunol* (2007) 179:1353–61. doi: 10.4049/jimmunol.179.2.1353

94. Greer AM, Wu N, Putnam AL, Woodruff PG, Wolters P, Kinet JP, et al. Serum IgE clearance is facilitated by human FcεRI internalization. *J Clin Invest* (2014) 124:1187–98. doi: 10.1172/JCI68964

95. Gomez G. Current strategies to inhibit high affinity FcεRI-mediated signaling for the treatment of allergic disease. *Front Immunol* (2019) 10:175. doi: 10.3389/fimmu.2019.00175

96. Feuille E, Nowak-Wegrzyn A. Allergen-specific immunotherapies for food allergy. *Allergy Asthma Immunol Res* (2018) 10(3):189–06. doi: 10.4168/aair.2018.10.3.189

97. Hwang DW, Nagler CR, Ciaccio CE. New and emerging concepts and therapies for the treatment of food allergy. *Immunother Adv* (2022) 2(1):ltac006. doi: 10.1093/immadv/ltac006

98. Pouessel G, Lezmi G. Oral immunotherapy for food allergy: Translation from studies to clinical practice? *World Allergy Org J* (2023) 16:100747. doi: 10.1016/j.waojou.2023.100747

99. Schworer SA, Kim EH. Sublingual immunotherapy for food allergy and its future directions. *Immunotherapy* (2020) 12:921–31. doi: 10.2217/imt-2020-0123

100. Bajowala SS. Sublingual immunotherapy as an option for effective food allergy treatment. *J Food Allergy* (2022) 4(2):106–11. doi: 10.2500/jfa.2022.4.220026

101. Pongracic JA, Gagnon R, Sussman G, Siri D, Oriel RC, Brown-Whitehorn TF, et al. Safety of epicutaneous immunotherapy in peanut-allergic children: realise randomized clinical trial results. *J Allergy Clin Immunol Pract* (2022) 10(7):1864–73.e10. doi: 10.1016/j.jaip.2021.11.017

102. Greenhawt M, Sindher SB, Wang J, O'Sullivan M, du Toit G, Kim EH, et al. Phase 3 trial of epicutaneous immunotherapy in toddlers with peanut allergy. *N Engl J Med* (2023) 388(19):1755–66. doi: 10.1056/NEJMoa2212895

103. Wong L, Kost L, Anderson B, Long A, Sindher SB, Chinthrajah RS, et al. Transitioning from epicutaneous to oral peanut immunotherapy. *Front Allergy* (2023) 4:1089308. doi: 10.3389/falgy.2023.1089308

104. Chan ES, Abrams EM, Hildebrand KJ, Watson W. Early introduction of foods to prevent food allergy. *Allergy Asthma Clin Immunol* (2018) 14(Suppl 2):57. doi: 10.1186/s13223-018-0286-1

105. Trogen B, Jacobs S, Nowak-Wegrzyn A. Early introduction of allergenic foods and the prevention of food allergy. *Nutrients* (2022) 14:2565. doi: 10.3390/nu14132565

106. Butler C, Reed MS, Schoenmann CL, Ousley L. Early introduction of allergenic foods in infants and children. *J Nurse Pract* (2023) 19(7):104668. doi: 10.1016/j.nurpra.2023.104668

107. Corica D, Aversa T, Caminiti L, Lombardo F, Wasniewska M, Pajno GB. Nutrition and avoidance diets in children with food allergy. *Front Pediatr* (2020) 8:518. doi: 10.3389/fped.2020.00518

108. Yulish EI. Antigistaminnyye sredstva v praktike lecheniya allergicheskikh zabolevaniy [Antihistamines in the practice of treating allergic diseases]. *Na Dopomogu Pediatru* (2011) 3(30):83–8.

109. Sidorovich OI. Preimushchestva antigistaminnykh preparatov pervogo pokoleniya [Benefits of first generation antihistamines]. *Effektivnaya Farmakoterapiya* (2015) 20:24–5.

110. Kawauchi H, Yanai K, Wang D-Y, Itahashi K, Okubo K. Antihistamines for allergic rhinitisTreatment from the viewpoint of nonsedative properties. *Int J Mol Sci* (2019) 20(1):213. doi: 10.3390/ijms20010213

111. Gorelov AV, Ursova NI. Sovremennyy vzglyad na problemu enterosorbtsii. Optimal'nyy podkhod k vyboru preparata [Modern view on the problem of enterosorption. The optimal approach to drug selection]. *Russkiy Meditsinskiy Zhurnal* (2006) 19:1391–96.

112. Paliy IG, Reznichenko IG. Sovremennyy vzglyad na problemu enterosorbtsii: vybor optimal'nogo preparata [Modern view on the problem of enteral sorption: selection of optimal medication]. *Liky Ukrayiny* (2008) 6(122):43–6.

113. Okhotnikova EN, Gladush Y, Ivanova TP. Ispol'zovaniye enterosorbenta Belyy ugol' pri allergicheskikh zabolevaniyakh u detey: rezul'taty sobstvennykh issledovaniy [Use of enterosorbent White coal in allergic diseases in children: results of own research]. *Sovremennaya Pediatriya* (2009) 4(26):39–43.

114. Smirnova GI. Enterosorbenty v kompleksnom lechenii atopicheskogo dermatita u detey [Enterosorbents in the complex treatment of atopic dermatitis in children]. *Rossiyskiy Pediatricheskiy Zhurnal* (2009) 1:41–4.

115. Kuznetsov SV. Effektivnost' enterosorbenta Belyy ugol' v kompleksnoy terapii gel'mintozov u detey [Efficiency of the enterosorbent White coal in the complex therapy of helminthiases in children]. *Zdorov'ye Rebenka* (2010) 4(25):30–3.

116. Garnik TP, Frolov VM, Sanzharevskaya IV, Bykadorov VI. Otsenka effektivnosti enterosorbenta na osnove dioksida kremniya «Belyy ugol'» pri lechenii bol'nykh nealkogol'nym steatogepatitom, sochetannym s khronicheskim nekal'kuleznym kholetsistitom i yego vliyaniye na kontsentratsiyu «srednikh molekul», soderzhaniye produktov lipoperoksidatsii i uroven' S-reaktivnogo belka v syvorotke krovi [Evaluation of the effectiveness of the enterosorbent based on silicon dioxide "White Coal" in the treatment of patients with non-alcoholic steatohepatitis associated with chronic non-calculous cholecystitis and its effect on the concentration of "medium molecules", the content of lipid peroxidation products and the level of C-reactive protein in the blood serum]. *Ukrayins'kyy Medychnyy Al'manakh* (2011) 14 (4):79–82.

117. Teryoshin VO, Kruglova OV. Vplyv suchasnoho kremnez'omnoho enterosorbentu aerosilu («Bile vuhillya») na pokaznyky syndromu imunotoksykozu u khvorykh na nealkohol'nyy steatohepatyt, spoluchenyy z khronichnym nekal'kul' oznym kholetsystytom na tli dysbiozu kyshechnyka [The effect of modern silica enterosorbent Aerosil ("White coal") on indicators of immunotoxicosis syndrome in patients with nonalcoholic steatohepatitis combined with chronic noncalculous cholecystitis against the background of intestinal dysbiosis]. *Ukrayins'kyy Morfolohichnyy Al'manakh* (2011) 9(4):102–06.

118. Teryoshin VO, Kruglova OV, Tyshchenko DV. Vplyv enterosorbentu Aerosil («Bile vuhillya») na kontsentratsiyu tsyrkulyuyuchykh imunnykh kompleksiv ta yikhniy molekulyarnyy sklad u krovi khvorykh z khronichnym bezkam"yanym kholetsystytom na tli vtorynnykhimunodefitsytnykh staniv [The effect of enterosorbent Aerosil ("White coal") on the concentration of circulating immune complexes and their molecular composition in the blood of patients with chronic noncalculous cholecystitis against the background of secondaryimmunodeficiency states]. *Ukrayins'kyy Morfolohichnyy Al'manakh* (2011) 14(5):193–97.

119. Protsak VV. Efektyvnist' zastosuvannya enterosorbentu enteroklin u kompleksi zi standartnoyu terapiyeyu khvorym z atopichnym dermatytom u stadiyi zahostrennya z moknuttyam [The effectiveness of the use of enteroclin enterosorbent in combination with standard therapy in patients with atopic dermatitis in the stage of exacerbation with wetting]. *Visnyk ProblemBiolohiyi i Medytsyny* (2019) 3(152):177–80. doi: 10.29254/2077-4214-2019-3-152-177-181

120. Bobyr VV, Stechenko LO, Shirobokov VP, Nazarchuk OA, Rymsha OV. The role of sorbents and probiotics in prevention of structural and morphological disorders in the small intestine of animals developing in dysbiosis. *Rep Morphol* (2020) 26(2):45–50. doi: 10.31393/morphology-journal-2020-26(2)-07

121. Markovinovic´ L, Knezovic´ I, Kniewald T, Maric´ LS, Trkulja V, Tešovic´ G. Enteroadsorbent Polymethylsiloxane Polyhydrate vs. Probiotic *Lactobacillus reuteri* DSM 17938 in the Treatment of Rotaviral Gastroenteritis in Infants and Toddlers, a Randomized Controlled Trial. *Front Pediatr* (2020) 8:553960. doi: 10.3389/fped.2020.553960

122. *WAO white book on allergy 2013 update, world allergy organization* (2013). Available at: https://www.worldallergy.org/wao-whitebook-on-allergy.

123. Baranova AA, Namazova-Baranova LS, Borovik TE, Makarova SG. *Pishchevaya allergiya. Rukovodstvo dlya vrachey [Food allergy. A Guide for Physicians]*. Moscow: Pediatr (2013). p. 160. redaktors.

124. Kucher SV, Lototska OV. Inclusion of enterosorbents in anti-inflammatory therapy improve treatment effectiveness in COPD patients during exacerbations. *Ukr Biochem J* (2021) 93(2):107–14. doi: 10.15407/ubj93.02.107

125. Braley S. Chemistry and properties of the medical-grade silicones. *J Macromol Sc Part A* (1970) 4(3):529–44. doi: 10.1080/00222337008074361

126. Bitar A, Ahmad NM, Fessi H, Elaissari A. Silica-based nanoparticles for biomedical applications. *Drug Discovery Today* (2012) 17(19-20):1147–54. doi: 10.1016/j.drudis.2012.06.014

127. Tang L, Cheng J. Nonporous silica nanoparticles for nanomedicine application. *Nano Today* (2013) 8(3):290–12. doi: 10.1016/j.nantod.2013.04.007

128. Howell CA, Mikhalovsky SV, Markaryan EN, Khovanov AV. Investigation of the adsorption capacity of the enterosorbent Enterosgel for a range of bacterial toxins, bile acids and pharmaceutical drugs. *Sci Rep* (2019) 9:5629. doi: 10.1038/s41598-019-42176-z

129. Howell CA, Markaryan EN, Mikhalovsky SV. Oral intestinal adsorbents - are they the next therapy for acute diarrhea in children: A mini review. *Ann Pediatr Child Health* (2020) 8(8):1202.

130. Kemppinen A, Howell C, Allgar V, Dodd M, Gregson J, Knowles C, et al. Randomised, double- blind, placebo controlled multi-centre study to assess the efficacy, tolerability and safety of Enterosgel® in the treatment of irritable bowel syndrome with diarrhoea (IBS-D) in adults. *Trials* (2020) 21:122. doi: 10.1186/s13063-020-4069-x

131. Ravasio R, Raimondo P. L'impatto economico di Enterosgel® nel trattamento ospedaliero delle sindromi diarroiche associate a diverse condizioni patologiche: un'analisi di minimizzazione dei costi. *AboutOpen* (2021) 8:99–105. doi: 10.33393/ao.2021.2345A

132. Jandosov J, Alavijeh M, Sultakhan S, Baimenov A, Bernardo M, Sakipova Z, et al. Activated carbon/pectin composite enterosorbent for human protection from intoxication with xenobiotics Pb(II) and sodium diclofenac. *Molecules* (2022) 27:2296. doi: 10.3390/molecules27072296

133. Jurkić LM, Cepanec I, Pavelić SK, Pavelić K. Biological and therapeutic effects of ortho-silicic acid and some ortho-silicic acid-releasing compounds: New perspectives for therapy. *Nutr Metab* (2013) 10:2. http://www.nutritionandmetabolism.com/content/10/1/2. doi: 10.1186/1743-7075-10-2

134. Chen L, Liu J, Zhang Y, Zhang G, Kang Y, Chen A, et al. The toxicity of silica nanoparticles to the immune system. *Nanomedicine* (2018) 13(15):1939–62. doi: 10.2217/nnm-2018-0076

135. Janczura M, Lulinski P, Sobiech M. Imprinting technology for effective sorbent fabrication: current state-of-art and future prospects. *Materials* (2021) 14(8):1850. doi: 10.3390/ma14081850

136. Seisenbaeva GA, Ali LMA, Vardanyan A, Gary-Bobo M, Budnyak TM, Kessler VG, et al. Mesoporous silica adsorbents modified with amino polycarboxylate ligands –

functional characteristics, health and environmental effects. *J Hazardous Materials* (2021) 406:124698. doi: 10.1016/j.jhazmat.2020.124698

137. Bergna HE, Roberts WO eds. *Colloidal silica. Fundamentals and applications*. Boca Raton: CRC Press (2005). 944 p. doi: 10.1201/9781420028706

138. Slavova TG, Radulova GM, Kralchevsky PA, Danov KD. Encapsulation of fragrances and oils by core-shell structures from silica nanoparticles, surfactant and polymer: Effect of particle size. *Colloids Surfaces A: Physicochemical Eng Aspects* (2020) 606:125558. doi: 10.1016/j.colsurfa.2020.125558

139. Arts JHE, Muijser H, Duistermaat E, Junker K, Kuper CF. Five-day inhalation toxicity study of three types of synthetic amorphous silicas in Wistar rats and post-exposure evaluations for up to 3 months. *Food Chem Toxicol* (2007) 45(10):1856–67. doi: 10.1016/j.fct.2007.04.001

140. Qian KK, Bogner RH. Application of mesoporous silicon dioxide and silicate in oral amorphous drug delivery systems. *J Pharm Sci* (2012) 101(2):444–63. doi: 10.1002/jps.2277

141. Slowing I, Viveroescoto J, Wu C, Lin V. Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers. *Adv Drug Delivery Rev* (2008) 60(11):1278–88. doi: 10.1016/j.addr.2008.03.012

142. Sripanyakorn S, Jugdaohsingh R, Dissayabutr W, Anderson SHC, Thompson RPH, Powell JJ. The comparative absorption of silicon from different foods and food supplements. *Br J Nutr* (2009) 102(06):825–34. doi: 10.1017/S0007114509311757

143. Lührs AK, Geurtsen W. The application of silicon and silicates in dentistry: a review. *Prog Mol Subcell Biol* (2009) 47:359–80. doi: 10.1007/978-3-540-88552-8_16

144. Aguilar F, Charrondiere UR, Dusemund B, Galtier P, Gilbert J, Gott DM, et al. Scientific opinion of the panel on food additives and nutrient sources added to food on calcium silicate, silicon dioxide and silicic acid gel added for nutritional purposes to food supplements following a request from the European Commission. *EFSA J* (2009) 1132:1–24. doi: 10.2903/j.efsa.2009.1132

145. Younes M, Aggett P, Aguilar F, Dusemund B, Filipič M, Frutos MJ, et al. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS). Re-evaluation of silicon dioxide (E 551) as a food additive. *EFSA J* (2018) 16(1):5088. doi: 10.2903/j.efsa.2018.5088

146. Gmoshinski IV, Shipelin VA, Shumakova AA, Trushina EN, Mustafina OK, Safenkova IV, et al. Toxicity evaluation of nanostructured silica orally administered to rats: influence on immune system function. *Nanomaterials* (2020) 10:2126. doi: 10.3390/nano10112126

147. Nabeshi H, Yoshikawa T, Akase T, Yoshida T, Tochigi S, Hirai T, et al. Effect of amorphous silica nanoparticles on in *vitro* RANKL-induced osteoclast differentiation in murine macrophages. *Nanoscale Res Lett* (2011) 6(1):464. doi: 10.1186/1556-276X-6-464

148. Kim HW, Ahn E-K, Jee BK, Yoon H-K, Lee KH, Lim Y. Nanoparticulate-induced toxicity and related mechanism in *vitro* and in vivo. *J Nanopart Res* (2009) 11 (1):55–65. doi: 10.1007/s11051-008-9541-6

149. Marcus DM. An analytical review of silicone immunology. *Arthritis Rheumatism* (1996) 39(10):1619–26. doi: 10.1002/art.1780391003

150. Fruijtier-Pölloth C. The toxicological mode of action and the safety of synthetic amorphous silica. A nanostructured material. *Toxicology* (2012) 294:61–79. doi: 10.1016/j.tox.2012.02.001

151. Fruijtier-Pölloth C. The safety of nanostructured synthetic amorphous silica (SAS) as a food additive (E 551). *Arch Toxicol* (2016) 90:2885–916. doi: 10.1007/s00204-016-1850-4

152. *Silicon dioxide: summary of the dossier. OECD environment, health and safety publications. Series on the safety of manufactured nanomaterials no. 71* Vol. 23. Paris: ENV/JM/MONO (2016). Available at: https://one.oecd.org/document/env/jm/mono(2016)23/en/pdf.

153. *Synthetic Amorphous Silica (CAS No. 7631-86-9). ECETOC JACC report No. 51*. Brussels: ECETOC AISBL (2006). Available at: https://www.ecetoc.org/wp-content/uploads/2021/10/JACC-051.pdf.

154. Babak OY, Fadeyenko GD, Frolov VM, Sotska YA, Kruglova OV. Otsinka efektyvnosti suchasnoho kremnez′omnoho enterosorbentu «Bile vuhillya» (aerosil) ta vitchyznyanoho hepatoprotektoru antralyu u likvidatoriv naslidkiv avariyi na Chornobyl′s′kiy AES z khronichnoyu spoluchenoyu patolohiyeyu hepatobiliarnoyi systemy [Evaluation of the effectiveness of the modern siliceous enterosorbent "White coal" (aerosil) and the domestic hepatoprotector antral in liquidators of the consequences of the accident at the Chernobyl nuclear power plant with chronic combined pathology of the hepatobiliary system]. *Ukrayins′kyy medychnyy al′manakh* (2011) 14(6):24–30.

155. Sotska YA, Frolov VM. Efektyvnist′ detoksykatsiynoyi terapiyi u khvorykh z khronichnym virusnym hepatytom S nyz′koho stupenya aktyvnosti, spoluchenym z khronichnym nekal′kul′oznym kholetsystytom, pry zastosuvanni suchasnykh kremnez′omnykh enterosorbentiv [The effectiveness of detoxification therapy in patients with chronic viral hepatitis C of a low degree of activity, combined with chronic noncalculous cholecystitis, with the use of modern silica enterosorbents]. *Ukrayins′kyy medychnyy al′manakh* (2012) 15(4):145–50.

156. Sotska YA, Frolov VM, Shpilevska SS. Pokaznyky adenilovoyi systemy krovi u khvorykh z khronichnym virusnym hepatytom S nyz′koho stupenya aktyvnosti, spoluchenym z khronichnym nekal′kul′oznym kholetsystytom pry zastosuvanni suchasnykh kremnez′omnykh enterosorbentiv [Indicators of the adenyl system of the blood in patients with chronic viral hepatitis C of a low degree of activity combined with chronic noncalculous cholecystitis when using modern siliceous enterosorbents. In: *Problems of environmental and medical genetics and clinical immunology*, vol. . 5. . Kyiv – Lugansk: Zbirnyk naukovykh prats′ (2012). p. 373–82.

157. Frolov VM, Sotska YA, Kruglova OV, Sanzharevska IV. Vplyv suchasnoho kremnez′omnoho enterosorbentu «Bile vuhillya» na aktyvnist′ fermentiv systemy antyoksydantnoho zakhystu u khvorykh z tsyrozamy pechinky riznoyi etiolohiyi [The influence of modern silica enterosorbent "White coal" on the activity of enzymes of the antioxidant defense system in patients with cirrhosis of the liver of various etiologies]. In: *Problems of environmental and medical genetics and clinical immunology*, vol. . 2. . Kyiv - Lugansk: Zbirnyk naukovykh prats′ (2012). p. 310–24.

158. Frolov VM, Sotskaya YA, Kruglova OV, Sanzarevskaya IV. Perspektivy detoksitsiruyushchey terapii u bol'nykh s tsirozami pecheni pri ispol'zovanii sovremennogo kremnezemnogo enterosorbenta «Belyy ugol'» [Perspectives of detoxic therapy at the patients with hepatic cirrhosis at the application modern silicon enterosorbent "White coal"]. *Ukrayins'kyy medychnyy al'manakh* (2012) 15 (2):184–90.

159. Frolov VM, Sotskaya YA, Peresadin NA. Effektivnost' enterosorbenta Belyy Ugol' v lechenii bol'nykh tsirozom pecheni [Efficacy of the enterosorbent White Coal in the treatment of patients with liver cirrhosis]. *Likars'ka sprava* (2012) 8:108–15.

160. Shapovalova IO. Vplyv suchasnoho kremnez′omnoho enterosorbentu «Bile vuhillya» (Aerosil) na pokaznyky endohennoyi «metabolichnoyi» intoksykatsiyi u khvorykh na khronichnyy toksychnyy hepatyt, poyednanyy z khronichnym nekal′kul ′oznym kholetsystytom na tli ozhyrinnya [The influence of modern silica enterosorbent "White coal" (Aerosil) on indicators of endogenous "metabolic" intoxication in patients with chronic toxic hepatitis combined with chronic noncalculous cholecystitis against the background of obesity. In: *Problems of environmental and medical genetics and clinical immunology*, vol. . 5. . Kyiv – Lugansk: Zbirnyk naukovykh prats′ (2011). p. 287–302.

161. Konecˇ P, Ehrlich R, Gulumian M, Jacobs M. Immunity to the dual threat of silica exposure and mycobacterium tuberculosis. *Front Immunol* (2019) 9:3069. doi: 10.3389/fimmu.2018.03069

162. Maier M, Hannebauer B, Holldorff H, Albers P. Does lung surfactant promote disaggregation of nanostructured titanium dioxide? *J Occup Environ Med* (2006) 48 (12):1314–20. doi: 10.1097/01.jom.0000215405.72714.b2

163. Suksaeree J, Monton C, Chankana N, Charoenchai L. Microcrystalline cellulose promotes superior direct compressed *Boesenbergia rotunda* (L.) Mansf. extract tablet properties to spray- dried rice starch and spray-dried lactose. *Arab J Basic Appl Sci* (2023) 30(1):13–25. doi: 10.1080/25765299.2022.2153527

164. Nsor-Atindana J, Chen M, Goff HD, Zhong F, Sharif HR, Li Y. Functionality and nutritional aspects of microcrystalline cellulose in food. *Carbohydr Polym* (2017) 172:159–74. doi: 10.1016/j.carbpol.2017.04.021

165. Younes M, Aggett P, Aguilar F, Crebelli R, Di Domenico A, Dusemund B, et al. (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)). Re-evaluation of celluloses E 460(i), E 460(ii), E 461, E 462, E 463, E 464, E 465, E 466, E 468 and E 469 asfood additives. *EFSA J* (2018) 16(1):e05047. doi: 10.2903/j.efsa.2018.5047

166. Kurchenko O (Study Director). *Efficacy of the administration of colloidal silicon dioxide in tablet dosage form (Carbowhite) in patients with acute diarrhea*. NLM, Bethesda, MD: U.S. FDA Resources. Clinical Trial (2016). clinicaltrials.gov/ct2/show/NCT03633344.

167. Ding M, Chen F, Shi X, Yucesoy B, Mossman B, Vallyathan V. Diseases caused by silica: mechanisms of injury and disease development. *Int Immunopharmacol* (2002) 2:173–82. doi: 10.1016/S1567-5769(01)00170-9

168. Dekkers S, Krystek P, Peters RJ, Lankveld DP, Bokkers BG, van Hoeven-Arentzen PH, et al. Presence and risks of nanosilica in food products. *Nanotoxicology* (2011) 5(3):393–05. doi: 10.3109/17435390.2010.519836

169. Leung CC, Yu IT, Chen W. Silicosis. *Lancet* (2012) 379:2008–18. doi: 10.1016/S0140-6736(12)60235-9

170. Allison AC, Harington JS, Birbeck M. An examination of the cytotoxic effects of silica on macrophages. *J Exp Med* (1996) 124:141–54. doi: 10.1084/jem.124.2.141

171. Fenoglio I, Croce A, Di Renzo F, Tiozzo R, Fubini B. Pure-silica zeolites (Porosils) as model solids for the evaluation of the physicochemical features determining silica toxicity to macrophages. *Chem Res Toxicol* (2000) 13:489–500. doi: 10.1021/acs.chemrestox.6b00409

172. Pavan C, Fubini B. Unveiling the variability of "quartz hazard" in light of recent toxicological findings. *Chem Res Toxicol* (2017) 30:469–85. doi: 10.1021/acs.chemrestox.6b00409

173. Turci F, Pavan C, Leinardi R, Tomatis M, Pastero L, Garry D, et al. Revisiting the paradigm of silica pathogenicity with synthetic quartz crystals: the role of crystallinity and surface disorder. *Part Fibre Toxicol* (2016) 13(1):32. doi: 10.1186/s12989-016-0136-6

174. Napierska D, Thomassen LCJ, Lison D, Martens JA, Hoet PH. The nanosilica hazard: another variable entity. *Part Fibre Toxicol* (2010) 7(1):39. doi: 10.1186/1743-8977-7-39

175. Hamilton RF Jr, Thakur SA, Mayfair JK, Holian A. MARCO mediates silica uptake and toxicity in alveolar macrophages from C57BL/6 mice. *J Biol Chem* (2006) 281:34218–26. doi: 10.1074/jbc.M605229200

176. Beamer GL, Seaver BP, Jessop F, Shepherd DM, Beamer CA. Acute exposure to crystalline silica reduces macrophage activation in response to bacterial lipoproteins. *Front Immunol* (2016) 7:49. doi: 10.3389/fimmu.2016.00049

177. Shkurupy VA, Nadeev AP, Karpov MA, Bugrimova YS. Experimental cytomorphological studies of the reaction of mononuclear phagocyte system in granulomatosis of mixed (silicotic and tuberculous) etiology. *Bull Exp Biol Med* (2010) 149:462–65. doi: 10.1007/s10517-010-0971-7

178. Dobrovolskaia MA, McNeil SE. Immunological properties of engineered nanomaterials. *Nat Nanotechnol* (2007) 2(8):469–78. doi: 10.1038/nnano.2007.223

179. Dobrovolskaia MA, Aggarwal P, Hall JB, McNeil SE. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. *Mol Pharm* (2008) 5(4):487–95. doi: 10.1021/mp800032f

180. De Oliveira Abrão C, de Araújo Filho JA. Mycobacterium sherrisii lung infection in a Brazilian patient with silicosis and a history of pulmonary tuberculosis. *Case Rep Infect Dis* (2015) 2015:498608. doi: 10.1155/2015/498608

181. Pérez JJB, González AP, Amado LEM, Vales JLG, Gallardo RV, Barreira AS, et al. Clinical significance of environmental mycobacteria isolated from respiratory specimens of patients with and without silicosis. *Arch Bronconeumol* (2016) 52(3):145–50. doi: 10.1016/j.arbres.2015.07.007

182. Yang H, Liu C, Yang D, Zhang H, Xi Z. Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition. *J Appl Toxicol* (2009) 29(1):69–78. doi: 10.1002/jat.1385

183. Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science* (2006) 311(5761):622–27. doi: 10.1126/science.1114397

184. Boraschi D, Fadeel B, Duschl A. Nanoparticles and the immune system. In: Fadeel B, Shvedova A, Pietroiusti A, editors. *Adverse effects of engineered nanomaterials: exposure, toxicology, and impact on human health*. London, UK: Academic Press (2017). p. 313–37.

185. Bhattacharya K, Andon FT, El-Sayed R, Fadeel B. Mechanisms of carbon nanotube-induced toxicity: focus on pulmonary inflammation. *Adv Drug Delivery Rev* (2013) 65(15):2087–97. doi: 10.1016/j.addr.2013.05.012

186. Oh W-K, Kim S, Choi M, Kim C, Jeong YS, Cho BR, et al. Cellular uptake, cytotoxicity, and innate immune response of silica-titania hollow nanoparticles based on size and surface functionality. *ACS Nano* (2010) 4(9):5301–13. doi: 10.1021/nn100561e

187. Nishijima N, Hirai T, Misato K, Aoyama M, Kuroda E, Ishii KJ, et al. Human scavenger receptor A1-mediated inflammatory response to silica particle exposure is size specific. *Front Immunol* (2017) 8:379. doi: 10.3389/fimmu.2017.00379

188. Lee S, Yun H-S, Kim S-H. The comparative effects of mesoporous silica nanoparticles and colloidal silica on inflammation and apoptosis. *Biomaterials* (2011) 32(35):9434–43. doi: 10.1016/j.biomaterials.2011.08.042

189. Di Cristo L, Movia D, Bianchi MG, Allegri M, Mohamed BM, Bell AP, et al. Proinflammatory effects of pyrogenic and precipitated amorphous silica nanoparticles in innate immunity cells. *Toxicol Sci* (2016) 150(1):40–53. doi: 10.1093/toxsci/kfv258

190. Xue Y, Chen Q, Ding T, Sun J. SiO2 nanoparticle-induced impairment of mitochondrial energy metabolism in hepatocytes directly and through a Kupffer cell-mediated pathway vitro. *Int J Nanomedicine* (2014) 9:2891–03. doi: 10.2147/IJN.S60661

191. Mendoza A, Torres-Hernandez JA, Ault JG, Pedersen-Lane JH, Gao D, Lawrence DA. Silica nanoparticles induce oxidative stress and inflammation of human peripheral blood mononuclear cells. *Cell Stress Chaperones* (2014) 19(6):777–90. doi: 10.1007/s12192-014-0502-y

192. Ravi Kumar MN, Sameti M, Mohapatra SS, Kong X, Lockey RF, Bakowsky U, et al. Cationic silica nanoparticles as gene carriers: synthesis, characterization and transfection efficiency in vitro and in *vivo*. *J Nanosci Nanotechnol* (2004) 4(7):876–81. doi: 10.1166/jnn.2004.120

193. Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdörster G, et al. Safe handling of nanotechnology. *Nature* (2006) 444(7117):267–69. doi: 10.1038/444267a

194. Johnston CJ, Driscoll KE, Finkelstein JN, Baggs R, O'Reilly MA, Carter J, et al. Pulmonary chemokine and mutagenic responses in rats after subchronic inhalation of amorphous and crystalline silica. *Toxicol Sci* (2000) 56(2):405–13. doi: 10.1093/toxsci/56.2.405

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Probiotic *Lactobacillus plantarum* GUANKE effectively alleviates allergic rhinitis symptoms by modulating functions of various cytokines and chemokines

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Background: Currently, the prevalence of allergic rhinitis (AR) remains high and there is a great need to develop better and safer ways to alleviate AR symptoms. The *Lactobacillus plantarum* GUANKE probiotic was reported as an immunomodulator through maintaining Th1/Th2 balance. This study aimed to determine the efficacy of GUANKE in AR subjects.

Methods: Adults aged from 18 to 60 years old and previously suffered from AR were recruited and received GUANKE probiotics treatment for 4 weeks. The questionnaires of Total nasal symptom scores (TNSS), total non-nasal symptom score (TNNSS), and rhinitis control assessment test (RCAT) were used to assess the effectiveness before and after treatment. The serum allergen-specific IgE and cytokines were also determined at baseline and after 4 weeks of probiotics administration.

Results: The results showed that TNSS and TNNSS were significantly reduced and the RCAT score was significantly increased compared to baseline. The sub-symptom score of rhinorrhea, itching, sneezing, and tearing in each questionnaire also showed significant changes, and the serum IgE level was markedly decreased. We further measured inflammatory-related proteins in serum and found that a total of 20 proteins (6 upregulated and 14 downregulated) were significantly changed compared to baseline, including IL-4, IL-7, IL-20, IL-33, CXCL1, CXCL5, CXCL6, CXCL11, CCL4, CCL23, TGF-alpha, LAP-TGF-beta-1, MMP-1, MMP-10, AXIN1, NT-3, OSM, SCF, CD6, and NRTN. Enrichment analysis showed that these significantly altered proteins were mainly enriched in cytokine and chemokine-related signaling pathways.

Conclusion: Taken together, this study demonstrated the *Lactobacillus plantarum* GUANKE can serve as an effective immunobiotic for the treatment of AR, which is realized through maintaining the Th1/Th2 balance by modulating the functions of various cytokines and chemokines.

KEYWORDS

allergic rhinitis, probiotic, *Lactobacillus plantarum*, GUANKE, cytokine

Introduction

Allergic rhinitis (AR) is a non-infectious inflammatory disease of the nasal mucosa, which is characterized by stuffy nose, itchy nose, sneezing, and runny nose. AR is accompanied by eye burning, itchy eyes, pharyngeal congestion, tears and other eye symptoms, and also causes nasal polyps, sinusitis and other complications (1). In recent years, the prevalence of AR has shown a significant increase globally, affecting 10–20% population and becoming one of the most common chronic respiratory inflammatory diseases worldwide (2). According to the epidemiological surveys, the average prevalence of AR in Europe and North America is about 25%, while the AR incidence trend in both adults and children in China is still growing in recent years and has a significant effect on the general public (3).

The pathogenesis of AR is complex and is influenced by genotypic, epigenetic, and environmental factors (4). As a complex human disorder, its severity is commonly assessed by measures such as total nasal symptom scores (TNSS), total non-nasal symptom score (TNNSS), and rhinitis control assessment test (RCAT) (5). Of these measures, they generally include the severity of symptoms such as nasal congestion, rhinorrhea, nasal itching, sneezing, or other sub-symptoms or whether the rhinitis-related symptoms were improved or controlled. Its pathological mechanism is that after the susceptible individuals are exposed to allergen, non-infectious inflammatory diseases of the nasal mucosa are mainly mediated by immune globulins E (IgE) and induced by a variety of immune active cells and cytokines. The production of AR is closely related to the occurrence of T helper 2 (Th2) immune response, which is mainly manifested in the shift of the immune response of the body to various allergens to the Th2 type, that is, the Th1 type cell response is suppressed, and the Th2 type cell response is enhanced. The interaction of multiple cytokines secreted by Th1 and Th2 cells disrupts the balance, leading to imbalanced T lymphocyte differentiation and the release of a large amount of histamines (6, 7).

Currently, there are many kinds of drugs for AR treatment, such as antihistamines, intranasal corticosteroids, anticholinergics, antileukotrienes, to name a few, but long-term use of these drugs will bring undesirable side effects (8). In recent years, several studies have reported that probiotics are an alternative strategy for the treatment of AR (9–11). Probiotics can act as an immunomodulator to activate the host defense and regulate the immune response in the respiratory system (10). Some *Lactobacillus* species have been reported to have excellent immunomodulatory ability in respiratory diseases (12). The probiotic of GUANKE strain belongs to *Lactobacillus plantarum*, originally isolated from the fecal sample of a healthy individual, which was first found to be able to promote SARS-CoV-2 specific immune responses through enhancing interferon signaling and suppressing apoptotic and inflammatory pathways by acting as an immunomodulator role via maintaining Th1/Th2 balance (13). However, whether GUANKE probiotic can be a candidate immunobiotic for the treatment of AR remains to be determined, which formed the main purpose of this study.

Materials and methods

Subjects

A total of 47 adult subjects with perennial AR were recruited from local community clinics or health service centers in the city of Hangzhou area of Zhejiang province, China for this study. The inclusion criteria of each participant were: (1) diagnosed with rhinitis or symptoms; (2) aged between 18 and 60 years; (3) no other diagnosed diseases at the time of recruitment; (4) did not take any drugs for rhinitis or other immune-related diseases. The exclusion criteria of subjects included those who: (1) suffered from other definite respiratory or diseases; (2) received systemic steroids or antihistamines within 3 days; (3) were pregnant. All subjects provided written informed consent. The study protocol was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine.

Study product

The products used in the study is GUANKE Immunobiotics (Maiyata Inc., Shaoxing, Zhejiang, China), which is a probiotic mixture of GUANKE (*Lactobacillus plantarum*, CGMCC No. 21720) freeze-dried with maltodextrin, cranberry fruit powder, erythritol, isomaltulose and vitamin C. All study products were produced at a Good Manufacturing Practice-certified manufacturing facility.

Study design

All recruited subjects were orally taken 2 packs of probiotics (1.5 g per stick pack contains 5.0×10^{10} CFU, Lot number: 22AR292) per day. Peripheral blood was collected and serum was isolated immediately before and after taking 4-week probiotics. The serum was stored at −80°C refrigerator for measuring IgE and cytokines. Three questionnaires were filled in by every participant at each visit.

Total nasal symptom score

TNSS is one of the validated AR questionnaires (14), which is expressed as the sum of the scores for four symptoms (nasal congestion, rhinorrhea, nasal itching, and sneezing). Each symptom was rated on a 4-point scale from 0 (no symptoms), 1 (mild symptoms), 2 (moderate symptoms), to 3 (severe symptoms), and recorded at 0 week and 4 weeks after taking probiotics. The sum score of each symptom was used to evaluate rhinitis-related symptoms before and after treatment with the probiotic.

Total non-nasal symptom score

TNNSS is the second questionnaire used in this study (14), which is based on the presence or absence of symptoms, such as post-nasal discharge, tearing, nasal or ocular itching, nasal or maxillary pain, and headache. Each item was assigned to two grades: symptomatic and asymptomatic. If no symptom occurs, the score is 0 point, otherwise the score is 1 point for each abovementioned symptom. The total score ranges from 0 to 5. The sum score of each symptom was used to evaluate non-rhinitis symptoms before and after treatment.

Rhinitis control assessment test

RCAT is the third questionnaire used in the study (15), which consists of six items: nasal congestion, sneezing, watery eyes, sleep

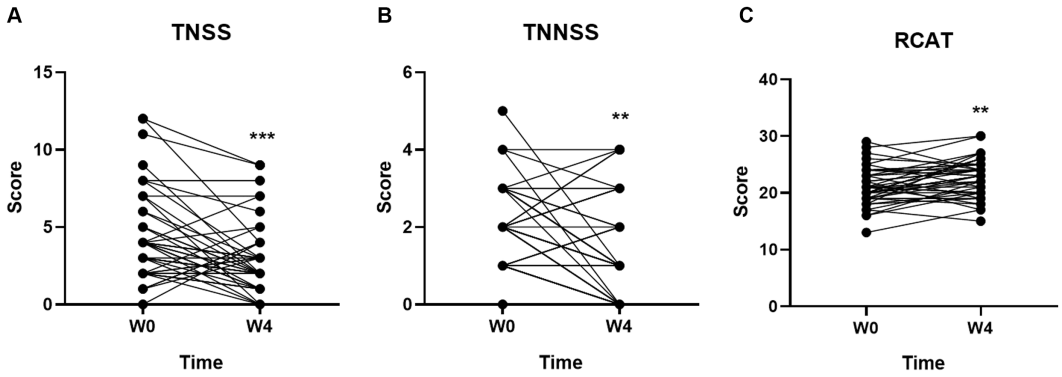

FIGURE 1
Effect of GUANKE probiotic on AR symptoms evaluated by three questionnaires **(A)** TNSS, **(B)** TNNSS, and **(C)** RCAT. The scores are presented as the sum of the all symptoms in each questionnaire. The significance was evaluated by performing paired *t*-test. *Indicates a statistically significant difference between the different time points. $^{**}p < 0.01$, $^{***}p < 0.001$.

interference, daily activities, and degree of rhinitis control. Each item is assigned 1–5 points, and the score of each item are summed up for evaluating the level of rhinitis control. A higher score indicates better rhinitis control.

Measurement of IgE in serum

Serum IgE level was determined by ELISA (E-EL-H6104, Elabscience Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's introduction. Briefly, a total of 100 µL standard or serum samples were added into the corresponding well, and the plate were coated and incubated at 37°C for 90 min. After draining each well, 100 µL Biotinized antibody working solution was added into each well and incubated at 37°C for 1 h with enzymic labeled plate and film coating. Then, 350 µL washing liquid was added into each well, incubated for 1–2 min, then discarded liquid and dried on a paper. After repeating this step for three times, 100 µL enzyme conjugate working solution was then added into each well, and incubated at 37°C for 30 min before draining the liquid and washing the plate for five times. Then, 90 µL substrate solution (TMB) was added to each well, and the plate was coated and incubated at 37°C for 15 min in the dark. Finally, 50 µL termination solution was added to each well to terminate the reaction. The optical density (OD value) of each well was immediately measured with the enzyme label instrument at the wavelength of 450 nm. The concentration of human IgE in each sample was calculated by comparing the OD of the samples to the standard curve.

Determination of inflammatory cytokines in serum

Inflammatory cytokines were measured using the Olink® Target 96 Inflammation Panel (Olink Proteomics AB, Uppsala, Sweden) according to the manufacturer's instructions. This panel enables 92 cytokines to be analyzed simultaneously by using 1 µL of each sample. In brief, pairs of oligonucleotide-labeled antibody probes bind to their targeted protein, and if the two probes are brought in close proximity the oligonucleotides will hybridize in a pair-wise manner. The addition of a DNA polymerase leads to a proximity-dependent DNA polymerization event, generating a unique PCR target sequence. The resulting DNA sequence is subsequently detected and quantified using a microfluidic real-time PCR instrument (Signature Q100, LC-Bio Technology Co., Ltd., Hangzhou, China). The resulting Ct-data is then quality-controlled and normalized using a set of internal and external controls. The final assay read-out is presented in Normalized Protein expression (NPX) values, which is an arbitrary unit on a log2-scale where a high value corresponds to a higher protein expression.

Statistical analysis

The normality was performed using the Kolmogorov–Smirnov test. If the data did not distribute normally, the difference was determined by the Wilcoxon matched-pairs *t*-test of the results before and after the treatment. All values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism v.8.0 Software (GraphPad Inc., San Diego, CA, United States), and the $p < 0.05$ was defined as statistical significance.

Results

Effects of GUANKE probiotic on rhinitis symptoms

Compared with the baseline (Week 0), both the TNSS (Figure 1A) and TNNSS (Figure 1B) were significantly reduced after 4-week probiotic treatment ($p < 0.001$ for both). Further, the total score of RCAT (Figure 1C) was markedly increased after taking 4-week probiotic ($p < 0.01$), indicating the rhinitis symptoms were well controlled by GUANKE probiotic.

Changes of the symptoms in TNSS from baseline

To further characterize the individual symptom alterations in each questionnaire, we first compared the individual symptoms in

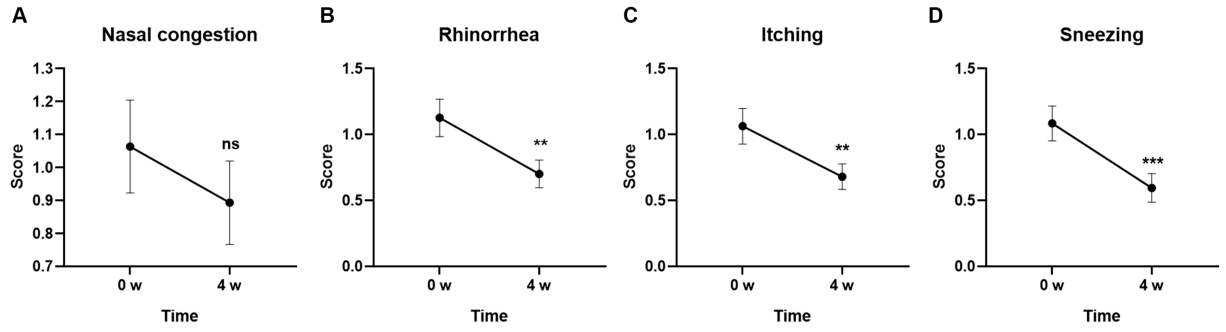

FIGURE 2
Effect of GUANKE probiotic on AR related sub-symptoms in TNSS questionnaire. **(A)** Nasal congestion, **(B)** rhinorrhea, **(C)** itching, **(D)** sneezing. The score of each sub-symptom was presented. The significance was evaluated by performing paired *t*-test. *Indicates a statistically significant difference between the different time points. $^{**}p < 0.01$, $^{***}p < 0.001$, ns indicates no statistical significance.

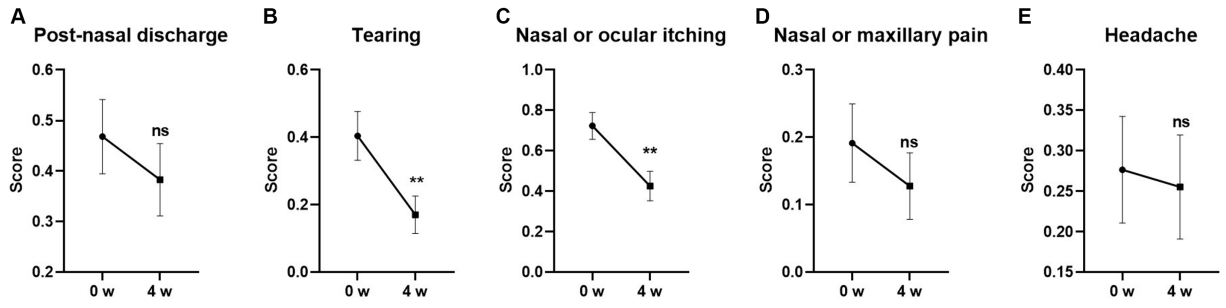

FIGURE 3
Effect of GUANKE probiotic on AR related sub-symptoms in TNNSS questionnaire. **(A)** Post-nasal discharge, **(B)** tearing, **(C)** nasal or ocular itching, **(D)** nasal or maxillary pain, **(E)** headache. The score of each sub-symptom was presented. The significance was evaluated by performing paired *t*-test. *Indicates a statistically significant difference between the different time points. $^{**}p < 0.01$, ns indicates no statistical significance.

TNSS. As shown in Figure 2, three of four symptoms including Rhinorrhea ($p < 0.01$), Itching ($p < 0.01$), and Sneezing ($p < 0.001$) were significantly changed after 4-week GUANKE probiotic treatment. Although the Nasal congestion score did not obtain significance (Figure 2A), it also showed a decrease trend at Week 4.

Changes of the symptoms in TNNSS from baseline

Among the five individual symptoms in TNNSS, the scores for Tearing and Nasal or Ocular itching were significantly decreased from baseline after 4-week GUANKE probiotic treatment (Figures 3B,C) ($p < 0.01$ for each). The other three symptoms including Post-nasal discharge, Nasal or maxillary pain, and Headache also showed a decreased trend compared to the baseline, however, no statistical significance was detected (Figures 3A,D,E) ($p > 0.05$ for each).

Changes of the symptoms in RCAT from baseline

Among the individual symptoms in RCAT, the score for Degree of rhinitis control was the most obvious improvement at Week 4. GUANKE probiotic treatment significantly controlled rhinitis symptoms compared to the baseline (Figure 4F) ($p < 0.001$). Nasal congestion was also significantly improved after 4-week treatment (Figure 4A) ($p < 0.05$). The other scores including Sneezing, Watery eyes, Interference of sleep, and Daily activities tended to be different between the week 0 and after 4-week of treatment but did not reach statistical significance (Figures 4B–E) ($p > 0.05$ for each).

Effects of GUANKE probiotic on serum IgE concentration

To further investigate the effects of GUANKE probiotic on allergic rhinitis, we determined the IgE concentration in serum. As shown in Figure 5, the level of IgE was significantly decreased after 4-week GUANKE treatment ($p < 0.01$), indicating the improvement of GUANKE on AR symptoms was indeed mediated by IgE.

Effects of GUANKE probiotic on serum cytokines level

Through performing inflammatory-related proteins determination by Olink, we found that a total of 20 proteins were significantly changed after 4-week GUANKE probiotic treatment

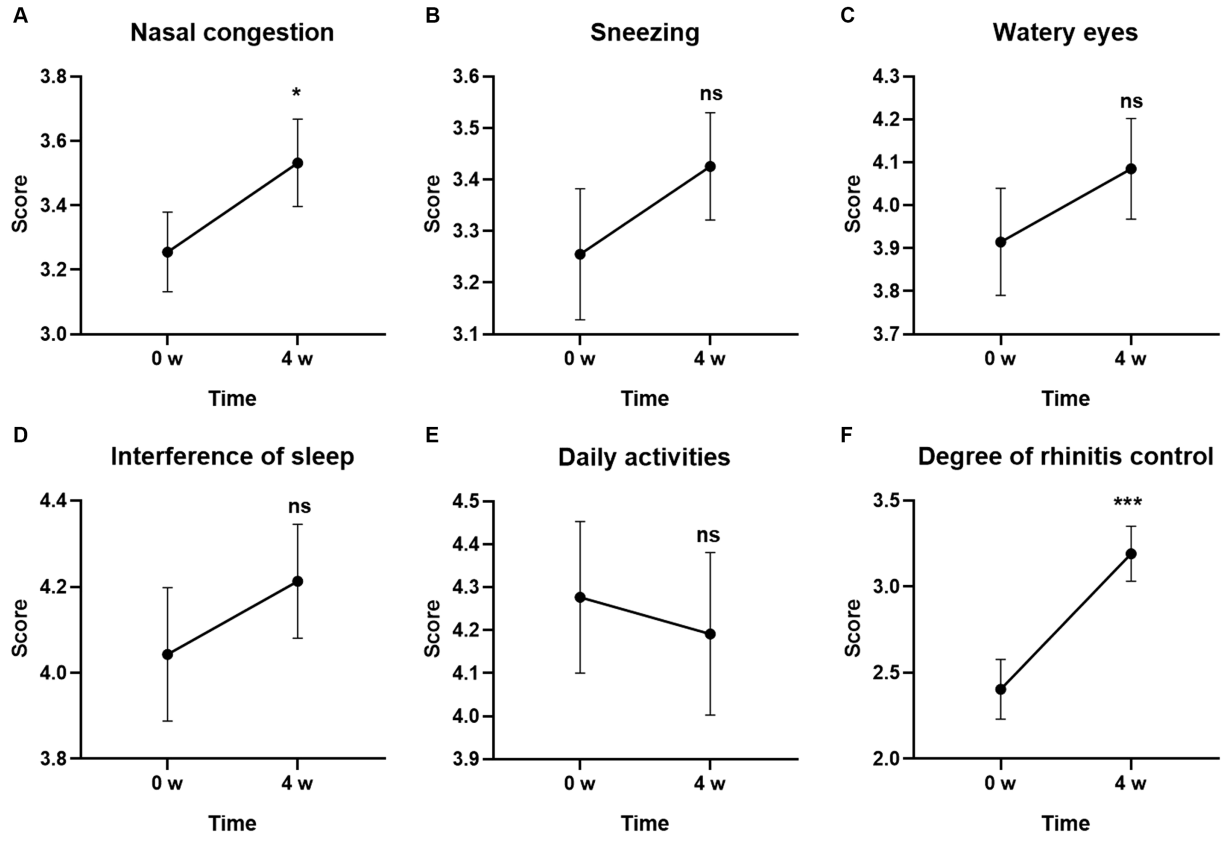

FIGURE 4
Effect of GUANKE probiotic on AR related sub-symptoms in RCAT questionnaire. **(A)** Nasal congestion, **(B)** sneezing, **(C)** watery eyes, **(D)** interference of sleep, **(E)** daily activities, **(F)** degree of rhinitis control. The score of each sub-symptom was presented. The significance was evaluated by performing paired *t*-test. *Indicates a statistically significant difference between the different time points. $*p < 0.05$, $***p < 0.001$, ns indicates no statistical significance.

(Supplementary Figure S1), which include six significantly upregulated proteins: NT-3, CXCL11, MMP-10, IL-4, CCL23, and AXIN1, and 14 significantly downregulated proteins: CXCL5, IL-7, CXCL1, CXCL6, OSM, CCL4, MMP-1, SCF, CD6, NRTN, LAP-TGF-beta-1, IL-33, TGF-alpha, and IL-20 (Figure 6A; $p < 0.05$). We further performed GO enrichment analysis (Figure 6B), which showed that most of these markedly changed proteins were enriched in extracellular region and space. Functionally, these proteins mainly enriched in growth factor activity, cytokine activity, and chemokine activity, as well as chemokine and cytokine-mediated signaling pathways. Further, KEGG enrichment analysis also indicated these proteins were enriched in cytokine and chemokine-related signaling pathways and function (Figure 6C). Taken together, this suggests that GUANKE probiotic mainly improves symptoms of AR through mediating cytokines and chemokines to maintain Th1/Th2 balance.

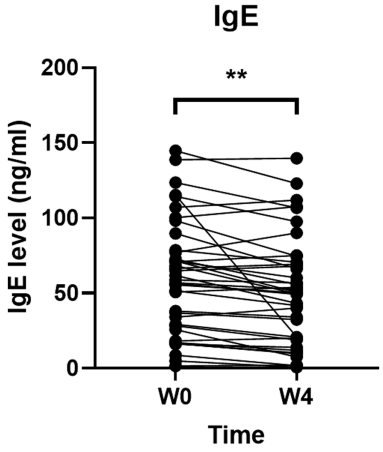

FIGURE 5
Effect of GUANKE probiotic on IgE level before (Week 0, baseline) and after (4 weeks) treatment. The significance was evaluated by performing paired *t*-test. *Indicates a statistically significant difference between the different time points. $**p < 0.01$.

Discussion

In the present study, we demonstrated the efficacy of GUANKE probiotic on the improvement of allergic rhinitis. Our results clearly indicated that GUANKE probiotic can significantly improve the symptoms of allergic rhinitis, such as rhinorrhea, itching, sneezing, and tearing, which are mainly modulated by IgE and inflammatory cytokines and chemokines.

Recently, a number of studies have reported that probiotics can serve as an alternative method in treating AR (16, 17). Both human and animal studies have demonstrated various probiotics could

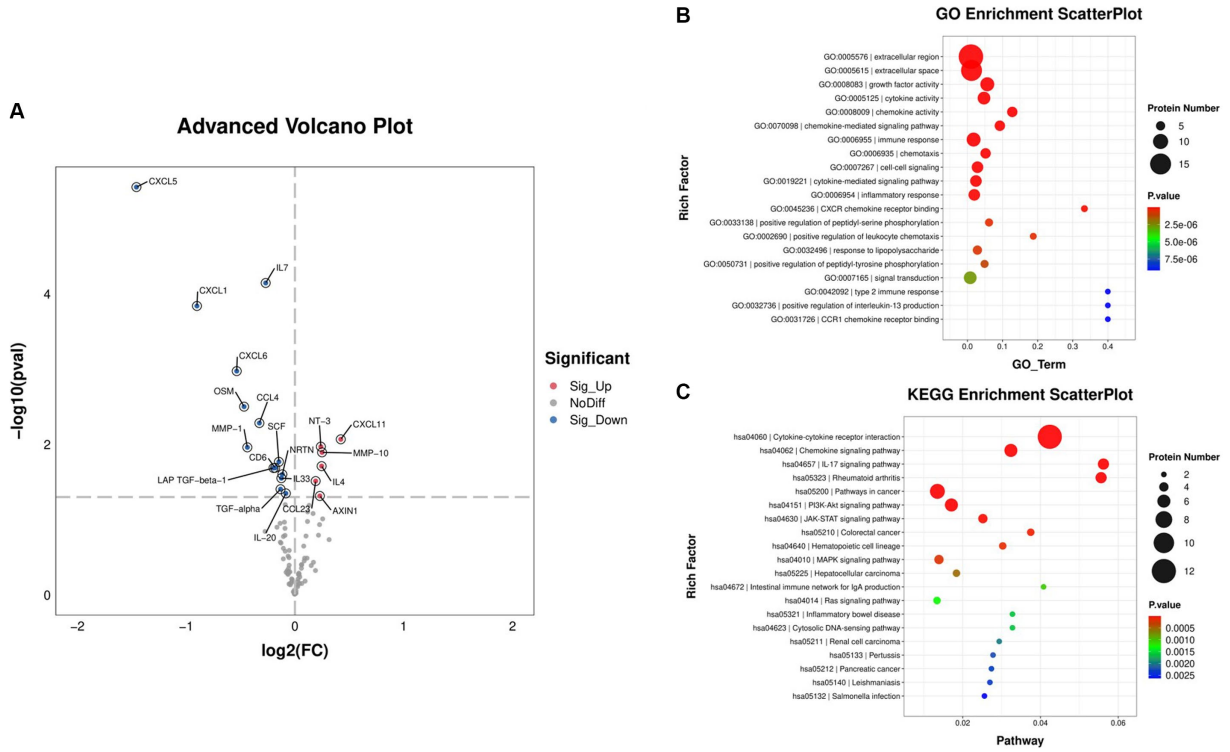

FIGURE 6
Differentially expressed cytokines before (week 0, baseline) and after 4 weeks of GUANKE treatment. **(A)** Volcano plot of the distribution of differentially expressed proteins. Red dots: significantly upregulated proteins, blue dots: significantly downregulated proteins, gray dots: non-significantly expressed proteins. The threshold is $p < 0.05$, and fold change >2. Scatter plot of **(B)** GO and **(C)** KEGG enrichment analysis for the above differentially expressed proteins. The color indicates the different p-value. The dot size represents the number of proteins.

alleviate AR symptoms and quality of life (10). Anania et al. (18) found that a mixture of *Bifidobacterium animalis* subsp. *Lactis* BB12 and *Enterococcus faecium* L3 probiotics could reduce symptoms of AR in children. Kang et al. (19) reported that 4 weeks treatment with probiotic NVP-1703 (a mixture of *Bifidobacterium longum* and *Lactobacillus plantarum*) alleviated perennial AR mediated by IgE and IL-10. Torre et al. (20) used iPROB® probiotic preparation (Anallergo SpA, Florence, Italy) to treat AR patients for 60 days and found a significant decrease in Average Rhinitis Total Symptom Score and improvement in quality of life. In animal studies, Choi et al. (21) found that oral administration of *Lactobacillus plantarum* (CJLP133 and CJLP243) improved the symptoms and reduced the inflammation in a birch pollen-induced AR mouse model. Similarly, oral administration of another *Lactiplantibacillus plantarum* NR16 reduced airway hyperresponsiveness and leukocyte infiltration in lesions of birch pollen-induced AR mice (22). Lin et al. (23) applied *Lacticaseibacillus paracasei* GM-080 in both ovalbumin (OVA)-induced AR mouse model and perennial AR children, and the results indicated that it significantly ameliorates allergic airway inflammation. Here, we are the first to apply *Lactobacillus plantarum* GUANKE probiotic in AR. In line with the above studies, we found that 4 weeks of GUANKE probiotic treatment can also effectively control the AR symptoms such as rhinorrhea, itching, sneezing, and tearing, demonstrating its efficacy in the improvement of AR.

Allergic rhinitis triggers a systemic increase of inflammation with various cytokines release (24). Recently, a large number of inflammatory cytokines have been found significantly changed in AR, although the molecules regulated by different probiotics appeared to vary (25). In the present results, GUANKE probiotic mainly regulated the levels of inflammatory cytokines, such as IL-4, IL-7, IL-20, and IL-33; chemokines, such as CXCL1, CXCL5, CXCL6, CXCL11, CCL4, and CCL23; as well as other cytokines, such as TGF-alpha, LAP-TGF-beta-1, MMP-1, MMP-10, AXIN1, NT-3, OSM, SCF, CD6, and NRTN. These significantly altered molecules were mainly enriched in cytokine and chemokine-related signaling pathways and corresponding functions.

The inflammatory cytokines are the main regulated molecules involved in AR. IL-4 plays a key role in inducing IgE production and acts as a therapeutic target in AR (26). In this study, we found that IL-4 level was significantly increased after GUANKE probiotic treatment. Although it was in contrast with the previous reports as a Th2 cytokine, here, we speculated it acted as an anti-inflammatory cytokine (27). IL-10 is another well-known anti-inflammatory cytokine, which also showed an increased trend after treatment although it did not obtain statistical significance, suggesting that GUANKE probiotic enhanced anti-inflammatory ability. IL-7 is of great significance in regulating the immune function of the body, which has been demonstrated to be necessary for the generation and maintenance of T and B cells, and lack of IL-7 would cause immature immune cell arrest (28, 29). IL-20 is a pro-inflammatory mediator, which can regulate cytokine and chemokine expression in different types of cells (30). Recent studies have shown that IL-20 plays an important role in the pathogenesis of bronchial asthma (31). It participates in T cell-mediated disease development by regulating cytokines secreted by T cells. Long-term exposure of T cells to IL-20

can cause an increase in initial T cell polarization toward Th2 cells, resulting in increased secretion of IL-4 and IL-13, and reduced secretion of IFN-γ (32) and participating in the occurrence of AR by affecting the Th1/Th2 balance. IL-33 plays a pro-inflammatory role in allergic diseases and is recognized as an important contributor to Th2-type immune responses (33). The elevated level of IL-33 in serum correlated significantly with the severe symptoms of AR patients (34), and immunotherapy can decrease IL-33 levels and improve AR symptoms (35). Here, we also found the dysregulation of these inflammatory cytokines after GUANKE probiotic administration for 4 weeks, suggesting GUANKE probiotic in AR treatment mainly functioned as an immunomodulator.

Except for inflammatory cytokines, the chemokines and chemokine receptors also play critical roles in AR pathogenesis. They participate in all of the three phases of AR by promoting inflammatory cell recruitment, differentiation, and allergic mediator release, and also be therapeutic targets for AR (36). The origin of chemokines and the inflammatory cells are different, but CCL and CXCL are two types of chemokines that are more closely related to the development of AR (37). Here, we found that 6 chemokines were significantly changed after GUANKE probiotic treatment. CXCL1, CXCL5, and CXCL6 were markedly decreased, which receptor is CXCR2, expressed in neutrophils, monocytes, NK cells, mast cells, and basophils, and the main function is related to B cell lymphopoiesis and neutrophil trafficking. While CXCL11 was significantly increased, which receptor is CXCR3, expressed in Th1 cells, CD8+ T cells, and NK cells, and the main function is related to Type I adaptive immunity (37). Previous studies also showed that CCL4 and CCL23 were also significantly changed in AR patients (38, 39). Taken together, these data demonstrated the importance of chemokines in AR.

In recent years, the regulatory role of probiotics in allergic rhinitis has expanded beyond the Th1/Th2 balance to other T cell subsets, such as regulatory T (Treg) cells. Foxp3 is a key regulatory factor for the normal development and function of Treg cells (40). *Lactobacillus rhamnosus* has been shown to maintain the Treg/Th2 balance by increasing the levels of Foxp3 + Treg cells (41). Additionally, spore-forming *Bacillus* species can induce Foxp3 + Treg cells to suppress inflammatory reactions (42). IL-10 is also a crucial anti-inflammatory factor, and probiotics can induce IL-10 production, promoting the differentiation of Treg cells (43–45). Furthermore, short-chain fatty acids (SCFAs), as major metabolites of intestinal microbiota fermentation, have been reported to regulate immune responses in multiple organs and maintain intestinal homeostasis (46). Gu et al. found a significant upregulation of SCFAs levels and a reduction in inflammation in a mouse model following gavage with *Lactobacillus plantarum* ZJ316 (47). Studies have shown that an increased abundance of microbiota-producing SCFAs in the gut can enhance the generation of Treg cells (48). Further research is needed to investigate whether the GUANKE strain affects allergic rhinitis symptoms through the regulation of these pathways.

Finally, the potential limitations of this study we want to address. First, the sample size was relatively small. Despite of this, we still observed significant changes of symptoms and cytokines before and after GUANKE probiotic treatment in AR patients. Second, we only compared two time points that were before and after 4-week GUANKE probiotic administration. It would be better to compare more time points such as shorter or longer treatment, which could characterize the process of dynamic changes after GUANKE treatment. Third, the randomized, double-blind, placebo-controlled study is better to demonstrate the efficacy of GUANKE probiotic in AR treatment, and this study is currently ongoing.

In conclusion, we provide a new probiotic *Lactobacillus plantarum* GUANKE for the improvement of AR. Our findings revealed that the GUANKE can effectively alleviate not only the symptoms of AR but also maintain the Th1/Th2 balance along with IgE, cytokines, and chemokines alterations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Institutional Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

HH: Project administration, Writing – review & editing, Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. GC: Data curation, Investigation, Methodology, Writing – original draft. BZ: Writing – review & editing. XZ: Data curation, Investigation, Writing – original draft. JH: Writing – original draft, Data curation, Investigation, Writing – review & editing. WD: Data curation, Investigation, Writing – review & editing. MDL: Funding acquisition, Project administration, Supervision, Writing – review & editing, Conceptualization, Investigation, Resources.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1291100/full#supplementary-material

References

1. Bousquet J, Anto JM, Bachert C, Baiardini I, Bosnic-Anticevich S, Walter Canonica G, et al. Allergic rhinitis. *Nat Rev Dis Primers*. (2020) 6:95. doi: 10.1038/s41572-020-00227-0

2. Meltzer EO. Allergic rhinitis: burden of illness, quality of life, comorbidities, and control. *Immunol Allergy Clin N Am*. (2016) 36:235–48. doi: 10.1016/j.iac.2015.12.002

3. Zhang Y, Zhang L. Increasing prevalence of allergic rhinitis in China. *Allergy Asthma Immunol Res*. (2019) 11:156–69. doi: 10.4168/aair.2019.11.2.156

4. Meng Y, Wang C, Zhang L. Recent developments and highlights in allergic rhinitis. *Allergy*. (2019) 74:2320–8. doi: 10.1111/all.14067

5. Wise SK, Damask C, Roland LT, Ebert C, Levy JM, Lin S, et al. International consensus statement on allergy and rhinology: allergic rhinitis - 2023. *Int Forum Allergy Rhinol*. (2023) 13:293–859. doi: 10.1002/alr.23090

6. Bernstein DI, Schwartz G, Bernstein JA. Allergic rhinitis: mechanisms and treatment. *Immunol Allergy Clin N Am*. (2016) 36:261–78. doi: 10.1016/j.iac.2015.12.004

7. Zhang Y, Lan F, Zhang L. Update on pathomechanisms and treatments in allergic rhinitis. *Allergy*. (2022) 77:3309–19. doi: 10.1111/all.15454

8. Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. *Lancet*. (2011) 378:2112–22. doi: 10.1016/S0140-6736(11)60130-X

9. Guvenc IA, Muluk NB, Mutlu FS, Eski E, Altintoprak N, Oktemer T, et al. Do probiotics have a role in the treatment of allergic rhinitis? A comprehensive systematic review and meta-analysis. *Am J Rhinol Allergy*. (2016) 30:157–75. doi: 10.2500/ajra.2016.30.4354

10. Huang J, Zhang J, Wang X, Jin Z, Zhang P, Su H, et al. Effect of probiotics on respiratory tract allergic disease and gut microbiota. *Front Nutr*. (2022) 9:821900. doi: 10.3389/fnut.2022.821900

11. Zajac AE, Adams AS, Turner JH. A systematic review and meta-analysis of probiotics for the treatment of allergic rhinitis. *Int Forum Allergy Rhinol*. (2015) 5:524–32. doi: 10.1002/alr.21492

12. Du T, Lei A, Zhang N, Zhu C. The beneficial role of probiotic Lactobacillus in respiratory diseases. *Front Immunol*. (2022) 13:908010. doi: 10.3389/fimmu.2022.908010

13. Xu J, Ren Z, Cao K, Li X, Yang J, Luo X, et al. Boosting vaccine-elicited respiratory mucosal and systemic COVID-19 immunity in mice with the oral *Lactobacillus plantarum*. *Front Nutr*. (2021) 8:789242. doi: 10.3389/fnut.2021.789242

14. Pfaar O, Demoly P, Gerth van Wijk R, Bonini S, Bousquet J, Canonica GW, et al. Recommendations for the standardization of clinical outcomes used in allergen immunotherapy trials for allergic rhinoconjunctivitis: an EAACI position paper. *Allergy*. (2014) 69:854–67. doi: 10.1111/all.12383

15. Meltzer EO, Schatz M, Nathan R, Garris C, Stanford RH, Kosinski M. Reliability, validity, and responsiveness of the rhinitis control assessment test in patients with rhinitis. *J Allergy Clin Immunol*. (2013) 131:379–86. doi: 10.1016/j.jaci.2012.10.022

16. Liu P, Hu T, Kang C, Liu J, Zhang J, Ran H, et al. Research advances in the treatment of allergic rhinitis by probiotics. *J Asthma Allergy*. (2022) 15:1413–28. doi: 10.2147/JAA.S382978

17. Das RR, Singh M, Shafiq N. Probiotics in treatment of allergic rhinitis. *World Allergy Organ J*. (2010) 3:239–44. doi: 10.1097/WOX.0b013e3181f234d4

18. Anania C, Di Marino VP, Olivero F, De Canditiis D, Brindisi G, Iannilli F, et al. Treatment with a probiotic mixture containing *Bifidobacterium animalis* Subsp. Lactis BB12 and *Enterococcus faecium* L3 for the prevention of allergic rhinitis symptoms in children: a randomized controlled trial. *Nutrients*. (2021) 13:1315. doi: 10.3390/nu13041315

19. Kang MG, Han SW, Kang HR, Hong SJ, Kim DH, Choi JH. Probiotic NVP-1703 alleviates allergic rhinitis by inducing IL-10 expression: a four-week clinical trial. *Nutrients*. (2020) 12:1427. doi: 10.3390/nu12051427

20. Torre E, Sola D, Caramaschi A, Mignone F, Bona E, Fallarini S. A pilot study on clinical scores, immune cell modulation, and microbiota composition in allergic patients with rhinitis and asthma treated with a probiotic preparation. *Int Arch Allergy Immunol*. (2022) 183:186–200. doi: 10.1159/000518952

21. Choi SP, Oh HN, Choi CY, Ahn H, Yun HS, Chung YM, et al. Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 alleviates birch pollen-induced allergic rhinitis in mice. *J Appl Microbiol*. (2018) 124:821–8. doi: 10.1111/jam.13635

22. Yang J, Bae J, Choi CY, Choi SP, Yun HS, Chun T. Oral administration of Lactiplantibacillus plantarum NR16 isolated from kimchi ameliorates murine allergic rhinitis. *Lett Appl Microbiol*. (2022) 75:152–60. doi: 10.1111/lam.13716

23. Lin EK, Chang WW, Jhong JH, Tsai WH, Chou CH, Wang IJ. Lacticaseibacillus paracasei GM-080 ameliorates allergic airway inflammation in children with allergic rhinitis: from an animal model to a double-blind, randomized, placebo-controlled trial. *Cells*. (2023) 12:768. doi: 10.3390/cells12050768

24. Borish L. Allergic rhinitis: systemic inflammation and implications for management. *J Allergy Clin Immunol*. (2003) 112:1021–31. doi: 10.1016/j.jaci.2003.09.015

25. Steiner NC, Lorentz A. Probiotic potential of Lactobacillus species in allergic rhinitis. *Int Arch Allergy Immunol*. (2021) 182:807–18. doi: 10.1159/000515352

26. Nur Husna SM, Md Shukri N, Mohd Ashari NS, Wong KK. IL-4/IL-13 axis as therapeutic targets in allergic rhinitis and asthma. *PeerJ*. (2022) 10:e13444. doi: 10.7717/peerj.13444

27. Opal SM, DePalo VA. Anti-inflammatory cytokines. *Chest*. (2000) 117:1162–72. doi: 10.1378/chest.117.4.1162

28. Barata JT, Durum SK, Seddon B. Flip the coin: IL-7 and IL-7R in health and disease. *Nat Immunol*. (2019) 20:1584–93. doi: 10.1038/s41590-019-0479-x

29. Lin J, Zhu Z, Xiao H, Wakefield MR, Ding VA, Bai Q, et al. The role of IL-7 in immunity and cancer. *Anticancer Res*. (2017) 37:963–7. doi: 10.21873/anticanres.11405

30. Lin TY, Hsu YH. IL-20 in acute kidney injury: role in pathogenesis and potential as a therapeutic target. *Int J Mol Sci*. (2020) 21:1009. doi: 10.3390/ijms21031009

31. Gong W, Wang X, Zhang Y, Hao J, Xing C, Chu Q, et al. Interleukin-20 promotes airway remodeling in asthma. *Inflammation*. (2014) 37:2099–105. doi: 10.1007/s10753-014-9944-8

32. Oral HB, Kotenko SV, Yilmaz M, Mani O, Zumkehr J, Blaser K, et al. Regulation of T cells and cytokines by the interleukin-10 (IL-10)-family cytokines IL-19, IL-20, IL-22, IL-24 and IL-26. *Eur J Immunol*. (2006) 36:380–8. doi: 10.1002/eji.200425523

33. Borgia F, Custurone P, Li Pomi F, Vaccaro M, Alessandrello C, Gangemi S. IL-33 and IL-37: a possible Axis in skin and allergic diseases. *Int J Mol Sci*. (2022) 24:372. doi: 10.3390/ijms24010372

34. Asaka D, Yoshikawa M, Nakayama T, Yoshimura T, Moriyama H, Otori N. Elevated levels of interleukin-33 in the nasal secretions of patients with allergic rhinitis. *Int Arch Allergy Immunol*. (2012) 158:47–50. doi: 10.1159/000337764

35. Cayrol C, Girard JP. Interleukin-33 (IL-33): a nuclear cytokine from the IL-1 family. *Immunol Rev*. (2018) 281:154–68. doi: 10.1111/imr.12619

36. Li Z, Yu S, Jiang Y, Fu Y. Chemokines and chemokine receptors in allergic rhinitis: from mediators to potential therapeutic targets. *Eur Arch Otorhinolaryngol*. (2022) 279:5089–95. doi: 10.1007/s00405-022-07485-6

37. Bao Y, Zhu X. Role of chemokines and inflammatory cells in respiratory allergy. *J Asthma Allergy*. (2022) 15:1805–22. doi: 10.2147/JAA.S395490

38. Barrenas F, Andersson B, Cardell LO, Langston M, Mobini R, Perkins A, et al. Gender differences in inflammatory proteins and pathways in seasonal allergic rhinitis. *Cytokine*. (2008) 42:325–9. doi: 10.1016/j.cyto.2008.03.004

39. Zissler UM, Jakwerth CA, Guerth F, Lewitan L, Rothkirch S, Davidovic M, et al. Allergen-specific immunotherapy induces the suppressive secretoglobin 1A1 in cells of the lower airways. *Allergy*. (2021) 76:2461–74. doi: 10.1111/all.14756

40. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. *Annu Rev Immunol*. (2012) 30:39–68. doi: 10.1146/annurev-immunol-020711-075024

41. Jia L, Wu R, Han N, Fu J, Luo Z, Guo L, et al. Porphyromonas gingivalis and *Lactobacillus rhamnosus* GG regulate the Th17/Treg balance in colitis via TLR4 and TLR2. *Clin Transl Immunol*. (2020) 9:e1213. doi: 10.1002/cti2.1213

42. Fu L, Peng J, Zhao S, Zhang Y, Su X, Wang Y. Lactic acid bacteria-specific induction of CD4(+)Foxp3(+)T cells ameliorates shrimp tropomyosin-induced allergic response in mice via suppression of mTOR signaling. *Sci Rep*. (2017) 7:1987. doi: 10.1038/s41598-017-02260-8

43. Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, et al. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci U S A*. (2010) 107:2159–64. doi: 10.1073/pnas.0904055107

44. Ludwig IS, Broere F, Manurung S, Lambers TT, van der Zee R, van Eden W. *Lactobacillus rhamnosus* GG-derived soluble mediators modulate adaptive immune cells. *Front Immunol*. (2018) 9:1546. doi: 10.3389/fimmu.2018.01546

45. Ghavami SB, Yadegar A, Aghdaei HA, Sorrentino D, Farmani M, Mir AS, et al. Immunomodulation and generation of Tolerogenic dendritic cells by probiotic Bacteria in patients with inflammatory bowel disease. *Int J Mol Sci*. (2020) 21:6266. doi: 10.3390/ijms21176266

46. Wang A, Li Z, Sun Z, Zhang D, Ma X. Gut-derived short-chain fatty acids bridge cardiac and systemic metabolism and immunity in heart failure. *J Nutr Biochem*. (2023) 120:109370. doi: 10.1016/j.jnutbio.2023.109370

47. Gu Q, Xia C, Liu N, Chen Z, Zhou Q, Li P. *Lactobacillus plantarum* ZJ316 alleviates ulcerative colitis by inhibiting inflammation and regulating short-chain fatty acid levels and the gut microbiota in a mouse model. *Food Funct*. (2023) 14:3982–93. doi: 10.1039/D2FO02567A

48. Zhang W, Cheng C, Han Q, Chen Y, Guo J, Wu Q, et al. Flos *Abelmoschus manihot* extract attenuates DSS-induced colitis by regulating gut microbiota and Th17/Treg balance. *Biomed Pharmacother*. (2019) 117:109162. doi: 10.1016/j.biopha.2019.109162

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Sustained silencing peanut allergy by xanthopurpurin is associated with suppression of peripheral and bone marrow IgE-producing B cell

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Introduction: Peanut allergy is an immunoglobulin E (IgE) mediated food allergy. *Rubia cordifolia* L. (*R. cordifolia*), a Chinese herbal medicine, protects against peanut-induced anaphylaxis by suppressing IgE production *in vivo*. This study aims to identify IgE-inhibitory compounds from the water extract of *R. cordifolia* and investigate the underlying mechanisms using *in vitro* and *in vivo* models.

Methods: Compounds were isolated from *R. cordifolia* water extract and their bioactivity on IgE production was assessed using a human myeloma U266 cell line. The purified active compound, xanthopurpurin (XPP), was identified by LC-MS and NMR. Peanut-allergic C3H/HeJ mice were orally administered with or without XPP at 200μg or 400μg per mouse per day for 4 weeks. Serum peanut-specific IgE levels, symptom scores, body temperatures, and plasma histamine levels were measured at challenge. Cytokines in splenocyte cultures were determined by ELISA, and IgE $^{+}$ B cells were analyzed by flow cytometry. Acute and sub-chronic toxicity were evaluated. IL-4 promoter DNA methylation, RNA-Seq, and qPCR analysis were performed to determine the regulatory mechanisms of XPP.

Results: XPP significantly and dose-dependently suppressed the IgE production in U266 cells. XPP significantly reduced peanut-specific IgE (>80%, $p < 0.01$), and plasma histamine levels and protected the mice against peanut-allergic reactions in both early and late treatment experiments ($p < 0.05$, n=9). XPP showed a strong protective effect even 5 weeks after discontinuing the treatment. XPP significantly reduced the IL-4 level without affecting IgG or IgA and IFN-γ production. Flow cytometry data showed that XPP reduced peripheral and bone marrow IgE $^{+}$ B cells compared to the untreated group. XPP increased

IL-4 promoter methylation. RNA-Seq and RT-PCR experiments revealed that XPP regulated the gene expression of CCND1, DUSP4, SDC1, ETS1, PTPRC, and IL6R, which are related to plasma cell IgE production. All safety testing results were in the normal range.

Conclusions: XPP successfully protected peanut-allergic mice against peanut anaphylaxis by suppressing IgE production. XPP suppresses murine IgE-producing B cell numbers and inhibits IgE production and associated genes in human plasma cells. XPP may be a potential therapy for IgE-mediated food allergy.

KEYWORDS

Rubia cordifolia L., food allergy, IgE, transcriptome, RNA-Seq

1 Introduction

Food allergy (FA) is an adverse immune response to food allergens. It can be life-threatening and the prevalence of FA has significantly increased over the past decades, affecting about 32 million people in the United States, of which 5.6 million are children under age 18 (1–9). Treatment options are extremely limited. Food avoidance and rescue medication after accidental exposure are the first lines of FA management. Peanut allergy (PNA) causes severe, and sometimes fatal reactions, often co-existing with other food allergies (1, 10–15). Food allergy is also increasing in other westernized and developing countries (2, 16). It negatively influences the quality of life for patients and their families. Unfortunately, there are currently limited treatment options for food allergies. Strict avoidance of allergens remains the standard treatment for the majority of food allergies, except for peanut (PN). Since 2020, an FDA approved drug (Palforzia) is available for peanut desensitization in children 4-17 years old (17). However, it is not approved for other age groups due to lack of efficacy, and requires a prolonged course of treatment with indefinite daily dosing (18, 19). Palforzia is associated with a high rate of adverse effects and relapse after treatment cessation (20). Moreover, real-world acceptance of Palforzia is reported to be low (21). Therefore, the need to develop treatments, particularly for milk and peanut (PN) allergies, is urgent and challenging.

Food allergies typically are IgE-mediated. IgE molecules binding to mast cells and basophils sensitize these cells. When IgE specifically binds to the epitopes of the food allergen, it activates mast cells and basophils to release histamine and many other mediators (22). These, in turn, elicit inflammation and immediate hypersensitivity (22). Since IgE plays a pivotal role in food allergies, the concept of decreasing IgE levels has been investigated as a therapeutic strategy for food allergy treatment. In 2003, the anti-IgE antibody, TNX901, was tested in a double-blind, randomized trial. The results showed that TNX-901 significantly increased the threshold of the sensitivity of patients to peanut (23, 24). In another study, omalizumab (Xolair), was also found to greatly reduce anaphylactic reactions during the oral immunotherapy (OIT) (25). These studies suggested potential for developing novel therapies to regulate IgE. However, the current form of anti-IgE therapy has limitations. It demands continuing bi-weekly or monthly injections to maintain effects, as this treatment neutralizes secreted IgE, but does not stop B-cell IgE production (26, 27). In addition, biologic therapies are very expensive. An alternative and cost-effective approach to inhibiting IgE production by B cells is highly desirable.

Previously, Lopez et al. screened 70 herbal medicines from traditional Chinese medicine (TCM) and found that *Rubia cordifolia* L. (*R. cordifolia*) inhibited IgE production in a dose-dependent manner *in vitro* (28). *R. cordifolia* also suppressed the PN-specific IgE levels and protected PN-allergic mice against anaphylactic reactions (28). In this study, we aimed to test the effect of a pure compound, xanthopurpurin (XPP), isolated from *R. cordifolia*, in a PN-allergic murine model and investigate underlying mechanisms. We, for the first time, demonstrate that XPP markedly reduced peanut-specific IgE, protected peanut-allergic mice from anaphylaxis, and reduced plasma histamine levels. Splenocytes and bone marrow IgE-producing B cells were significantly reduced, which was associated with reduction of IL-4 and epigenetic modulation of IL-4 gene promoter. RNA-Seq analysis of plasma cells revealed that XXP has the capacity to regulate genes associated with antibody production.

2 Materials and methods

2.1 General materials

Water extract of *R. cordifolia* was purchased from E-Fong Herbs Inc. (South EI Monte, CA). Methanol, acetonitrile, and other

chemicals were purchased from Fisher Scientifics (Pittsburgh, PA). Freshly ground, whole roasted PN (White Rose brand, NJ) were used to prepare the crude PN extract (CPE) as previously described (29, 30). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, CA). The human myeloma cell line, U266 B cells, was purchased from American Type Culture Collection (Manassas, Virginia).

2.2 Isolation of active compounds from *Rubia cordifolia* L.

In order to isolate individual components from *R. cordifolia*, liquid-liquid extraction was used with different organic solvents. In brief, 200g of *R. cordifolia* water extract was first dissolved into 4L of DDH_2O and extracted with 4L of dichloromethane for 24 hrs using a liquid-liquid extractor (Sigma-Aldrich, St. Louis, MO) at 100°C with a 2L heating mantle (EM2000/CX1, Barnstead International, Dubuque, IA). The dichloromethane extract of *R. cordifolia* was first dried using a rotary evaporator (Rotavapor R-210, BÜCHI, Switzerland), then redissolved into methanol and loaded onto Sephadex LH20 (Sigma-Aldrich, St. Louis, MO) column for further separation.

2.3 Liquid chromatography-mass spectrometer and NMR

Liquid chromatography was performed using a Waters 2690 HPLC-PDA system (Waters, Milford, MA). *R. cordifolia* water extract (30 mg/mL) was first filtered through a 0.8µm Nalgene™ syringe filter (Thermofisher, Waltham, MA) and separated on a ZORBAX SB-C18 (4.6 × 150mm, 5µm) column (Agilent, Santa Clara, CA) on the HPLC system. The separation was carried out using mobile phase A (0.1% aqueous formic acid) and mobile phase B (acetonitrile with 0.1% formic acid) at a flow rate of 1mL/min. The separation gradient started at 2% of mobile phase B to 25% within 45 min, to 35% within 25 min, to 55% mobile phase B within 15 min, to 75% in 10 min and maintained at 75% mobile phase B for another 5 min. Data was collected and processed using Waters' Empower 2 software. Mass spectra data was collected using Waters Alliance 2695 HPLC system coupled with Waters LCT premier TOF mass spectrometer in both positive mode and negative mode to characterize the molecular weight of unknown constituent. The parameters were set as: Capillary Voltage: 3200 v; Cone Voltage: 15 v; Aperture I: 25 v; Desolvation Temperature: 300 °C; Source Temperature: 110 °C; Desolvation gas: 500 L/h; Nebulizing gas: 40 L/h; Ionization mode: Electrospray; Positive Ion Acquisition Range: m/z 50-1000. The results were collected and analyzed by Empower and Masslynx software. ^{1}H NMR (at 300 MHz) and ^{13}C NMR (at 75 MHz) spectra were obtained on a JOEL instrument using DMSO-d_6 as the solvent.

2.4 Human myeloma cell culture and IgE antibody measurement

The human myeloma cell line (U266), purchased from ATCC (American Type Culture Collection; Rockville, MD), has been used to test different herb extracts and purified compounds for their IgE inhibitory effects (31). 2×10^5 cells/mL of cells were cultured at 37°C under 5% CO_2 in complete media containing RPMI 1640 medium supplemented, 10% FBS, 1 mM sodium pyruvate, 1×10^{-5} M β-ME and 0.5% penicillin-streptomycin. XPP at 0, 2.5, 5, 10, 20 and 40 µg/mL were added at day 0. After 6 days, supernatants were harvested and IgE levels were measured by using an ELISA Kit (Mabtech Inc, OH). Briefly, samples and standards were added into prewashed 96-well plate coated with capture monoclonal antibodies (mAbs). After 2-hour incubation at room temperature, plate was washed 5 times and incubated with detection mAb for 1 hour. The plate was then washed for 5 times and incubated with streptavidin-HRP for 1hour at room temperature. The plate was then washed and developed with TMB substrate for 15 min. The optical density was measured using ELISA reader at 450nm. IgE concentrations were calculated based on the standard curve.

2.5 Animals

Six-week-old female C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained on PN-free chow under specific pathogen-free conditions according to standard guidelines for the care and use of animals (32). The study protocol was approved by institutional animal care and use committee (IACUC) at Icahn Mount Sinai School of Medicine, New York (IACUC #LA11-00082). The institutional Public Health Service (PHS) animal welfare assurance number is D16-00069 (A3111-01).

2.6 Induction of PN allergic murine model and XPP treatment

As described previously (33), mice were systemically sensitized by intraperitoneal (i.p.) injection of 500 µg of crude PN extract with 2 mg of alum in PBS (Model #1). Sensitization was given in three weekly injections at weeks 0, 1 and 2. XPP treatment started at week 3, representing an early treatment protocol as PN allergy is not yet established at this time point. Treatment with XPP (400 µg/day dissolved in drinking water) was given once daily for a period of 8 weeks. Sham mice were orally given water daily. Naïve mice were used as controls which were not sensitized or treated. Post therapy oral challenge was given at week 18 with 200 mg of PN (Figure 1A).

Late treatment with XPP was tested in an oral sensitization model (Model #2). As described previously (34, 35), C3H/HeJ mice were intragastrically (i.g.) sensitized three times at week 0 with 10 mg of

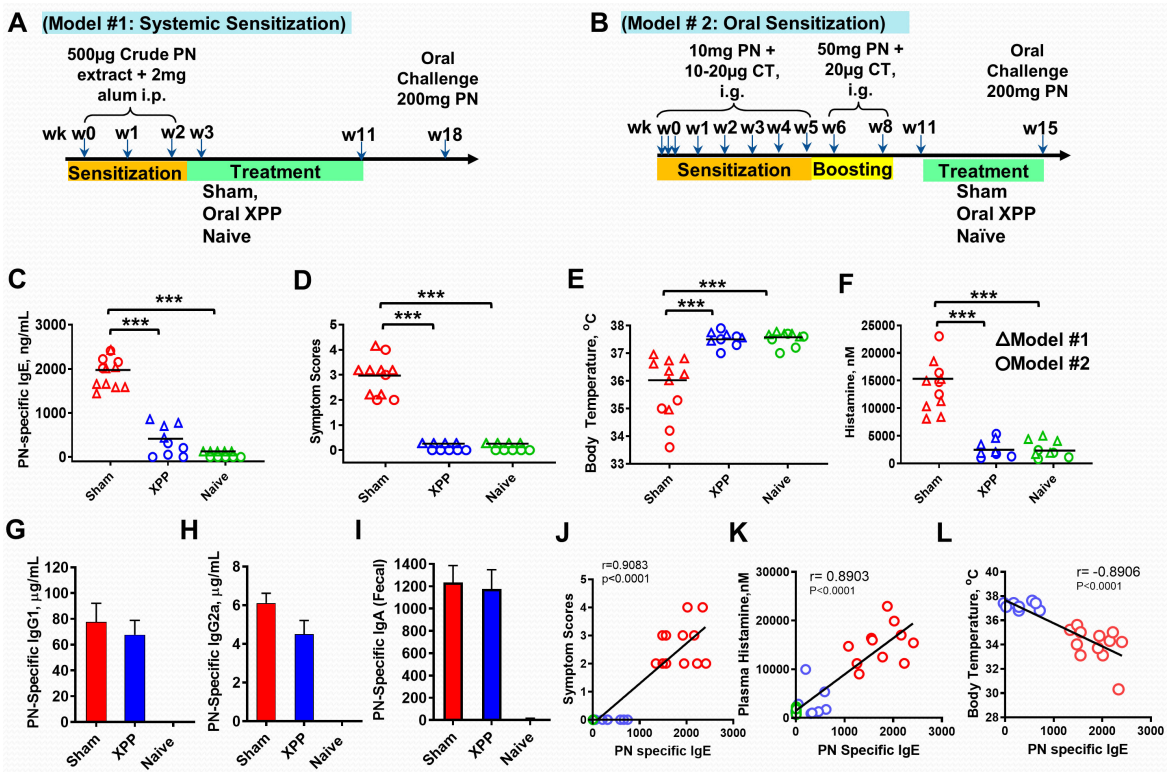

FIGURE 1
XPP prevented peanut-induced anaphylactic reactions in the systemic (triangle symbols) and Oral (circle symbols) sensitization protocols. **(A)** Experimental design for systemic i.p. PN sensitization; **(B)** Experimental design for oral PN sensitization; **(C)** Post-therapy PN-specific IgE in measure by ELISA; **(D)** Symptom Scores; **(E)** Body temperatures measured 30 minutes after PN challenge using a rectal probe; **(F)** Histamine levels in plasma obtained after PN challenge measured by ELISA; **(G)** Serum PN-specific IgG1 (Model #2); **(H)** Serum PN-specific IgG2a (Model #2); **(I)** Fecal PN-specific IgA (Model #2); **(J)** Correlation between PN specific IgE and Symptom score; **(K)** Correlation between Plasma histamine and PN specific IgE; **(L)**. Correlation between Body Temperature and PN specific IgE; Pearson correlation coefficient (r) was calculated. Bars indicate group means. *** = $P < 0.001$ *vs.* Sham. N = 4~5 mice/group from 2 separate experiments.

homogenized PN extract in 0.5 mL PBS containing 75 mg sodium bicarbonate, 10 µg of the mucosal adjuvant cholera toxin (List Laboratories, CA), and 16.5 µL (1.1 µL/g body weight) of 80 proof of Stolichnaya Vodka®. After the first week sensitization, mice were given the sensitization solution weekly as above with 20 µg CT for 5 weeks. 50 mg PN with 20 µg CT was given as the boosting dose at weeks 6 and 8 using the same gavage solution. Naïve mice were not sensitized. Oral XPP treatment (400 µg/day dissolved in drinking water) started at week 11 and continued through week 15. This treatment protocol represents late treatment as PN allergy is established and mice are reactive to challenge at this timepoint as shown by us previously (34). Sham group of mice received water daily.

Oral challenge with 200 mg PN was given at the end of week 15. Mice were fasted for 2 hrs prior to sensitization and challenge (Figure 1B).

2.7 Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30-40 minutes following oral PN challenge as described previously (34, 35). Visually observed symptoms were scored utilizing the scoring system described previously (34): 0 - no symptoms; 1 - scratching and rubbing around the snout and head; 2 - puffiness around the eyes and snout, pilar erection, reduced activity, and/or decreased activity with increased respiratory rate; 3 - wheezing, labored respiration, cyanosis around the mouth and the tail; 4 - no activity after prodding, or tremor and convulsion; 5 - death. Cage identities were concealed during visual assessment of anaphylactic symptoms. Rectal temperatures were measured using a rectal probe (Harvard Apparatus, NJ).

2.8 Measurement of PN specific-IgE, IgG1 and IgG2a

PN specific-IgE in serum was measured as reported previously (34–36). Briefly, 96-well microtiter plates were coated with crude PN extract (sample wells) and rat anti-mouse IgE (reference wells) and held overnight at 4°C. After washing three times, plates were blocked for 2 hours at room temperature with 2% BSA-PBS. After three washes plates were incubated with diluted serum samples and purified mouse IgE antibody (in reference wells) overnight at 4°C. Biotinylated anti-IgE was added to the plates followed with the

addition of avidin-peroxidase and ABTS substrate. For PN specific-IgG1 or IgG2a ELISAs, plates were coated with DNP-HSA in the reference wells and CPE in the sample wells. After the blocking, anti DNP-IgG1 or anti DNP-IgG2a were added to the reference wells, while samples were added into the sample wells. Plates were developed with biotinylated Rat anti-mouse IgG1 or IgG2a (BD Biosciences, CA) and ABTS (LGC, Searcare, MA). All plates were read using a spectrophotometer (Molecular Devices, San Jose, CA) using SoftMax software (Molecular Devices, San Jose, CA).

2.9 Measurement of plasma histamine levels

As previously performed (34, 35), blood samples were collected via sub-mandibular bleeding at 30 minutes after scoring and measurement of body temperature and plasma was isolated immediately after the blood collection. All samples were stored at -80°C for further usage. Histamine levels in the serum were measured using a commercial available ELISA kit (Fisher Scientific, Waltham, MA) as described by the manufacturer.

2.10 Safety assays

For acute toxicity analysis, naïve C3H/HeJ mice were fed with 4 mg of XPP per mouse, which is10 times of the daily dose. Mice were observed for 14 days. In the sub-chronic toxicity assay, naïve C3H/HeJ mice were fed with 5 times their daily therapeutic dose (2 mg/day) for 14 days. Control group mice (sham) were fed with water. Blood samples were collected at the end of each experiment. Blood urea nitrogen (BUN) and alanine aminotransferase (ALT) measurements for the evaluation of kidney and liver functions respectively and complete blood count (CBC) testing were performed by ALX laboratories, NY.

2.11 Splenocytes cultures and cytokines measurement

Mice were sacrificed at the end of each experiment. The splenocytes (SPCs) and mesenteric lymph node (MLN) cells of each mouse was collected as previously described (37). Cells (4 x10^6/well/mL) were cultured in 24 well plates in the presence or absence of CPE (200 µg/mL). After 72 hrs of incubation at 37°C under 5% CO_2, supernatants were collected. Cytokine levels were measured using ELISA kits in triplicate according to the manufacturer's instructions (BD Biosciences, San Jose, CA and R&D systems, Minneapolis, MN).

2.12 Cell viability

Cell viability was evaluated using a trypan blue exclusion as previously described (31). Briefly, a 10µL of cells suspension from each culture was mixed with equal volume of trypan blue dye. The mixture was loaded into a hemocytometer and cells were counted under a microscope. The percentage of viable cells was calculated as follows: Viable cells (%) = (total number of viable cells)/(total number of cells) × 100.

2.13 Flow cytometry

Single cell suspensions of spleen, bone marrow from two femurs and heparinized peripheral blood were isolated in AIM-V medium. Erythrocytes were lysed with red cell lysing buffer (Sigma-Aldrich, St. Louis, MO). For staining, cells were suspended in ice cold staining buffer (PBS including 0.5 mM EDTA, 0.05 mM Sodium Azide, 0.5% BSA). Surface staining was performed by incubating cells with unlabeled anti-IgE (to block membrane IgE), BV605 anti-B220, BV711-anti-CD3, anti-CD16/32 (Fc-block), all from BD Biosciences, CA). Live-dead discriminating dye (Live-Dead Aqua, Invitrogen, CA) was included. After incubation, cells were washed and suspended in fixation/permeabilization buffer for 15 mins. Cells were washed with permeabilization buffer and incubated with Fitc-anti IgE in permeabilization buffer for 30 mins in the dark on ice. After washing, cells were treated with Cytofix buffer for 15 mins for post-fixation and then washed 3 times with staining buffer and finally resuspended in 200 µL staining buffer for cell acquisition on LSRII flow cytometer (BD Bioscience, San Jose, CA). Flow cytometry analysis was performed using Flow Jo 5.4 software (Tree Star Inc., Ashland, Oregon) as follows. Singlet cells were selected on the basis of FSC-A/FSC-H profile and used to generate lymphocyte gate based on forward and side scatter properties (Supplementary Figure 1). Live cells were selected as cells negative for Live-Dead stain. From live cell gate, all IgE+ (Fitc-IgE) cells were gated and subsequently analyzed for IgE+ B cells (Fitc-IgE +; BV605-B220+ cells).

2.14 DNA methylation

Genomic DNA was isolated from mouse splenocytes collected from orally sensitized mouse model#2 using Qiagen mini DNA/RNA extraction kit (Qiagen, Valencia, CA). Isolated DNA was bisulfite-modified using an Epitect plus DNA Bisulfite kit (Qiagen) following the manufacturer's instructions. Bisulfite converted DNA was then amplified with the IL-4 primers listed below by PCR. The PCR products were subsequently purified and pyrosequenced using sequencing primers on a Pyromark Q24 system (Qiagen) as described previously (38). Primers for IL-4; Fwd: GTTTTTAAGGGGTTTTTATAGTAGGAAG; Rev-Biotin-AATTACCACTAACTCTCCTCCTACA. Sequencing (CpG -393) AGATTTTTTTGATATTATTTTGTT.

2.15 RNA sequencing and identification of molecular targets underlying IgE regulation

U266 cells were treated with XPP at 10 µg/mL. After 3 days of treatment, cells were collected and total RNA was isolated using Qiagen RNeasy MiniKits following the manufacturer's instructions. The purity of total RNA was analyzed and the RNA-Seq was

performed with an Illumina HiSeq2500 instrument at the Genomics Core facility at Icahn School of Medicine at Mount Sinai. Counts were converted to Log2 counts per million and normalized. A log2 fold change was calculated and the gene list with values ≥ 2 or ≤ -2 were uploaded to DAVID bioinformatic system for functional analysis.

2.16 Real time polymerase chain reaction

RT-PCR analysis of genes related to Cell cycle, B cell differentiation, and IgE production was performed by QuantStudio 5 real-time PCR system (Thermo Fisher, Waltham, MA). For each PCR reaction, 12.5 µL maxima SYBR Green/ROX qPCR Master Mix 2x (Thermo Fisher, Waltham, MA) was mixed with 1.8 µL of 0.3 µM target primers and 300 ng of template DNA to make the total volume as 25 µL. The PCR procedure was set as 40 cycles at 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. GAPDH was used as the housekeeping gene. The Delta Ct values for each gene were calculated by normalizing the Ct values with the housekeeping gene. The relative fold change in mRNA expression between different groups was calculated and expressed as $2^{-\Delta\Delta CT}$. The primer sequence used are shown in Supplementary Table 1.

2.17 Statistics

Data were analyzed using GraphPad Prism software (version 8.2.1, GraphPad Software. La Jolla, CA). Differences between two groups were analyzed using unpaired Student's t-test. Differences between multiple groups were analyzed by One Way Analysis of Variance (One way ANOVA) followed by pair wise testing using Bonferroni's adjustment. Pearson's correlation coefficients were analyzed using GraphPad Prism software, and r values were generated. P values ≤0.05 were considered statistically significant.

3 Results

3.1 XPP isolated from *R. cordifolia* dose-dependently inhibited IgE production by human IgE producing myeloma cell line

R. cordifolia water extract was first extracted using dichloromethane, and further fractionated. Five fractions were collected and the fifth fraction (Fr.5) was found to have the highest anti-IgE effect at 10 µg/mL in human myeloma cell line (U266 cells) *in vitro* (Supplementary Figure 2). The major compound from this fraction was further collected and purified. The purity of the isolated compound was more than 95% as determined by analytical HPLC. The structure of this compound was identified using LC-MS (Figure 2A) and ^{1}H and ^{13}C NMR spectroscopy (Supplementary Table 2) (39). Mass spectra data showed a $[M+H]^+$ ion peak of *m/z* 241 and $[M-H]^-$ ion peak of *m/z* 239 (Figure 2A). The molecular weight (MW) of this compound was then determined to be 240 g/mol. The ^{1}H NMR data and the ^{13}C NMR data of this compound was consistent with previously reported data of xanthopurpurin (Supplementary Table 2).

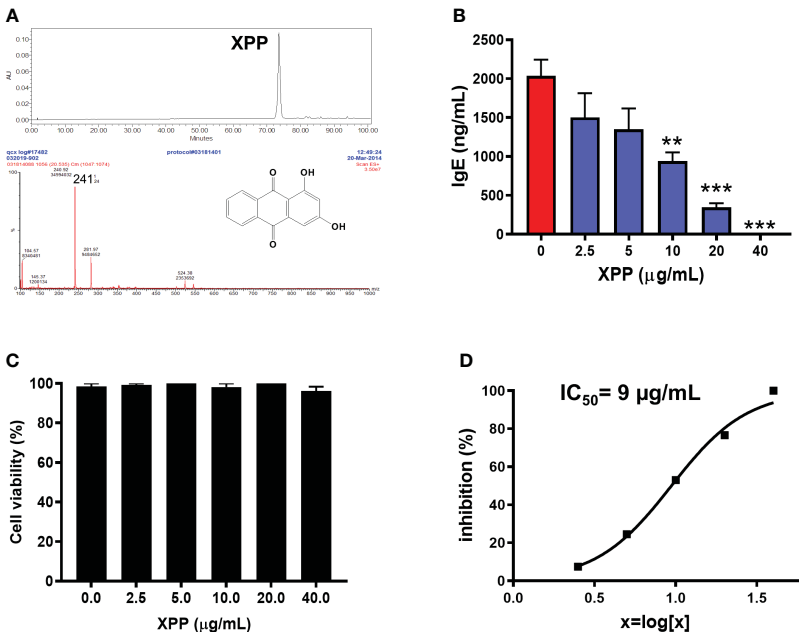

FIGURE 2
Characterization of xanthopurpurin (XPP) and the *in vitro* inhibitory effect of XPP on IgE by U266 cells. **(A)** HPLC chromatogram and Mass spectrum of XPP. The chemical structure of XPP was presented. **(B)** XPP dose-dependently inhibited IgE production by U266 cells (human IgE was measured by ELISA). **(C)** Cell viability assay of XPP on U266 cells by trypan blue exclusion. **(D)** IC50 values of XPP on IgE production in U266 cells. **p<0.01; ***p<0.001 vs. Untreated control (0 µg/mL). n=4-6.

Thus, based on the MS and NMR data, this compound was identified as xanthopurpurin (XPP).

We determined the biological effect of XPP on IgE production *in vitro* on U266 cells at various concentrations. XPP inhibited the IgE production by U266 cells in a dose-dependent manner (Figure 2B). Inhibition of IgE was first observed at XPP concentration of at 2.5 μg/mL, reaching statistically significant inhibition at 10 μg/mL ($p < 0.01$), and completely inhibition of IgE production was observed at 40 μg/mL. Cell viability was analyzed using trypan blue staining as previously published (31). No toxicity was observed at any concentration tested (Figure 2C). The IC_{50} value was calculated as 9 μg/mL (Figure 2D). To our knowledge, this is the first report that XPP inhibits IgE production. Notably, there was no cytotoxicity.

3.2 XPP inhibited peanut specific IgE production and protected PN allergic mice against PN anaphylaxis

The *in vitro* data above prompted us to test whether XPP inhibits IgE production *in vivo*. We employed a well-established PN allergy model in which B and T cell responses and clinical reactions resemble human peanut- allergy. This model has been used by us and many other investigators (29, 36). We tested XPP effect in two separate experiments in which mice were either systemically sensitized with PN+ alum adjuvant (Model # 1, Figure 1A) or orally sensitized with PN + cholera toxin (Model #2, Figure 1B). Both sensitization protocols are well established (28, 29, 36, 40). To elicit anaphylaxis, all mice were challenged orally and XPP treatment was given i.g. as indicated (Figures 1A, B). PN-specific IgE levels at challenge in XPP-treated mice were significantly and markedly reduced compared to the sham-treated PNA mice in both models (75% reduction, $p < 0.001$ *vs.* Sham, Figure 1C). Impressively, all XPP-treated mice were completely protected from anaphylaxis following PN challenge, evidenced by symptom scores of 0 (Figure 1D, $p < 0.001$ *vs.* Sham), normal body temperatures (Figure 1E, $p < 0.001$ *vs.* Sham), and essentially normal plasma histamine levels (Figure 1F, $p < 0.001$). Protection in model #1 was observed 7 weeks after stopping therapy with significantly reduced symptom scores and PN-specific IgE levels and increased body temperatures, which all indicated a persistent effect (Supplementary Figure 3). Results in model #2 represent efficacy of late treatment with XPP as it was administered post-boosting, at a time when mice had established food allergy and were capable of anaphylaxis (34). Finally, XPP did not reduce PN-IgG1 (Figure 1G), PN-IgG2a (Figure 1H) or PN-IgA (Figure 1I) in the orally sensitized mouse model #2, which demonstrated its selectivity for IgE reduction. Furthermore, correlation analysis revealed that PN specific IgE showed significant positive correlation with symptom score and plasma histamine level ($r = 0.9083$, $p < 0.0001$; $r = 0.8903$, $p < 0.0001$, Figures 1J and K) and negatively correlated with body temperatures ($r = -0.8906$, $p < 0.0001$, Figure 1L).

3.3 Oral administration of XPP has a high safety profile

Given that XPP effectively suppressed IgE production and protected peanut allergic mice from anaphylaxis, it may have a potential for clinical use. It is important to understand its safety profile. Acute toxicity and sub-chronic toxicity experiments of XPP were performed. In the acute-toxicity assay, C3H/HeJ mice were fed with 10 times (4 mg/day) of the regular XPP treatment dose once and observed daily for 14 days. No deaths and abnormal behavior or diarrhea were observed (Table 1). In the sub-chronic toxicity assay, mice were fed 5 times (2 mg/day) of the regular treatment dose for 14 consecutive days. No deaths and abnormal behavior or diarrhea were observed either, and all mice appeared healthy. Mice were then sacrificed, and blood samples were obtained for chemical analysis by ALX laboratories (NY). Results showed that the serum ALT and BUN levels were similar to the control group and within the normal range (Table 1). The complete blood count (CBC) tests showed that

TABLE 1 Safety evaluation of XPP.

	Naïve	XPP (Acute)	XPP (sub-chronic)	Reference
Treatment	Vehicle	10x	5x	
Mortality (%)	0.00	0.00	0.00	N/A
Morbidity (%)	0.00	0.00	0.00	N/A
BUN	27.0 ± 2.8	19.01.2	19.86±2.19	9~36
ALT	27.5 ± 10.6	24.8±11.7	23.57±9.57	22~400
RBC (M/uL)	7.17±0.43	6.93±1.11	7.34±0.40	2.8~10.8
HGB (g/dL)	11.2±0.49	10.8±1.56	11.83±0.65	3.8~10.2
PLT(K/pL)	505.00±342.52	407.25±252.86	553.67±79.34	6.3~16.3
WBC (K/uL)	6.225±2.93	4.04±0.71	2.91±0.70	115~840

For acute toxicity experiment, C3H/HeJ mice were fed 10 times normal dose (4mg/day/mouse) and observed for 14 days. For sub-chronic toxicity experiment, C3H/HeJ mice were fed 5 times of normal dose (2mg/day/mouse) for 14 days. Naive group of mice were fed with water as controls. Mice were sacrificed and blood samples were collected after each experiment. Blood urea nitrogen (BUN) and alanine aminotransferase (ALT) measurements for evaluation of kidney and liver functions respectively and complete blood count (CBC) testing were performed by ALX laboratories, NY. BUN, Blood Urea Nitrogen; ALT, Alanine Aminotransferase; RBC, Red Blood Cells; HGB, Hemoglobin; PLT, Platelets; WBC, White Blood Cells.

the white blood cell, red blood cell, hemoglobin and platelet levels in the XPP treated group were also within the normal range and similar to the control group (Table 1). These results demonstrate that XPP has a high safety profile. These data are in line with the *in vitro* data that demonstrated no cytotoxicity of XPP or global immune suppression.

3.4 XPP decreased the IgE-expressing memory B cells in spleen and bone marrow

Since XPP significantly reduced the PN-specific IgE levels compared to sham-treated mice, we, therefore, addressed questions of whether the IgE-producing B cells numbers are modified by XPP treatment. IgE-producing B cells from the spleen and bone marrow (samples collected from orally sensitized mouse model #2) were evaluated using flow cytometry of cells stained for intracellular IgE (Figures 3A, B). The percentage of the IgE+B cells (over all IgE+ cells) was significantly reduced by XPP in both spleen and bone marrow cells ($p < 0.01$; $p < 0.001$, Figures 3C, D).

3.5 XPP treatment reduced production of IL-4 but not IFN-γ or IL-10

IL-4 is the key Th2 cytokine promoting IgE antibody switching and memory, while IFN- γ and IL-10 inhibit IL-4 production (41). We determined IL-4 production by splenocytes (SPCs) and MLN cells harvested from each group of mice from the orally sensitized mouse model #2 following the last challenge. XPP-treated mice showed significantly inhibited IL-4 level in both splenocyte and MLN cultures after the crude peanut extract (CPE) stimulation (Figures 4A, D). There were no significant differences in IL-10 (Figures 4B, E) or IFN-γ production (Figures 4C, F) in XPP-treated group in both the SPC or MLN cultures compared with the sham group. These data suggest that XPP may have a direct effect on IL-4 regulation.

3.6 XPP increased the methylation at murine IL-4 promoter

Methylation status at the IL-4 promoter directly regulates IL-4 transcription; i.e. increased methylation suppresses IL-4 transcription

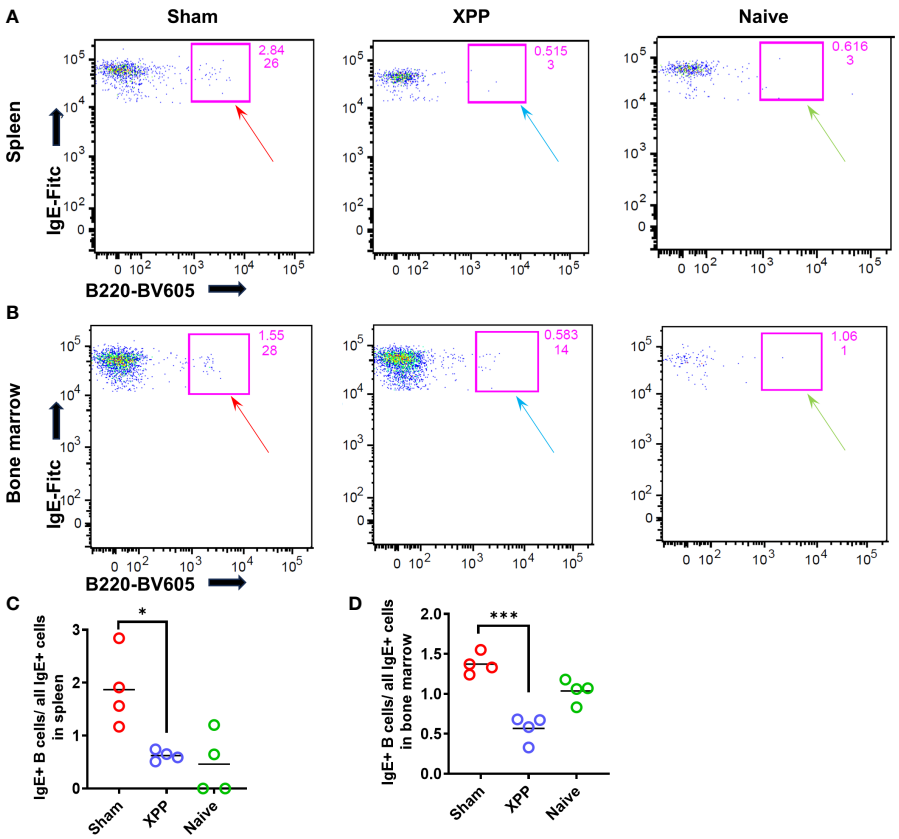

FIGURE 3
IgE+ B cells population in spleen and bone marrow samples. Splenocytes and bone marrow cells from oral sensitized model #2 were stained with antibodies to CD45R/B220 and IgE and analyzed by flow cytometry. **(A)** IgE+ B cells were found in the spleen of Sham, XPP treated, and naive mice; **(B)**; IgE+ B cells were found in the bone marrow of Sham, XPP treated, and naive mice; **(C)** Percent of IgE+ B cells in spleen; **(D)** Percent of IgE+ B cells in bone marrow; *$p \leq 0.05$; ***$p < 0.001$, n=4.

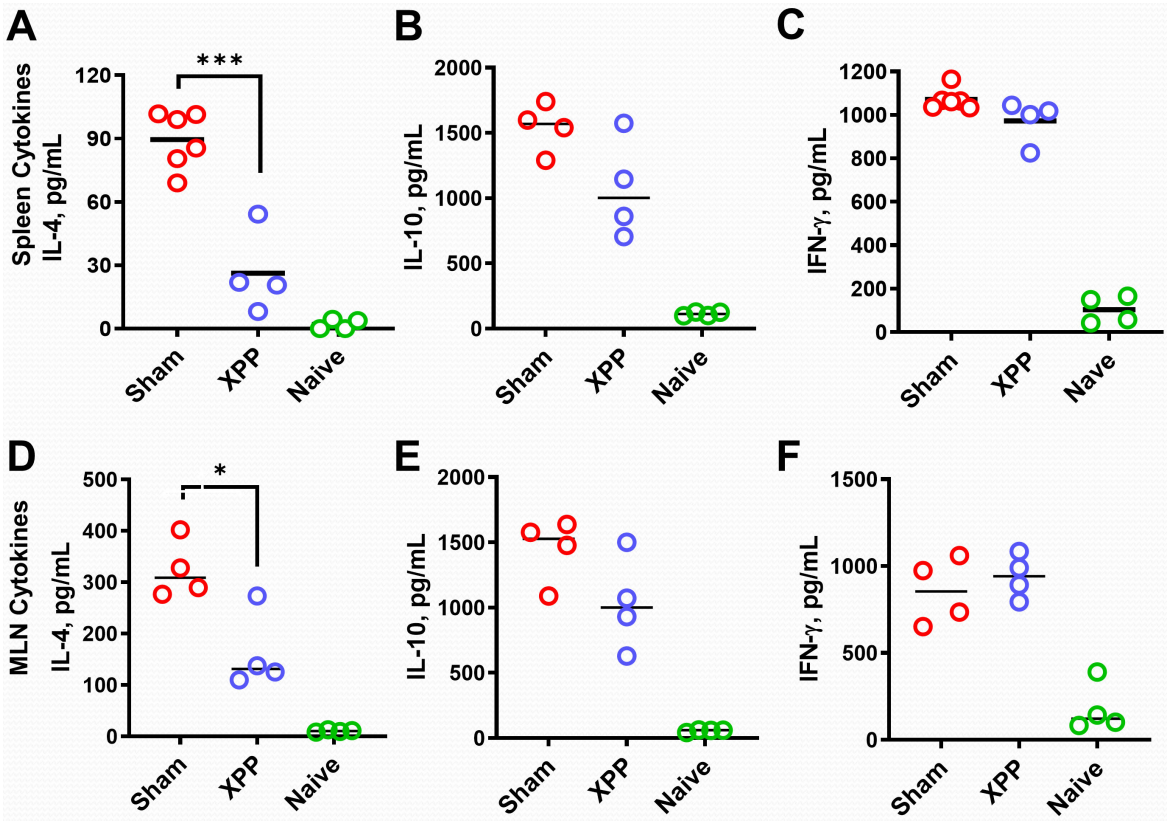

FIGURE 4
Effect of XPP on cytokine levels and IgE-producing B cell numbers. Splenocytes culture and the MLN cell cultures (from oral sensitized model #2) were stimulated with protein extracts of peanut (CPE). IL-4 **(A)**, IL-10 **(B)** and IFN-γ **(C)** production in murine splenocytes; IL-4 **(D)**, IL-10 **(E)** and IFN-γ **(F)** production in murine MLN were measured by ELISA. Bars indicate group means. N = 4-5 mice/group. *: $p \leq 0.05$, ***: $P<0.001$ *vs.* Sham.

whereas decreased methylation increases IL-4 transcription (42, 43). We therefore determined methylation levels at CpG-393 of IL-4 promoter as previously described (34). Percentages of CpG methylation for CpG-393 showed an increase in XPP-treated mice compared to sham (Figure 5A) suggesting that XPP treatment leads to a more closed IL-4 promoter. In all groups, methylation of IL-4 promoter CpG site showed robust significant inverse correlation with IL-4 production (Figure 5B, $r = -0.6338$, $p < 0.05$) and PN-specific IgE (Figure 5C, $r = -0.6960$, $p < 0.0082$). These data suggest that regulation of IL-4 promoter CpG methylation status may be a contributing mechanism of XPP suppression of IL-4 production.

3.7 Transcriptional profiling of IgE producing plasma cells treated with XPP

RNA-Seq was used to evaluate the full gene-expression profiles of XPP-treated IgE producing human plasma cells. Overall, 4009 genes were upregulated, and 5094 genes were downregulated (Figure 6A). To further analyze the RNA-Seq results, pathway enrichment was investigated using DAVID. 53 related genes were matched to cell cycle process using David database. 65 related genes were matched to B cell differentiation- related genes, and 26 related genes were matched to IgE-related genes from Pubmed database (Figure 6B). The overlap graph showed the number of genes found in our RNA-Seq data using each method. Six pathways were involved in the regulation of XPP-treated IgE-producing plasma cells. These pathways are related to plasma B cells, IgE production, B cell differentiation, cell cycle, P53 pathway, and DNA replication. The heatmap for each pathway is shown in Figure 6C. As hundreds of genes were identified in the six enriched pathways, we further narrowed down candidate regulatory genes by filtering for differential expression using Log2C values > 2 or < -2 as cut-off values. Upregulated genes are shown in yellow whereas downregulated genes are shown in green (Figure 6D).

3.8 PCR validation of candidate genes related to cell cycle, B cell differentiation, and IgE production in XPP treated IgE-plasma cells

In order to further verify XPP mechanism of action at the molecular level, the expression levels of some of these target genes were validated by qRT-PCR. We chose the CCND1, DUSP4, and PTPARC from the cell cycle related genes, SCD1 and ETS1 from the

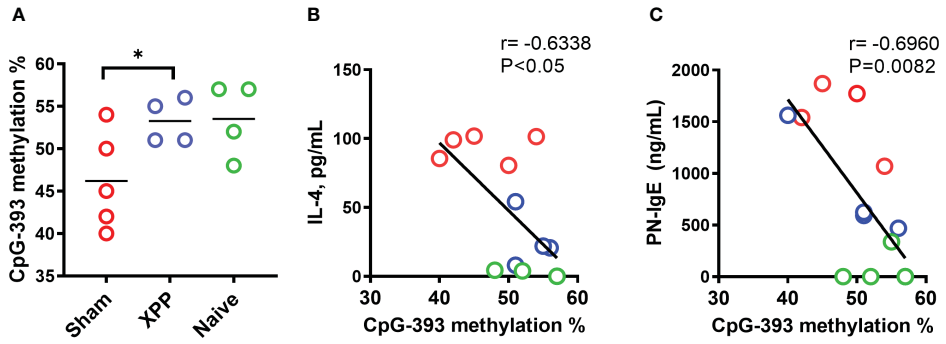

FIGURE 5
Percentage methylation of CpG sites in IL-4 promoter. Percentages of DNA methylation at the CpG-393 **(A)** site of IL-4 gene promoters in splenocytes collected from oral sensitized model #2 was assessed by pyrosequencing of bisulfite converted genomic DNA. Bars in A is group means. Pearson correlation between splenocyte IL-4 production vs. methylation percentage of CpG-393 **(B)** and PN-specific serum IgE vs. methylation percentage of CpG-393 **(C)** were calculated. N = 4-5 mice/group. * P<0.05.

B cell terminal differentiation gene list, and IL6R and PTPRC from the IgE production related gene list as the targets for RT-PCR validation. The RT-PCR confirmed that the CCND1, SCD1, and IL6R genes expression were significantly reduced in U266 cells with the treatment of XPP at the concentration of 20 μg/mL (Figures 7A–C, $p < 0.001$), while the DUSP4 and PTPRC gene expression was significantly increased by XPP at the same concentration (Figures 7D, F, $p < 0.01$). ETS1 gene expression also showed the upregulation trend with the increasing concentration of XPP, but no significant value was observed (Figure 7E). Overall, we were able to identify key genes associated with different pathways involving disease progression, and successfully validated some of these genes by qRT-PCR.

4 Discussion

Efficacious reduction of IgE for the treatment of food allergy remains elusive. Herbal medicines have been investigated for their inhibition effect on IgE production. We previously showed that the herbal formula FAHF-2 and its refined form showed significant IgE inhibition, both *in vitro* and *in vivo* (44–46). The active compound, berberine, was isolated from *P. chinensis*, which is one of the nine herbal constituents in FAHF-2 formula (31). We showed that berberine significantly inhibited the IgE production *in vitro* and modulated the ε-germline transcript expression through regulation of the phosphorylation of IκBα, STAT-3 and T-bet (31). However, the bioavailability of berberine is poor. Oral berberine alone did not reduce PN-specific IgE levels and failed to protect PN-allergic mice from anaphylaxis, while the whole formula showed significant suppression of IgE levels (47).

Previously, our lab determined that the herbal medicine, *R. cordifolia*, inhibited the IgE production both *in vitro* and *in vivo* (28). *R. cordifolia* also reduced the anaphylactic symptoms in PN allergic mouse model (28). Historically, *R. cordifolia* is a widely used herbal medicine in Asia. Recent studies have shown that *R. cordifolia* roots have antibacterial, antioxidant and anti-inflammatory activities (28). *R. cordifolia* is enriched for anthraquinones, which have been isolated and identified, such as, alizarin (1,3-dihydroxy-2-ethoxymethyl-9,10 anthraquinone), purpurin (1,2,4-trihydroxyanthraquinone), mollugin (1-hydroxy-2-methy-9,10-anthraquinone) (48–50). We, for the first time, investigated the effect of xanthopurpurin on PN specific food allergy using murine models. Importantly, complete protection from anaphylaxis persisted for 7 weeks after stopping treatment which is longer than observed for OIT approaches in murine models (Supplementary Figure 3) (34). Protection was also observed when late treatment XPP was given after establishing PN allergy. Other compounds from *R. cordifolia*, such as purpurin and alizarin, inhibited IgE production only *in vitro*, but failed *in vivo* (Data not shown). Xanthopurpurin (XPP) significantly inhibited IgE production not only *in vitro*, but also *in vivo*, similar to the whole *R. cordifolia* herbal medicine (28). Thus, XPP has promise as a therapy for food allergy.

Food-induced anaphylaxis is an IgE-dependent type-I hypersensitivity reaction. Allergen-specific IgE produced by B cells is increased in response to allergen stimulation due to increased number of IgE-producing B cells. Regulation of IgE levels using anti-IgE molecules has been employed in many studies targeting various allergic diseases (26, 51). However, omalizumab can neutralize the free serum IgE (52), but less information about targeting to IgE production B cells and plasma cells. Compared with anti-IgE therapies, the XPP treatment not only inhibited IgE production, but it also suppressed the number of IgE producing B cells in our animal model. Downregulation of IgE-producing B cells provides evidence of a direct effect on IgE production.

Food allergy is associated with the imbalance of Th1and Th2 response. Th2 response was dramatically increased while Th1 response was diminished in food allergy (53). Th2 cytokines play important role in the pathogenesis of food allergy. IL-4 is pivotal for the activation of Th2 response and INF-γ is associated with the active Th1 response. To investigate the effect of XPP on PN allergic, we utilized two mouse models, systemic model and oral model, in this study. Both systemic model (i.p. sensitization) and oral model

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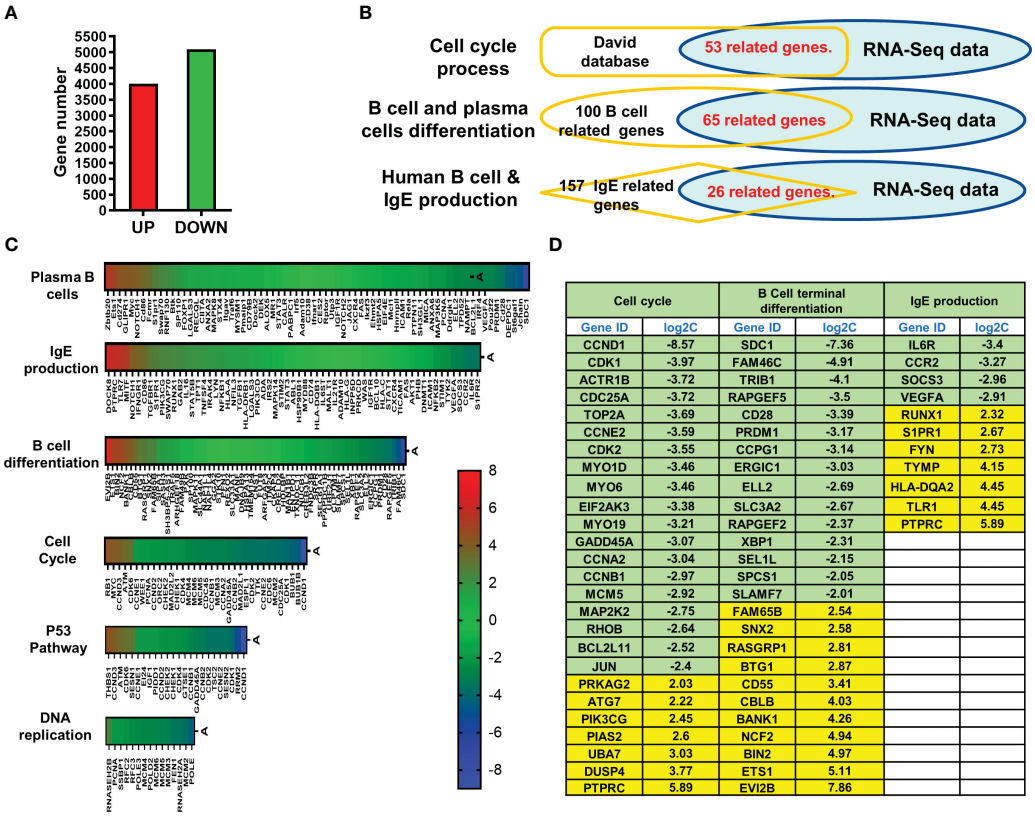

Cell cycle		B Cell terminal differentiation		IgE production	
Gene ID	log2C	Gene ID	log2C	Gene ID	log2C
CCND1	-8.57	SDC1	-7.36	IL6R	-3.4
CDK1	-3.97	FAM46C	-4.91	CCR2	-3.27
ACTR1B	-3.72	TRIB1	-4.1	SOCS3	-2.96
CDC25A	-3.72	RAPGEF5	-3.5	VEGFA	-2.91
TOP2A	-3.69	CD28	-3.39	RUNX1	2.32
CCNE2	-3.59	PRDM1	-3.17	S1PR1	2.67
CDK2	-3.55	CCPG1	-3.14	FYN	2.73
MYO1D	-3.46	ERGIC1	-3.03	TYMP	4.15
MYO6	-3.46	ELL2	-2.69	HLA-DQA2	4.45
EIF2AK3	-3.38	SLC3A2	-2.67	TLR1	4.45
MYO19	-3.21	RAPGEF2	-2.37	PTPRC	5.89
GADD45A	-3.07	XBP1	-2.31		
CCNA2	-3.04	SEL1L	-2.15		
CCNB1	-2.97	SPCS1	-2.05		
MCM5	-2.92	SLAMF7	-2.01		
MAP2K2	-2.75	FAM65B	2.54		
RHOB	-2.64	SNX2	2.58		
BCL2L11	-2.52	RASGRP1	2.81		
JUN	-2.4	BTG1	2.87		
PRKAG2	2.03	CD55	3.41		
ATG7	2.22	CBLB	4.03		
PIK3CG	2.45	BANK1	4.26		
PIAS2	2.6	NCF2	4.94		
UBA7	3.03	BIN2	4.97		
DUSP4	3.77	ETS1	5.11		
PTPRC	5.89	EVI2B	7.86		

FIGURE 6
RNA sequencing of XPP treated U266 cells and pathway analysis. **(A)** Bar graph of total up-regulated a down-regulated genes; **(B)** RNA-Seq expression profile of most up- or down-regulated genes in cell cycle, B cell differentiation, and IgE production. **(C)** Heatmap of RNA-Seq results resulted to 6 pathways; **(D)** Table of candidate genes which have most significant changes. The threshold of log2C values was set as lower than -2.0 or higher than 2.0.

(i.g. sensitization) induced persistent peanut hypersensitivity. Systemic model using i.p. sensitization method in C3H/HeJ mice is most efficient, fast and simple to achieve, with high levels of PN sIgE and hypersensitivity. Oral model with i.g sensitization is more similar to the real food allergy scenario on human who was exposed to the PN allergen by food consumption. The oral model required to use the cholera toxin as the adjuvant, which is toxic, and need a longer time to introduce the allergic reactions. In this study, we first tested XPP on a fast established systemic sensitization model. Then, we investigated the effect of XPP on oral sensitization model which mimic the peanut allergy in reality. XPP treatment reduced IgE production, but did not show obvious effects on PN-sIgG1, -sIgG2a, and -sIgA levels (Figures 1G–I). IgG2a is Th1-associated immunoglobulin. Our data showed that the XPP treatment significantly suppressed the IL-4 production, but did not significantly inhibit the production of IL-10 and INF-γ. We also performed the *in vitro* experiment of XPP on human IgG producing myeloma cell line, ARH-77 (ATCC, Manassas, VA). XPP didn't show significant inhibition on the IgG production. No toxicity was observed in this experiment (Supplementary Figure 4). All these data together suggest that XPP treatment selectively regulates Th2 response, without global immunosuppression. However, whether XPP has an effect on other IgG-producing myeloma cells lines, or on IgA- IgM- or IgD-isotype antibody producing myeloma cells are unknown, requiring further investigation.

Epigenetic mechanisms have been suggested in food allergy studies (54). Prior studies reported that allergic subjects showed significantly lower methylation level at IL-4 promoter region compared with health subjects (55). We found that XPP treatment increased DNA methylation status of CpG at site -393 compared with the sham group ($p = 0.05$). Decreased DNA methylation in Sham group is consistent with higher IL-4 gene expression, whereas higher DNA methylation in XPP-treated group implies decreased transcription of IL-4, which in turn reduces production of IL-4. Correlation data also confirmed that the increased DNA methylation at CpG-393 was negatively correlated with IL-4 expression and IgE production.

Additional mechanisms contributing to the regulatory effect of XPP on IgE production may involve targeting of genes related to other signal pathways. Our RNA-seq data of XPP-treatment on U266 cell identified 6 pathways/processes as being enriched, including Plasma/B cells and IgE production, among others. Significantly up or down regulated genes (log 2C value > 2 or < 2) were selected as candidates for the validation using qPCR. Top 3 down regulated and 3 upregulated genes showed CCND1, SDC1 and IL-6R were significantly reduced whereas DUSP4 and PTPRC

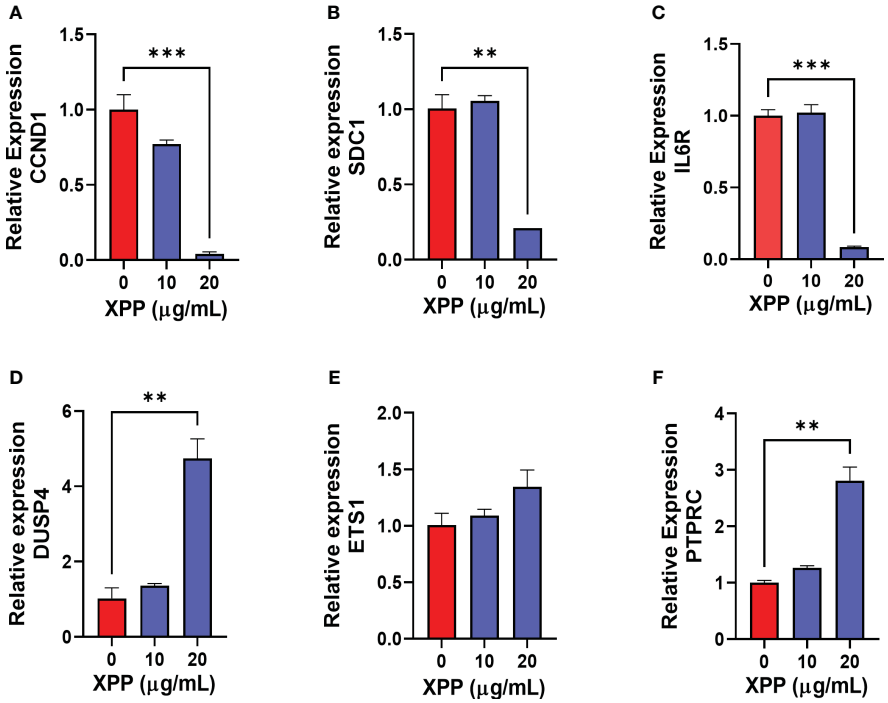

FIGURE 7
Relative gene expression of 6 down-or up-regulated genes in XPP treated U266 cells. The relative expression level of CCND1 **(A)**, SDC1 **(B)**, IL6R **(C)**, DUSP4 **(D)**, ETS1 **(E)**, and PTPRC **(F)** were determined by comparing with GAPDH mRNA expression. **p<0.01 ***p<0.001.

expressions were significantly increased. The ETS1 gene showed mild non-significant increase at 20 μg/mL of XPP. Previous reports of these genes in the context of food allergy are scarce. CCND1 and IL-6R have been reported to be associated with asthma inflammation and high serum IgE in patients (56–58). IL-6 signaling directly related to the survival and maturation of B cells and Plasma cells (59, 60). SDC1 is a maker of long-lived plasma cells and myeloma pathogenesis (61, 62). DUSP4, on the other hand, was reported as a tumor suppressor (63, 64). DUSP4 expression reduced the expansion of antigen-specific B cells and the production of antibodies (65). While the direct immunoregulatory targets of XPP remain to be identified, our data suggests that the XPP treatment decreases Th2-immune responses and pathways related to IgE production.

In conclusion, we, for the first time, demonstrate that xanthopurpurin, an active compound isolated from *R. cordifolia*, suppressed the IgE production *in vivo* and protected peanut allergic mice from anaphylactic reactions. Furthermore, xanthopurpurin also exhibits regulatory effects on IL-4 promoter DNA methylation, and on expression of several genes related to B cell survival and IgE production.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Institutional animal care and use committee (IACUC) at Icahn Mount Sinai School of Medicine, New York. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. KS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – review & editing. YC: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. HL: Data curation, Investigation, Methodology, Writing – review & editing. AM: Data curation, Investigation, Methodology, Writing – review & editing. PY: Data curation, Investigation, Methodology, Writing – review & editing. XL: Conceptualization, Writing – review & editing. RT: Writing – review & editing. JG: Writing – review & editing. AN: Writing – review & editing. JZ: Data curation, Writing – review & editing. X-ML: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

X-ML received research support to her institution from the National Institutes of Health (NIH)/National Center for Complementary and Alternative Medicine (NCCAM) # 1P01 AT002644725-01 "Center for Chinese Herbal Therapy (CHT) for Asthma", and grant #1R01AT001495-01A1 and 2R01 AT001495-05A2, NIH/NIAID R43AI148039, NIH/NIAID 1R21AI176061-01, NIH/NIAID 1R44AI177183-01, NIH/NIAID 1R41AI172572-01A1, Food Allergy Research and Education (FARE), Winston Wolkoff Integrative Medicine Fund for Allergies and Wellness, the Parker Foundation and Henan University of Chinese Medicine; received consultancy fees from FARE and Johnson & Johnson Pharmaceutical Research & Development, L.L.C. Bayer Global Health LLC; received royalties from UpToDate; received travel expenses from the NCCAM and FARE; share US patent US7820175B2 (FAHF-2), US10500169B2 (XPP), US10406191B2 (S. Flavescens), US10028985B2 (WL); US11351157B2 (nanoBBR): take compensation from her practice at Center for Integrative Health and Acupuncture PC; US Times Technology Inc is managed by her related party; is a member of General Nutraceutical Technology LLC and Health Freedom LLC. NY received research support from the National Institutes of Health (NIH)/National Center for Complementary and Alternative Medicine (NCCAM), NIH/NIAID R43AI148039, NIH/NIAID 1R21AI176061-01, NIH/NIAID 1R44AI177183-01, NIH/NIAID 1R41AI172572-01A1; shares US patent: US10500169B2 (XPP), US10406191B2 (S. Flavescens), US10028985B2 (WL); and is a member of General Nutraceutical Technology, LLC and Health Freedom LLC; receives a salary from General Nutraceutical Technology, LLC. KS shares US patent: US11351157B2 (nanoBBR).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2024.1299484/full#supplementary-material

References

1. Cianferoni A, Muraro A. Food-induced anaphylaxis. *Immunol Allergy Clin North Am* (2012) 32(1):165–95. doi: 10.1016/j.iac.2011.10.002

2. Gupta RS, Warren CM, Smith BM, Jiang J, Blumenstock JA, Davis MM, et al. Prevalence and severity of food allergies among US adults. *JAMA Netw Open* (2019) 2 (1):e185630. doi: 10.1001/jamanetworkopen.2018.5630

3. Sasaki M, Koplin JJ, Dharmage SC, Field MJ, Sawyer SM, McWilliam V, et al. Prevalence of clinic-defined food allergy in early adolescence: The SchoolNuts study. *J Allergy Clin Immunol* (2018) 141(1):391–398.e4. doi: 10.1016/j.jaci.2017.05.041

4. Dunlop JH, Keet CA. Epidemiology of food allergy. *Immunol Allergy Clin North Am* (2018) 38(1):13–25. doi: 10.1016/j.iac.2017.09.002

5. Gupta RS, Springston EE, Warrier MR, Smith B, Kumar R, Pongracic J, et al. The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* (2011) 128(1):e9–17. doi: 10.1542/peds.2011-0204

6. Bunyavanich S, Rifas-Shiman SL, Platts-Mills TA, Workman L, Sordillo JE, Gillman MW, et al. Peanut allergy prevalence among school-age children in a US cohort not selected for any disease. *J Allergy Clin Immunol* (2014) 134(3):753–5. doi: 10.1016/j.jaci.2014.05.050

7. Gupta R, Holdford D, Bilaver L, Dyer A, Holl JL, Meltzer D. The economic impact of childhood food allergy in the United States. *JAMA Pediatr* (2013) 167(11):1026–31. doi: 10.1001/jamapediatrics.2013.2376

8. Gupta R, Holdford D, Bilaver L, Dyer A, Meltzer D. The high economic burden of childhood food allergy in the United States. *J Allergy Clin Immunol* (2013) 131(2): AB223. doi: 10.1016/j.jaci.2012.12.1464

9. FARE. *Food Allergy Research and Education*. Facts and Statistics. Available at: https://www.foodallergy.org/resources/facts-and-statistics#:~:text=How%20Many%20People%20Have%20Food,roughly%20two%20in%20every%20classroom.

10. Savage J, Sicherer S, Wood R. The natural history of food allergy. *J Allergy Clin Immunol Pract* (2016) 4(2):196–203;quiz 204. doi: 10.1016/j.jaip.2015.11.024

11. Brough HA, Caubet JC, Mazon A, Haddad D, Bergmann MM, Wassenberg J, et al. Defining challenge-proven coexistent nut and sesame seed allergy: A prospective multicenter European study. *J Allergy Clin Immunol* (2020) 145(4):1231–9. doi: 10.1016/j.jaci.2019.09.036

12. Warren CM, Jiang J, Gupta RS. Epidemiology and burden of food allergy. *Curr Allergy Asthma Rep* (2020) 20(2):6. doi: 10.1007/s11882-020-0898-7

13. Wang J, Sampson HA. Food anaphylaxis. *Clin Exp Allergy* (2007) 37(5):651–60. doi: 10.1111/j.1365-2222.2007.02682.x

14. *FDA Encourages Manufacturers to Clearly Declare All Uses of Sesame in Ingredient List on Food Labels* (2020). Available at: https://www.fda.gov/news-events/press-announcements/fda-encourages-manufacturers-clearly-declare-all-uses-sesame-ingredient-list-food-labels.

15. Motosue MS, Bellolio MF, Van Houten HK, Shah ND, Campbell RL. National trends in emergency department visits and hospitalizations for food-induced anaphylaxis in US children. *Pediatr Allergy Immunol* (2018) 29(5):538–44. doi: 10.1111/pai.12908

16. Tang ML, Mullins RJ. Food allergy: is prevalence increasing? *Intern Med J* (2017) 47(3):256–61. doi: 10.1111/imj.13362

17. Smith SS, Hilas O. Peanut (Arachis hypogaea) allergen powder-dnfp: the first FDA-approved oral immunotherapy for desensitization of peanut allergy in children. *J Pediatr Pharmacol Ther* (2021) 26(7):669–74. doi: 10.5863/1551-6776-26.7.669

18. Vickery BP, Vereda A, Casale TB, Beyer K, du Toit G, Hourihane JO, et al. AR101 oral immunotherapy for peanut allergy. *N Engl J Med* (2018) 379(21):1991–2001. doi: 10.1056/NEJMoa1812856

19. Bird JA, Spergel JM, Jones SM, Rachid R, Assa'ad AH, Wang J, et al. Efficacy and safety of AR101 in oral immunotherapy for peanut allergy: results of ARC001, a randomized, double-blind, placebo-controlled phase 2 clinical trial. *J Allergy Clin Immunol Pract* (2018) 6(2):476–485.e3. doi: 10.1016/j.jaip.2017.09.016

20. Perkin MR. Palforzia for peanut allergy: Panacea or predicament. *Clin Exp Allergy* (2022) 52(6):729–31. doi: 10.1111/cea.14145

21. Mustafa SS, Patrawala S. Real world adoption of FDA-approved peanut oral immunotherapy with palforzia. *J Allergy Clin Immunol* (2021) 147(2):1. doi: 10.1016/j.jaci.2020.12.401

22. Li X-M. *Alternative/Integrative Medical Approaches in Allergy and Sleep: Basic Principles and Clinical Practice, in Allergy and Sleep*. Fishbein A, Sheldon SH, editors. Switzerland: Springer Nature (2019).

23. Leung DY, Shanahan WR, Li XM, Sampson HA. New approaches for the treatment of anaphylaxis. *Novartis Found Symp* (2004) 257:248–60;discussion 260-4,276-85. doi: 10.1002/0470861193.ch20

24. Leung DY, Sampson HA, Yunginger JW, Burks AW, Schneider LC, Wortel CH, et al. Effect of anti-IgE therapy in patients with peanut allergy. *N Engl J Med* (2003) 348 (11):986–93. doi: 10.1056/NEJMoa022613

25. Umetsu DT, Rachid R, Schneider LC. Oral immunotherapy and anti-IgE antibody treatment for food allergy. *World Allergy Organ J* (2015) 8(1):20. doi: 10.1186/s40413-015-0070-3

26. Hendeles L, Sorkness CA. Anti-immunoglobulin E therapy with omalizumab for asthma. *Ann Pharmacother* (2007) 41(9):1397–410. doi: 10.1345/aph.1K005

27. Luger EO, Wegmann M, Achatz G, Worm M, Renz H, Radbruch A. Allergy for a lifetime? *Allergol Int* (2010) 59(1):1–8. doi: 10.2332/allergolint.10-RAI-0175

28. López-Expósito I, Castillo A, Yang N, Liang B, Li XM. Chinese herbal extracts of *Rubia cordifolia* and *Dianthus superbus* suppress IgE production and prevent peanut-induced anaphylaxis. *Chin Med* (2011) 6:35. doi: 10.1186/1749-8546-6-35

29. Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* (2000) 106(1 Pt 1):150–8. doi: 10.1067/mai.2000.107395

30. Beyer K, Morrow E, Li XM, Bardina L, Bannon GA, Burks AW, et al. Effects of cooking methods on peanut allergenicity. *J Allergy Clin Immunol* (2001) 107(6):1077–81. doi: 10.1067/mai.2001.115480

31. Yang N, Wang J, Liu C, Song Y, Zhang S, Zi J, et al. Berberine and limonin suppress IgE production by human B cells and peripheral blood mononuclear cells from food-allergic patients. *Ann Allergy Asthma Immunol* (2014) 113(5):556–564.e4. doi: 10.1016/j.anai.2014.07.021

32. Institute of laboratory animal resources commission of life sciences NRC. In: *Guide for the Care and Use of Laboratory Animals*. Washington DC: National Academic Press.

33. Kulis M, Li Y, Lane H, Pons L, Burks W. Single-tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts. *J Allergy Clin Immunol* (2011) 127(1):81–8. doi: 10.1016/j.jaci.2010.09.014

34. Srivastava KD, Song Y, Yang N, Liu C, Goldberg IE, Nowak-Wegrzyn A. B-FAHF-2 plus oral immunotherapy (OIT) is safer and more effective than OIT alone in a murine model of concurrent peanut/tree nut allergy. *Clin Exp Allergy* (2017) 47(8):1038–49. doi: 10.1111/cea.12936

35. Srivastava KD, Siefert A, Fahmy TM, Caplan MJ, Li XM, Sampson HA. Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. *J Allergy Clin Immunol* (2016) 138(2):536–43.e4. doi: 10.1016/j.jaci.2014.12.1701

36. Srivastava KD, Bardina L, Sampson HA, Li XM. Efficacy and immunological actions of FAHF-2 in a murine model of multiple food allergies. *Ann Allergy Asthma Immunol* (2012) 108(5):351–358.e1. doi: 10.1016/j.anai.2012.03.008

37. Qu C, Srivastava K, Ko J, Zhang TF, Sampson HA, Li XM. Induction of tolerance after establishment of peanut allergy by the food allergy herbal formula-2 is associated with up-regulation of interferon-gamma. *Clin Exp Allergy* (2007) 37(6):846–55. doi: 10.1111/j.1365-2222.2007.02718.x

38. Song Y, Liu C, Hui Y, Srivastava K, Zhou Z, Chen J, et al. Maternal allergy increases susceptibility to offspring allergy in association with TH2-biased epigenetic alterations in a mouse model of peanut allergy. *J Allergy Clin Immunol* (2014) 134 (6):1339–1345.e7. doi: 10.1016/j.jaci.2014.08.034

39. Berger Y, Castonguay A. The carbon-13 nuclear magnetic resonance spectra of anthraquinone, eight polyhydroxyanthraquinones and eight polymethoxyanthraquinones. *Organic Magnetic Resonance* (1978) 11(8):3. doi: 10.1002/mrc.1270110802

40. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* (2003) 112(11):1666–77. doi: 10.1172/JCI19785

41. Elser B, Lohoff M, Kock S, Giaisi M, Kirchhoff S, Krammer PH, et al. IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. *Immunity* (2002) 17(6):703–12. doi: 10.1016/S1074-7613(02)00471-5

42. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol* (2009) 9(2):91–105. doi: 10.1038/nri2487

43. Kwon NH, Kim JS, Lee JY, Oh MJ, Choi DC. DNA methylation and the expression of IL-4 and IFN-gamma promoter genes in patients with bronchial asthma. *J Clin Immunol* (2008) 28(2):139–46. doi: 10.1007/s10875-007-9148-1

44. Srivastava KD, Kattan JD, Zou ZM, Li JH, Zhang L, Wallenstein S, et al. The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. *J Allergy Clin Immunol* (2005) 115(1):171–8. doi: 10.1016/j.jaci.2004.10.003

45. Srivastava K, Yang N, Chen Y, Lopez-Exposito I, Song Y, Goldfarb J, et al. Efficacy, safety and immunological actions of butanol-extracted Food Allergy Herbal Formula-2 on peanut anaphylaxis. *Clin Exp Allergy* (2011) 41(4):582–91. doi: 10.1111/j.1365-2222.2010.03643.x

46. Yang N, Maskey AR, Srivastava K, Kim M, Wang Z, Musa I, et al. Inhibition of pathologic immunoglobulin E in food allergy by EBF-2 and active compound berberine associated with immunometabolism regulation. *Front Immunol* (2023) 14:1081121. doi: 10.3389/fimmu.2023.1081121

47. Yang N, Srivastava K, Song Y, Liu C, Cho S, Chen Y, et al. Berberine as a chemical and pharmacokinetic marker of the butanol-extracted Food Allergy Herbal Formula-2. *Int Immunopharmacol* (2017) 45:120–7. doi: 10.1016/j.intimp.2017.01.009

48. Itokawa H, Ibraheim ZZ, Qiao YF, Takeya K. Anthraquinones, naphthohydroquinones and naphthohydroquinone dimers from *Rubia cordifolia* and their cytotoxic activity. *Chem Pharm Bull (Tokyo)* (1993) 41(10):1869–72. doi: 10.1248/cpb.41.1869

49. Murthy HN, Joseph KS, Paek KY, Park SY. Anthraquinone production from cell and organ cultures of. *Metabolites* (2022) 13(1):39. doi: 10.3390/metabo13010039

50. Yang HS, Wang J, Guo C, Liu W, Chen YY, Wei JF, et al. Simultaneous determination of alizarin and rubimaillin in *Rubia cordifolia* by ultrasound-assisted ionic liquid-reversed phase liquid chromatography. *Zhongguo Zhong Yao Za Zhi* (2015) 40(13):2617–23.

51. Gauvreau GM, Arm JP, Boulet LP, Leigh R, Cockcroft DW, Davis BE, et al. Efficacy and safety of multiple doses of QGE031 (ligelizumab) versus omalizumab and placebo in inhibiting allergen-induced early asthmatic responses. *J Allergy Clin Immunol* (2016) 138(4):1051–9. doi: 10.1016/j.jaci.2016.02.027

52. Nyborg AC, Zacco A, Ettinger R, Jack Borrok M, Zhu J, Martin T, et al. Development of an antibody that neutralizes soluble IgE and eliminates IgE expressing B cells. *Cell Mol Immunol* (2016) 13(3):391–400. doi: 10.1038/cmi.2015.19

53. Romagnani S. Immunologic influences on allergy and the TH1/TH2 balance. *J Allergy Clin Immunol* (2004) 113(3):395–400. doi: 10.1016/j.jaci.2003.11.025

54. Cañas JA, Núñez R, Cruz-Amaya A, Gómez F, Torres MJ, Palomares F, et al. Epigenetics in food allergy and immunomodulation. *Nutrients* (2021) 13(12):4345. doi: 10.3390/nu13124345

55. Berni Canani R, Paparo L, Nocerino R, Cosenza L, Pezzella V, Di Costanzo M, et al. Differences in DNA methylation profile of Th1 and Th2 cytokine genes are associated with tolerance acquisition in children with IgE-mediated cow's milk allergy. *Clin Epigenet* (2015) 7:38. doi: 10.1186/s13148-015-0070-8

56. Li CH, Chiu KL, Hsia TC, Shen TC, Chen LH, Yu CC, et al. Significant association of cyclin D1 promoter genotypes with asthma susceptibility in Taiwan. *In Vivo* (2021) 35(4):2041–6. doi: 10.21873/invivo.12473

57. Thun GA, Imboden M, Berger W, Rochat T, Probst-Hensch NM. The association of a variant in the cell cycle control gene CCND1 and obesity on the development of asthma in the Swiss SAPALDIA study. *J Asthma* (2013) 50(2):147–54. doi: 10.3109/02770903.2012.757776

58. Wang Y, Hu H, Wu J, Zhao X, Zhen Y, Wang S, et al. The IL6R gene polymorphisms are associated with sIL-6R, IgE and lung function in Chinese patients with asthma. *Gene* (2016) 585(1):51–7. doi: 10.1016/j.gene.2016.03.026

59. Vazquez MI, Catalan-Dibene J, Zlotnik A. B cells responses and cytokine production are regulated by their immune microenvironment. *Cytokine* (2015) 74 (2):318–26. doi: 10.1016/j.cyto.2015.02.007

60. Tiburzy B, Kulkarni U, Hauser AE, Abram M, Manz RA. Plasma cells in immunopathology: concepts and therapeutic strategies. *Semin Immunopathol* (2014) 36(3):277–88. doi: 10.1007/s00281-014-0426-8

61. Halliley JL, Tipton CM, Liesveld J, Rosenberg AF, Darce J, Gregoretti IV, et al. Long-lived plasma cells are contained within the CD19(-)CD38(hi)CD138(+) subset in human bone marrow. *Immunity* (2015) 43(1):132–45. doi: 10.1016/j.immuni.2015.06.016

62. Ren Z, Spaargaren M, Pals ST. Syndecan-1 and stromal heparan sulfate proteoglycans: key moderators of plasma cell biology and myeloma pathogenesis. *Blood* (2021) 137(13):1713–8. doi: 10.1182/blood.2020008188

63. Hanna A, Nixon MJ, Estrada MV, Sanchez V, Sheng Q, Opalenik SR, et al. Combined Dusp4 and p53 loss with Dbf4 amplification drives tumorigenesis via cell cycle restriction and replication stress escape in breast cancer. *Breast Cancer Res* (2022) 24(1):51. doi: 10.1186/s13058-022-01542-y

64. Kim GC, Lee CG, Verma R, Rudra D, Kim T, Kang K, et al. ETS1 suppresses tumorigenesis of human breast cancer via trans-activation of canonical tumor suppressor genes. *Front Oncol* (2020) 10:642. doi: 10.3389/fonc.2020.00642

65. Yu M, Li G, Lee WW, Yuan M, Cui D, Weyand CM, et al. Signal inhibition by the dual-specific phosphatase 4 impairs T cell-dependent B-cell responses with age. *Proc Natl Acad Sci USA* (2012) 109(15):E879–88. doi: 10.1073/pnas.1109797109

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Research progress on the mechanism of probiotics regulating cow milk allergy in early childhood and its application in hypoallergenic infant formula

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Some infants and young children suffer from cow's milk allergy (CMA), and have always mainly used hypoallergenic infant formula as a substitute for breast milk, but some of these formulas can still cause allergic reactions. In recent years, it has been found that probiotic nutritional interventions can regulate CMA in children. Scientific and reasonable application of probiotics to hypoallergenic infant formula is the key research direction in the future. This paper discusses the mechanism and clinical symptoms of CMA in children. This review critically ex- amines the issue of how probiotics use intestinal flora as the main vector to combine with the immune system to exert physiological functions to intervene CMA in children, with a particular focus on four mechanisms: promoting the early establishment of intestinal microecological balance, regulating the body's immunity and alleviating allergic response, enhancing the intestinal mucosal barrier function, and destroying allergen epitopes. Additionally, it overviews the development process of hypoallergenic infant formula and the research progress of probiotics in hypoallergenic infant formula. The article also offers suggestions and outlines potential future research directions and ideas in this field.

KEYWORDS

cow's milk allergy, probiotics, regulation, hypoallergenic infant formula, mechanism

1 Introduction

Food allergy refers to the abnormal immune reaction to food proteins, which leads to the disorder of physiological function or tissue damage of the body, thus causing a series of clinical symptoms (1). According to the statistics of the World Health Organization (WHO), at present, food allergic reaction has risen to the sixth place in the global diseases, and the number of people suffering from such diseases has increased exponentially (2), affecting more than 20% of the world's population, especially children (3), and becoming the most important non-infectious disease affecting children's health. "Big-8 allergenic foods" had been identified, including gluten containing grains, crustaceans, fish, eggs, peanuts, soybeans, milk, nuts and products of the above 8 categories of substances (4). A series of investigation results show that the early life allergic reaction is mainly milk, egg allergy (5–11).

CMA is an allergic immune response to cow milk protein (CMP) that usually develops in the first few months after birth (12, 13). Cow's milk is an important source of nutrients when breastfeeding is insufficient (14). For children with CMA, different types of hydrolyzed formulas (HF) are recommended, extensively hydrolyzed formula (eHF) as the first choice for CMA treatment, and amino acid formulas (AAF) for more severe cases or those with reaction to eHF (15–18). In recent years, the infant formula adding probiotics were developed, and whether probiotics can reduce the risk of CMA, the present manuscript summarizes and discusses the mechanism and application of probiotics in early life to regulate CMA in children.

2 Mechanism and clinical symptoms of CMA in children

Cow milk contains large allergenic proteins. Most important of them are casein, β-lactoglobulin (BLG), and α-lactalbumin (19). CMA is one of the most common food allergies and ranks third among all food allergies leading to anaphylaxis (8–15% of cases) especially in childhood, affecting about 3–8% of children in different countries (20, 21). Infants are prone to CMA, which is mainly caused by the immature development of intestinal barrier and the incomplete development of immune system (22, 23). The intestinal mucosal cells of infants are sparsely arranged, the intestinal osmotic pressure is increased, and allergens are easy to enter the blood through mucosal cells to cause allergy.

Based on immunological mechanisms, CMA can be divided into three types, including immunoglobulin E (IgE) -mediated, non IgE -mediated, and combined (Figure 1). In a Brazilian referral center of Allergy and Clinical Immunology, clinical history, laboratorial findings and test results were collected from 115 pediatric patients with CMA in 2017 through electronic medical record, the results showed that 57% of the reactions were IgE-mediated, 20% were non-IgE-mediated and 23%, mixed reactions (24). An IgE-mediated CMA is a type I hypersensitivity reaction or immediate CMA, and the clinical manifestations occur within minutes to 2 h after milk ingestions, which involves mast cell degranulation (25). Tang et al. shown that among the 234 participants who were measured by an allergen array, 9 were boiled milk sIgE-positive, 50 were yogurt sIgE-positive, 17 were buttermilk sIgE-positive, and 158 were only raw milk sIgE-positive (26). Non IgE-mediated CMA often present symptoms 2 h to even several days induced by exposure to cow milk, involving respiratory tract, gastrointestinal tract and other parts (27, 28), including type II or type III hypersensitivity reactions mediated by IgG or IgM and tissue damage caused by complement, basophils and neutrophils, and type IV hypersensitivity reactions mediated by T lymphocytes (29). The allergic mechanism of non-IgE-mediated immune response is currently under debate and still needs further research. Combined CMA may be related to the cross-inhibitory response of Th1 and Th2 in the immune system of newborn infants (30). This cross-inhibitory response may have humoral and/or cell-mediated mechanisms and may present with symptoms such as atopic dermatitis, allergic eosinophilic esophagitis, and eosinophilic gastritis.

In recent years, more and more studies have shown that the imbalance of Treg and pro-inflammatory Th17 cells (Treg/Th17) is also one of the key factors causing allergic diseases. When milk protein allergic reaction occurs, Th17 is dominant, and the number of Treg decreases (31).

3 The development of hypoallergenic infant formula

Hypoallergenic foods are those that are well tolerated in at least 90% (95% of confidence interval) individuals with allergies in double-blind, placebo-controlled trials (32, 33). Hypoallergenic infant formula is a kind of infant formula for special medical use, and it can be divided into partially hydrolyzed formulas (pHF), eHF, and AAF (see Table 1) (34). Hydrolysis may destroy the epitopes of CMA by hydrolyzing part or all of the milk proteins into small molecular peptides and amino acids, which reduces the antigenicity of CMP. pHF is mainly used for dietary management of infants with functional gastrointestinal disorders and can also be used for initial intervention feeding of non-breastfed infants at high risk of milk protein allergy (whose parents or siblings have a history of allergy). eHF and AAF are mainly used in the dietary management of infants with CMA (35–39).

In some pHF, B cell epitopes of cow milk allergens are still present, which can cause CMA (40). It has been shown that there is a significant difference in BLG residues between pHF and eHF. The BLG level of pHF is 40,000 times higher than that of eHF (41). pHF seems to be a better alternative to infant formula based on CMP (42). However, pHF outperforms most eHF in terms of cost and taste preference (43). Although there is no clear definition of eHF and pHF, these two infant formulas have been developed and commercially available. Hydrolyzed infant formula varies due to protein source, degree of hydrolysis, protease species, auxiliary processing techniques (such as thermal processing) and peptide profile, and allergen residues may still be present (42). The antigenicity of allergen residues in infant formula depends on the degree of hydrolysis and filtration techniques applied during the preparation; therefore, it is recommended that the safety of the hydrolyzed infant formula be first confirmed before it is introduced into the diet of CMA infants (41). The main criterion for labeling infant formula as hypoallergenic is that 90% of children or infants with CMA confirmed by double-blind, placebo-controlled trials do not exhibit allergic reactions (44).

Based on pHF and eHF research, AAF was subsequently developed. Studies have shown that the use of AAF can greatly reduce sensitization reactions (45). AAF supplementation is recommended for infants with pHF or eHF allergy, growth retardation, or multiple food allergies (45). The main problem with AAF not being widely applied is the high cost and rather unpleasant taste (46). In some studies, eHFs, and AAFs were noted to be hypoallergenic, while pHFs was not included because the allergic reaction caused by it was unpredictable (47).

Up to now, the impact of intestinal flora diversity and/or dysfunction (dysbiosis) on food allergy has attracted more people's attention, and the addition of probiotics to infant formula to develop new products has become a research hotspot. At present, there are mainly the following probiotics that are

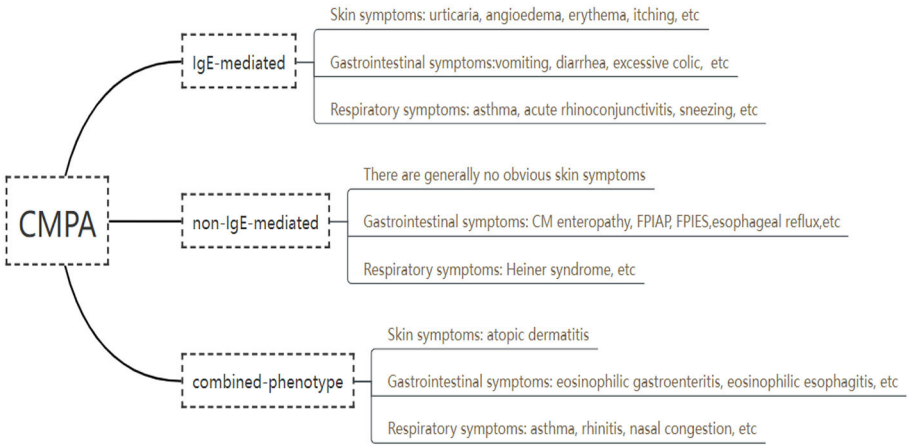

FIGURE 1
Clinical symptoms of cow's milk allergy in children.

TABLE 1 Classification and characteristics of infant formula for special medical purposes (34).

Type	Molecular size	Main characteristics of the formula	Effectiveness of immunization
pHF	<5,000 Da	1. Some epitopes of the allergen remain. 2. Peptides of sufficient size were retained to stimulate the induction of oral tolerance based on deeply hydrolyzed milk protein formulations	1. It is beneficial to the normal development of intestinal tract 2. Ingestion of cow's milk protein over a period of time can stimulate infant tolerance to cow's milk protein
eHF	<1,500 Da	1. The antigenicity is reduced, and the molecular mass is small 2. Trace amounts of allergens were present	1. It reduces the allergic reaction and stimulates the development of immune tolerance 2. Long-term consumption affects the development of intestinal function in infants, and gastrointestinal reactions still occur
AAF	<500 Da	1. Based on oligopeptide and purified amino acid, the effect of milk allergens can be avoided 2. It contains normal carbohydrates and fats, providing nutrients for infant growth and development	1. It has a significant improvement effect on the condition 2. It is not clear whether immune tolerance to cow's milk protein can be established

permitted to be used in infant food in China, such as *Lactobacillus acidophilus* NCFM, *Bifidobacterium animalis subsp. Lactis* Bb-12, *Bifidobacterium animalis subsp. Lactis* HN019, *Bifidobacterium animalis subsp. Lactis* Bi-07, *Lacticaseibacillus rhamnosus* GG (LGG), *Lacticaseibacillus rhamnosus* HN001, *Lacticaseibacillus rhamnosus* MP108, *Limosilactobacillus reuteri* DSM 17938, *Limosilactobacillus fermentum* CECT 5716, *Bifidobacterium breve* M-16V, *Lactobacillus helveticus* R0052, *Bifidobacterium bifidum* R0071, *Bifidobacterium longum subsp. longum* BB536, *Bifidobacterium longum subsp. infantis* R0033, etc. (48–50). The conditions under which these probiotics are used in hypoallergenic formulas need to be researched in depth.

4 Physiological functions of probiotics on children's health

As for the concept of probiotics, the most widely used is the definition of Food and Agriculture Organization (FAO) and WHO (51): Probiotics are living microorganisms that, when ingested in sufficient quantities, produce one or more demonstrated physiological functional benefits to the host.

The intestinal flora of infants is mainly dominated by Bifidobacterium, which maintains a dynamic balance with the changes of environment, diet and lifestyle in the later period (52). When the balance of intestinal flora is broken, the disordered intestinal flora will affect the occurrence and development of many diseases (53). Probiotics can play a beneficial role by regulating the abundance of intestinal flora and its metabolites. Clinical studies have found that Probio-M8 can improve asthma symptoms by regulating intestinal flora (54). In addition to directly acting on intestinal flora, probiotics can also play a role by indirectly regulating metabolites of intestinal flora, such as short-chain fatty acids (SCFA) (55), bile acids, lipids, and neurotransmitters, so as to improve the health of the body.

Different probiotic strains and doses can have different effects on health outcomes, and no one-size-fits-all strain addresses all health outcomes. However, probiotic supplementation is thought to trigger numerous immunological benefits through signaling pathways, cytokine expression. Induction of cytokine secretion by probiotic bacteria exhibited strain specificity, and the response may also vary in the presence of different species of probiotic bacteria or a mixture of probiotic bacteria (56). Ingested probiotics

have been reported to interact with enterocytes and dendritic cells, Th1, Th2, and regulatory T cells (Tregs) in the gut. It has been shown that probiotics can reduce inflammation by stimulating anti-inflammatory cytokines and reducing pro-inflammatory cytokines, which in turn modulate NK cell activity and inhibit Toll-like receptor (TLR), which in turn inhibits the nuclear factor-kappa B (NF-κB) pathway (57). Probiotics have been shown to suppress intestinal inflammation by down-regulating TLR expression. Depending on the type of TLR, reduced expression of TLR can lead to multiple benefits, such as reduced NF-κB activity and other proinflammatory expressions (58).

One of the important functional indicators to monitor the immunity enhancement of probiotics is its ability to inhibit the growth of pathogenic bacteria, which can secrete peptides and organic acids to inhibit the production of harmful substances such as amines and indole, thus having the effect of inhibiting pathogenic bacteria and relieving inflammation (59). Lactobacillus rhamnosus, the most common type of lactic acid bacteria in probiotics, can produce SCFA such as acetic acid, propionic acid and butyric acid through fermentation, thereby changing the osmotic pressure inside and outside the cells, forming an acidic environment, and having a synergistic effect on inhibiting pathogenic bacteria (60). Studies have also shown that probiotics can also secrete bacteriocins for wall membrane and intracellular to inhibit the growth of pathogenic bacteria (61–63). This is undoubtedly advantageous for children whose immune defense mechanisms are not well developed.

Due to the particularity of the children population and the complexity of CMA mechanism, in the industrial production of hypoallergic infant formula, how to improve the physiological function of probiotics is a key aspect of future research (64).

5 Mechanism of probiotics in the prevention and regulation of CMA in children

Since CMA children have shown differences in their gut microbiome composition (number and diversity of species), modulation of the intestinal microbiota seems a promising strategy for the control of allergic reactions. In addition, there are some evidences on the beneficial effects of probiotics on the natural history of CMA, recovery from CMA and the appearance of other allergic manifestations in pediatric age (65–68). Hence, there is increasing interest in the use of probiotics for the prevention and treatment of food allergies.

Based on the recent studies on the effects of probiotics and their metabolites on CMA in children, we speculate that probiotics may play an important role in CMA by promoting the early establishment of intestinal microecological balance, regulating the body's immunity, and enhancing the function of intestinal mucosal barrier. In addition, lactic acid bacteria, as one of the important probiotics, also have the potential to destroy allergen epitopes and thus reduce milk sensitization.

5.1 Promote the early establishment of intestinal microecological balance

The human microbiota is a complex microbial ecosystem composed of commensal, symbiotic, and pathogenic microorganisms that can be found in the gut, skin, oral cavity, nasal passages, and urogenital tract (69, 70). Early microbiota establishment is essential for proper immune development and is beneficial for overall health status (71). The colonization of the gut is a dynamic process that is thought to begin at the fetal stage, progressing through an ecologically ordered succession of species until reaching a steady and balanced composition (which occurs approximately 1,000 days after birth) (69, 70, 72–74).

Clinical studies of eHF supplemented with probiotics showed improved symptoms in infants with CMA (75–78). The addition of *Bifidobacterium* and LGG is beneficial to the early establishment of intestinal microecological balance in children with CMA (79). Candy et al. (80) showed that AAF including a prebiotic blend of fructo-oligosaccharides and the probiotic strain Bifidobacterium breve M-16V improves gut microbiota in non-IgE-mediated allergic infants. Canani et al. (81) found LGG-supplemented casein formula could cause enrichment of butyric acid-producing bacteria in gut for infants with CMA, thereby promoting tolerance and reducing the risk of allergy. Yanru (82) studied the structure of intestinal flora and SCFAs in feces of children with CMA and found that the presence of *Clostridium* and *Firmicutes* was related to infant's CMA, and the composition of SCFAs in feces of children was significantly different.

Probiotics can also promote the establishment of children's intestinal microecological balance by resisting pathogen colonization, because they may temporarily occupy the vacant functional niche in the resident microbiota and secrete reactive oxygen species to inhibit pathogen growth, thereby preventing opportunistic infections and reducing the occurrence of allergies (58).

5.2 Regulating the body's immunity and alleviating the allergic response

The addition of probiotics to infant formula to assist the management of CMA has become a research hotspot, this indicates that probiotics can regulate the body's immunity. As reviewed by Servin, different *Lactobacilli* and *Bifidobacteria* strains were reported to be capable of stimulating immune cells to secrete cytokines or shifting the Th2-type response back to a Th1-type response (83, 84). Song et al. (85) found that Lactobacillus rhamnosus2016SWU.05.0601 regulated immune balance in ovalbumin-sensitized mice by modulating expression of the immune-related transcription factors and gut microbiota, decreasing the levels of Th2 and Th17 but increasing the levels of Th1 and Treg cytokines.

Zhang's study indicates that oral administration of Bifidobacteria has the capacity to suppress the skewed Th2 response in allergic mice, increasing the number of Treg and IL-10-positive cells and improve the impaired intestinal epithelial barrier function (86), and Inoue et al. (87) showed that Bifidobacterium

breve M-16V modulated the systemic Th1/Th2 balance, suppressed the IgE production and reducing IL-4 levels by the *in vitro* and *in vivo* experiments. Lactobacillus casei strain Shirota (LcS) was administered intraperitoneally to ovalbumin-specific T cell receptor transgenic (OVA-TCRTg) mice, which increased IL-12 levels, decreased IgE and IgG1 levels, and restored the Th1/Th2 balance (88). The same immunological responses were also induced by Lactobacillus plantarum L-137 which could stimulate IL-12 production and reduce serum IgE and IgG levels (89).

The immunomodulatory effect of LGG on CMA has been extensively studied. Incidence of allergic symptoms decreased in children with CMA after taking extensively hydrolyzed casein formula (EHCF) containing LGG not only in mice but also in humans (90, 91). It has been reported that supplementing EHCF with LGG is more effective compared to EHCF alone in reducing CMA (44, 92). Similar results were found in another study by Thang et al., who used 3-week-old newly weaned Balb/c mice with adjuvant-free LGG sensitization to simulate CMA (93). In LGG-treated mice, Th2 responses were suppressed, resulting in remarkably lower hypersensitivity scores and CMP-specific IgG1 levels, and Th1 responses were promoted, resulting in increased levels of IFN and CMP-specific IgG2a (94). Moreover, lactic acid bacteria and its surface molecules can also affect the production of immune cells and cytokines (95). Therefore, the use of lactic acid bacteria fermentation to reduce milk sensitization has a good prospect (96–98).

Probiotics can also regulate the inflammatory signaling of intestinal epithelial cells to alleviate allergy. NF-κB and mitogen-activated protein kinase (MAPK) are two important inflammatory pathways. Studies have found that probiotics can inhibit the activation of NF-κB. The lactic acid bacteria could inhibit the phosphorylation of p38 MAPK and p65 NF-κB to mediate inflammatory responses (99). L. acidophilus L-92 could activate Th1 and Treg cells by participating in MAPK and NOD-like receptor pathways (100). In addition, DeMuri et al. (101) found that Lactobacillus acidophilus NCFM/Bifidobacterium lactis Bi-07 may alter inflammation by decreasing expression of E-selectin. Li et al. (102) indicates that Bifidobacterium breve M-16V may alter the gut microbiota to alleviate the allergy symptoms by IL-33/ST2 signaling. Wang et al. (103) showed that surface layer protein (Slp) of Lactobacillus acidophilus NCFM prevents TNF-α-stimulated cell apoptosis, as well as inhibits IL-8 secretion via inhibiting NF-κB activity, thereby exerting its anti-inflammatory activity. Chen et al. (104) found that LGG could effectively alleviate the allergic response, restore the levels of HIS, IgE, MCP, MCT, specific IgG, specific IgG1, specific IgG2a, and other inflammatory factors, and restore CD4+ T cell infiltration and the status of intestinal villi.

5.3 Enhance intestinal mucosal barrier function

The mucus layer of the intestinal mucosa is a mechanical barrier against pathogens (105). The intestinal mucosal immune system is the most complex part of the body's immune system. Intestinal commensal bacteria can stimulate the development and maturation of the intestinal mucosal immune system in the early stage, activate Th1 immune response, and inhibit IgE production to prevent allergic reactions. Moreover, research have shown that probiotics can enhance the host intestinal immune barrier and improve the immune regulation ability of Treg cells in the immune system (106). Colonized lactic acid bacteria can enhance the tight junction between epithelial cells, reduce intestinal permeability, support intestinal barrier function, and thus reduce the stimulation of allergens (107, 108). LGG could produce both a biofilm that can mechanically protect the mucosa, and different soluble factors beneficial to the gut by enhancing intestinal crypt survival, diminishing apoptosis of the intestinal epithelium, and preserving cytoskeletal integrity. Because the polysaccharides and pili present on LGG surface allow it to adhere to and temporarily colonize the intestinal mucosa (109).

5.4 Destruction of allergen epitopes thereby reducing CMA

As one of the important probiotics, lactic acid bacteria can not only regulate the composition of intestinal flora to play an immunomodulatory function or produce a variety of stimulus signals to activate immune cells, thereby triggering systemic immune response (110). On the other hand, lactic acid bacteria have a complex protease system (111), which can produce peptidase and protease to hydrolyze milk protein, destroy allergen epitopes (112), and thus reduce milk allergy (113).

Therefore, probiotic use not only emerges as a safe microbiological strategy in pediatrics for the promotion of intestinal immunity, but also becomes an important research direction for future CMA management.

6 Overviews in the application of probiotics in hypoallergenic infant formula

The application of probiotics to modulate the gut microbiome-immune axis to alleviate CMA has become a research hotspot. However, the mechanism by which the gut microbiota regulates CMA and the efficacy of probiotics are still in the preliminary exploration stage, and there are no clear and specific conclusions (114). Therefore, it is very important to locate specific strains in hypoallergenic infant formula.

Because of the different target proteins of hydrolysis, hydrolyzed infant formula is divided into hydrolyzed casein formula and hydrolyzed whey formula. However, some brands of pHF or eHF still have allergen B cell epitopes and can cause allergic reactions (115). In recent years, the combination of hydrolyzed proteases and probiotics has developed hypoallergenic milk protein hydrolysates, which have a broader application field. Probiotics have been shown to be beneficial in reducing symptoms in allergic patients, and adding probiotics to hypoallergenic infant formula is an innovative way to prevent and treat CMA (116). If a certain amount of LGG is added to eHF, it will bring many benefits to the infant, LGG more quickly induces the tolerance of infants with CMA, reduces the incidence of allergic dermatitis in infants, improves inflammation in the intestine to a certain extent, but also improves the recovery of allergic colitis (117). However,

the European Society of Pediatric Nutrition and the Society of Gastroenterology believe that these new infant formulas are still not entirely satisfactory because the real safety of the probiotics added to the formulas has not been fully evaluated (118).

The research on the anti-allergic mechanism of probiotics will be carried out through *in vitro* and *in vivo* experiments and establish a safety evaluation mechanism is the key research content in the future. Moreover, for the research and development of probiotic hypoallergenic formulations, the specific probiotics used alone or in combination, the timing of the start and end of treatment, and the appropriate dose are needed to be determined deeply.

7 Suggestions and prospects

For the management of CMA in children, probiotics combined with hypoallergenic infant formula to establish immune tolerance are recommended as the main route in the future. But the data on probiotics themselves as a CMA prevention strategy are imperfect. In-depth exploration of the mechanism of probiotics regulating CMA is the focus of current research.

The application of probiotics as functional ingredients in hypoallergenic infant formula by major brands in the dairy industry has become a research hotspot. However, the health effect of probiotics has high strain tolerance, and infants with different constitutions, different genetic backgrounds and different intestinal flora should be different, and the research on probiotics can be located on the individual strains. At present, researchers have evaluated the safety and the efficacy of EHCF with LGG by randomized, double-blind trial, and the results showed that EHCF + LGG could be tolerated by the vast majority of IgE-mediated CMA children, and that the step-down approach from AFF to EHCF + LGG could promote a faster acquisition of immune tolerance (119). Although the use of probiotics is beneficial to promote immune regulation and alleviate clinical symptoms, more methodologically based and homogenized research is needed to more specifically study each type, dose, and time of probiotic supplementation for the establishment of definitive care protocols.

An in-depth comparison of the mechanism of action of probiotics added to whey protein hydrolysate vs. casein hydrolysate to reduce CMA. Based on clinical data or the real-world evidence, the mechanism of probiotics in hypoallergenic formula powders was studied. Nevertheless, there is an increased risk of functional gastrointestinal disorders among infants with CMA which could be reduced among those fed with EHCF+LGG (120), and further research is required to fully elucidate the mechanism of action of the probiotics.

Author contributions

ML: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing—original draft. CYJ: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Project administration, Visualization, Writing—review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Van Ree R, Poulsen LK, Wong GW, Ballmer-Weber BK, Gao Z, Jia X. Food allergy: definitions, prevalence, diagnosis and therapy. *Chinese J Prevent Med.* (2015) 49:87–92. doi: 10.3760/cma.j.issn.0253-9624.2015.01.020

2. Jonathan MS, Amy SP. Atopic dermatitis and the atopic march. *J Allergy Clin Immunol.* (2003) 112:S118–27. doi: 10.1016/j.jaci.2003.09.033

3. Zheng T, Yu J, Oh MH, Zhu Z. The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. *Allergy, Asthma Immunol Res.* (2011) 3:67–73. doi: 10.4168/aair.2011.3.2.67

4. Candreva AM, Smaldini PL, Curciarello R, Cauerhff A, Fossati CA, Docena GH. Cross-reactivity between the soybean protein P34 and bovine caseins. *Aller Asthma Immun.* (2015) 7:60–8. doi: 10.4168/aair.2015.7.1.60

5. Jie HL. Epidemiology of food allergy in children from 31 cities in China. *Int J Pediatr.* (2017) 44:637–41.

6. Wang S, Jiang JX, Wang Y, Wang ZH, Wang T, Wang HS. Survey on prevalence of allergic symptoms among 0 to 24 months old children in Chinese cities. *Chin J Child Health Care.* (2016) 24:119–22.

7. Sha L, Shao M, Liu C, Wu Y, Chen Y. A cross-sectional study of the prevalence of food allergies among children younger than ages 14 years in a Beijing urban region. In: *Allergy and Asthma Proceedings*, (2019). doi: 10.2500/aap.2019.40.4193

8. Xiao YL, Pan JF, Wang LP, Duan YL. Food allergy status of infants and young children in community in Shanghai and influencing factors. *J Clin Med Pract.* (2018) 22:72–78. doi: 10.7619/jcmp.201811020

9. Nie J, Ran YC, Zhang YG, Chen J. The prevalence of food hypersensitivity in 0-24 months old children in Chengdu. *Chin J Woman Child Health Res.* (2017) 28:364–365.

10. Chen J, Niao Y, Zhang HZ, Zhao H. To investigate the prevalence of food allergy in children under 2 years of age in three cities of China. *Chin J Pediatr.* (2012) 50:5. doi: 10.1097/01.WOX.0000411608.35185.f5

11. Zou Y, Xu YL, Shen XM. Prevalence of food allergy in children under 3 years of age in Panzhihua city. *Chin J Public Health.* (2013) 29:1813–5. doi: 10.11847/zgggws2013-29-12-30

12. Johansson S, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF. Revised nomenclature for allergy for global use: report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immun.* (2004) 113:832–6. doi: 10.1016/j.jaci.2003.12.591

13. Fiocchi J, Brozek H, Schünemann S, Bahna L, von Berg A, Beyer K. World Allergy organization (WAO) diagnosis and rationale for action against cow's milk allergy (DRACMA) guidelines. *Pediat Allerg Immn.* (2010) 21:1–125. doi: 10.1111/j.1399-3038.2010.01068.x

14. Zepeda-Ortega B, Goh A, Xepapadaki P, Sprikkelman A, Nicolaou N, Hernandez REH. Strategies and future opportunities for the prevention, diagnosis, and management of cow milk allergy. *Front Immunol.* (2021) 12:608372. doi: 10.3389/fimmu.2021.608372

15. Meyer R, Groetch M, Venter C. When should infants with cow's milk protein allergy use an amino acid formula? A practical guide. *J Aller Clin Immunol.* (2018) 6:383–99. doi: 10.1016/j.jaip.2017.09.003

16. Groetch M, Baker MG, Durban R, Meyer R, Venter C, Muraro A. The practical dietary management of food protein-induced enterocolitis syndrome. *Ann Aller Asthma Immunol.* (2021) 127:28–35. doi: 10.1016/j.anai.2021.03.007

17. D'Auria E, Salvatore S, Acunzo M, Peroni D, Pendezza E, Di Profio E, et al. Hydrolysed formulas in the management of cow's milk allergy: new insights, pitfalls and tips. *Nutrients.* (2021) 13:2762. doi: 10.3390/nu13082762

18. Salvatore S, Agosti M, Baldassarre M E, D'Auria E, Pensabene L, Nosetti L, et al. Cow's milk allergy or gastroesophageal reflux disease-can we solve the dilemma in infants? *Nutrients.* (2021) 13:297. doi: 10.3390/nu13020297

19. Thompson G, Zhelev Z, Peters J, Khalid S, Briscoe S, Shaw L, et al. Symptom scores in the diagnosis of pediatric cow's milk protein allergy: a systematic review. *Pediatr Aller Immunol.* (2021) 32:1497–507. doi: 10.1111/pai.13537

20. Cianferoni A, Muraro A. Food-induced anaphylaxis. *Immunol Aller Clin.* (2012) 32:165–95. doi: 10.1016/j.iac.2011.10.002

21. Lajnaf R, Attia H, Ayadi MA. Technological properties and biological activities of camel a-lactalbumine: a review. *Int Dairy J.* (2023) 139:105563. doi: 10.1016/j.idairyj.2022.105563

22. Takiishi T, Fenero CI, Câmara NO. Intestinal barrier and gut microbiota: shaping our immune responses throughout life. *Tissue Barr.* (2017) 5:e1373208. doi: 10.1080/21688370.2017.1373208

23. Wang L, Zhu L, Qin S. Gut microbiota modulation on intestinal mucosal adaptive immunity. *J Immunol Res.* (2019) 2019:4735040. doi: 10.1155/2019/4735040

24. Marino LM, Rozalem-Reali AC, Cancado BL, Gontijo JC, Pereira RA, Manhaes IB, et al. Oral tolerance versus immunological mechanism in children with cow's milk allergy. *J Allergy Clin Immun.* (2019) 143:AB167. doi: 10.1016/j.jaci.2018.12.509

25. Xin L, Xu ZH, Huang MJ, Wu Y, Hu LM, Chen HB. Progress on the reduction of allergenicity of bovine milk proteins by lactic acid bacteria. *J Food Sci Biotechnol.* (2021) 40:12–9.

26. Tang R, Lyu X, Liu Y, Zhu M, Yang X, Wu Z, et al. Four clinical phenotypes of cow's milk protein allergy based on dairy product specific IgE antibody types in North China. *Front Immunol.* (2022) 13:949629. doi: 10.3389/fimmu.2022.949629

27. Venter C, Pereira B, Grundy J, Clayton CB, Roberts G, Higgins B, et al. Incidence of parentally reported and clinically diagnosed food hypersensitivity in the first year of life. *J Aller Clin Immunol.* (2006) 117:1118–24. doi: 10.1016/j.jaci.2005.12.1352

28. Zopf Y, Hahn EG, Raithel M, Baenkler HW, Silbermann A. The differential diagnosis of food intolerance. *Deutsches Ärzteblatt Int.* (2009) 106:359. doi: 10.3238/arztebl.2009.0359

29. Morita H, Nomura I, Matsuda A, Saito H, Matsumoto K. Gastrointestinal food allergy in infants. *Allergol Int.* (2013) 62:297–307. doi: 10.2332/allergolint.13-RA-0542

30. Liu D, Cong YJ. Application of non-thermal processing technology in hypo- or non-allergenic infant formula. *Sci Technol Food Ind.* (2021) 42, 395–402. doi: 10.13386/j.issn1002-0306.2020080026

31. Chen J, Zhang XN, Huo QW Li MH, Shang YN, Wang JG. Progress in the mechanisms of cow's milk protein allergy in infants and its treatments. *J Chin Inst Food Sci Technol.* (2020) 20:289–98. doi: 10.16429/j.1009-7848.2020.07.035

32. Mahler V, Goodman RE. Definition and design of hypoallergenic foods. In: Kleine-Tebbe, J, Jakob, T. editors *Molecular Allergy Diagnostics,* Cham: Springer (2017). doi: 10.1007/978-3-319-42499-6_27

33. Roberts G, Grimshaw K, Beyer K, Boyle R, Lack G, Austin M, et al. Can dietary strategies in early life prevent childhood food allergy? A report from two iFAAM workshops. *Clin Exper Aller.* (2019) 49:1567–77. doi: 10.1111/cea.13515

34. Ministry of Health of the People's Republic of China. *National Food Safety Standard - General Rules of Infant Formula for Special Medical Purposes: GB 25596-2010[S].* Beijing: Standards Press of China (2010).

35. Shi J, Zhou YD, Luo YK. Detection and analysis of binding activities of lgE antibodies from child patients allergic to cow's milk protein. *J China Agric Univ.* (2017) 22:40–4. doi: 10.11841/j.issn.1007-4333.2017.09.05

36. Ren WJ. Clinical efficacy of extensively hydrolyzed protein formula in the treatment of cow's milk protein allergy in infants. *Maternal Child Health Care China.* (2018) 33:3729–3731. doi: 10.7620/zgfybj.j.issn.1001-4411.2018.16.42

37. Wei M, Li WB, Chen PJ. Neonatal cow's milk protein allergy: analysis of misdiagnosis of 4 neonates. *Chin J Appl Clin Pediatr.* (2018) 33:145–7. doi: 10.3760/cma.j.issn.2095-428X.2018.02.015

38. Zhao LF, Zhang MJ, Zhu LJ. To study the clinical characteristics of infants with cow's milk protein allergy and the efficacy and safety of amino acid free formula replacement/extensively hydrolyzed formula sequential intervention. *Maternal Child Health Care China.* (2020) 35:4526–9. doi: 10.19829/j.zgfybj.issn.1001-4411.2020.23.040

39. Nutten S, Maynard F, Järvi A, Rytz A, Simons PJ, Heine RG, et al. Peptide size profile and residual immunogenic milk protein or peptide content in extensively hydrolyzed infant formulas. *Allergy.* (2020) 75:1446. doi: 10.1111/all.14098

40. Meulenbroek LA, Oliveira S, den Hartog Jager CF, Klemans RJ, Lebens AF, Van Baalen T, et al. The degree of whey hydrolysis does not uniformly affect in vitro basophil and T cell responses of cow's milk-allergic patients. *Clin Exper Aller.* (2014) 44:529–39. doi: 10.1111/cea.12254

41. Kuslys M, Nutten S, Anette J, Maynard F, Affolter M, Fryer P, et al. Extensively hydrolyzed formulas for the management of cow's milk protein allergy in infants: is extensive hydrolysis sufficient to guarantee success? *Clin Exper Aller.* (2018) 11:48.

42. Meltretter J, Wust J, Pischetsrieder M. Modified peptides as indicators for thermal and nonthermal reactions in processed milk. *J Agr Food Chem.* (2014) 62:10903–15. doi: 10.1021/jf503664y

43. Verduci E, D'Elios S, Cerrato L, Comberiati P, Calvani M, Palazzo S, et al. Cow's milk substitutes for children: Nutritional aspects of milk from different mammalian species, special formula and plant-based beverages. *Nutrients.* (2019) 11:1739. doi: 10.3390/nu11081739

44. Parekh H, Bahna SL. Infant formulas for food allergy treatment and prevention. *Pediatr Ann.* (2016) 45:E150–6. doi: 10.3928/00904481-20160225-01

45. Dupont C, Kalach N, Soulaines P, Bradatan E, Lachaux A, Payot F. Safety of a new amino acid formula in infants allergic to cow's milk and intolerant to hydrolysates. *J Pediatr Gastr Nutr.* (2015) 61:456–63. doi: 10.1097/MPG.0000000000000803

46. Borschel MW, Baggs GE, Oliver JS. Comparison of growth of healthy term infants fed extensively hydrolyzed protein-and amino acid-based infant formulas. *Nutrients.* (2018) 10:289. doi: 10.3390/nu10030289

47. Sackesen C, Altintas DU, Bingol A, Bingol G, Buyuktiryaki B, Demir E, et al. Current trends in tolerance induction in cow's milk allergy: from passive to proactive strategies. *Front Pediatr.* (2019) 7:372. doi: 10.3389/fped.2019.00372

48. Gorbach SL. Application and safety of probiotics in infant milk powder. Chinese Institute of Food Science and Technology. In: *Proceedings of the seventeenth International Symposium on Probiotics and Health*. (2022) 2.

49. Fields D, Czerkies L, Sun S, Storm H, Saavedra J, Sorensen R. A randomized controlled trial assessing growth of infants fed a 100% whey extensively hydrolyzed formula compared with a casein-based extensively hydrolyzed formula. *Global Pediatr Health.* (2016). 3:2333794X16636613. doi: 10.1177/2333794X16636613

50. Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, et al. *Lactobacillus rhamnosus* GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. *ISME J.* (2016) 10:742–50. doi: 10.1038/ismej.2015.151

51. Littlejohns P, Cluzeau F. Guidelines for evaluation. *Fam Pract.* (2000) 17:S3–6. doi: 10.1093/fampra/17.suppl_1.S3

52. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature.* (2018) 562:583. doi: 10.1038/s41586-018-0617-x

53. Ren CJ, Wu LM, Wangai K. Research progress about the effects of dietary polyphenols on the intestinal microbiota. *Sci Technol Food Ind.* (2022) 43:400–9. doi: 10.13386/j.issn1002-0306.2020090112

54. Liu A, Ma T, Xu N, Jin H, Zhao F, Kwok LY, et al. Adjunctive probiotics alleviates asthmatic symptoms via modulating the gut microbiome and serum metabolome. *Microbiol Spectr.* (2021) 9:e00859–21. doi: 10.1128/Spectrum.00859-21

55. Ma N, Chen X, Johnston LJ, Ma X. Gut microbiota-stem cell niche crosstalk: A new territory for maintaining intestinal homeostasis. *Imeta.* (2022) 1:e54. doi: 10.1002/imt2.54

56. Ashraf R, Shah NP. Immune system stimulation by probiotic microorganisms. *Crit Rev Food Sci.* (2014) 54:938–56. doi: 10.1080/10408398.2011.619671

57. Pourrajab B, Fatahi S. The effects of probiotic/synbiotic supplementation compared to placebo on biomarkers of oxidative stress in adults: a systematic review

and meta-analysis of randomized controlled trials. *Crit Rev Food Sci Nutr.* (2022) 62:490–507. doi: 10.1080/10408398.2020.1821166

58. Plaza-Diaz J, Ruiz-Ojeda FJ, Gil-Campos M, Gil A. Mechanisms of action of probiotics. *Nutrient.* (2019) 10:S49–66. doi: 10.1093/advances/nmy063

59. Xiaoxu Z, Huan L, Miao X, et al. Progress in the study of antibacterial effect of probiotic metabolites on pathogens. *Food Ferment Ind.* (2023) 49:297–302. doi: 10.13995/j.cnki.11-1802/ts.032474

60. Kathayat D, Closs G, Helmy YA, Deblais L, Srivastava V, Rajashekara G. In vitro and in vivo evaluation of Lacticaseibacillus rhamnosus GG and Bifidobacterium lactis Bb12 against avian pathogenic Escherichia coli and identification of novel probiotic-derived bioactive peptides. *Probiot Antimicrob Prot.* (2022) 1:1–7. doi: 10.1007/s12602-021-09840-1

61. Xiang YZ Li XY, Zheng HL, Chen JY, Lin LB, Zhang QL. Purification and antibacterial properties of a novel bacteriocin against *Escherichia coli* from Bacillus subtilis isolated from blueberry ferments. *LWT.* (2021) 146:111456. doi: 10.1016/j.lwt.2021.111456

62. Mardirossian M, Pérébaskine N, Benincasa M, Gambato S, Hofmann S, Huter P, et al. The dolphin proline-rich antimicrobial peptide Tur1A inhibits protein synthesis by targeting the bacterial ribosome. *Cell Chem Biol.* (2018) 25:530–9. doi: 10.1016/j.chembiol.2018.02.004

63. Oakley JL, Weiser R, Powell LC, Forton J, Mahenthiralingam E, Rye PD, et al. Phenotypic and genotypic adaptations in Pseudomonas aeruginosa biofilms following long-term exposure to an alginate oligomer therapy. *Msphere.* (2021) 6:10–128. doi: 10.1128/mSphere.01216-20

64. Chao M, Weidong G, Yu W, Yun Q. Functional studies of probiotics and their application in functional foods. Chinese Nutrition Society. In: *Proceedings of the 15th National Nutrition Science Congress of Chinese Nutrition Society*. (2022) 1.

65. Canani RB, Di Costanzo M. Gut microbiota as potential therapeutic target for the treatment of cow's milk allergy. *Nutrients.* (2013) 5:651–62. doi: 10.3390/nu5030651

66. Scalabrin D, Harris C, Johnston WH, Berseth CL. Long-term safety assessment in children who received hydrolyzed protein formulas with *Lactobacillus rhamnosus* GG: a 5-year follow-up. *Eur J Pediatr.* (2017) 176:217–24. doi: 10.1007/s00431-016-2825-4

67. Nocerino R, Bedogni G, Carucci L, Cosenza L, Cozzolino T, Paparo L, et al. The impact of formula choice for the management of pediatric cow's milk allergy on the occurrence of other allergic manifestations: the atopic march cohort study. *J Pediatr-Us.* (2021) 232:183. doi: 10.1016/j.jpeds.2021.01.059

68. Tan WF, Zhou ZC, Li W, Lu H, Qiu ZM. *Lactobacillus rhamnosus* GG for cow's milk allergy in children: a systematic review and meta-analysis. *Front Pediatr.* (2021) 9:727127. doi: 10.3389/fped.2021.727127

69. Vinderola G, Burns P. Chapter 1 - The Biotics Family. In: Gomes Da Cruz A, Ranadheera CS, Nazzaro F, Mortazavian A, editors. *Probiotics and Prebiotics in Foods*. Academic Press (2021) 1–11. doi: 10.1016/B978-0-12-819662-5.00014-8

70. Renz H, Skevaki C. Early life microbial exposures and allergy risks: opportunities for prevention. *Nat Rev Immunol.* (2021) 21:177–91. doi: 10.1038/s41577-020-00420-y

71. Kong HH, Morris A. The emerging importance and challenges of the human mycobiome. *Virulence.* (2017) 8:310–2. doi: 10.1080/21505594.2017.1279780

72. Cukrowska B, Bierła JB, Zakrzewska M, Klukowski M, Maciorkowska E. The relationship between the infant gut microbiota and allergy. The role of Bifidobacterium breve and prebiotic oligosaccharides in the activation of anti-allergic mechanisms in early life. *Nutrients.* (2020) 12:946. doi: 10.3390/nu12040946

73. Kumar H, Collado MC, Wopereis H, Salminen S, Knol J, Roeselers G. The bifidogenic effect revisited—ecology and health perspectives of bifidobacterial colonization in early life. *Microorganisms.* (2020) 8:1855. doi: 10.3390/microorganisms8121855

74. Vandenplas Y, Carnielli VP, Ksiazyk J, Luna MS, Migacheva N, Mosselmans JM, et al. Factors affecting early-life intestinal microbiota development. *Nutrition.* (2020) 78:110812. doi: 10.1016/j.nut.2020.110812

75. Canani RB, Nocerino R, Terrin G, Coruzzo A, Cosenza L, Leone L, et al. Effect of *Lactobacillus* GG on tolerance acquisition in infants with cow's milk allergy: a randomized trial. *J Aller Clin Immunol.* (2012) 129:580–2. doi: 10.1016/j.jaci.2011.10.004

76. Dupont C, Hol J, Nieuwenhuis EE. An extensively hydrolysed caseinbased formula for infants with cows' milk protein allergy: tolerance/hypoallergenicity and growth catch-up. *Br J Nutr.* (2015) 113:1102–2. doi: 10.1017/S000711451500015X

77. Muraro A, Hoekstra MO, Meijer Y, Lifschitz C, Wampler JL, Harris C, et al. Extensively hydrolysed casein formula supplemented with *Lactobacillus rhamnosus* GG maintains hypoallergenic status: randomised double-blind, placebo-controlled crossover trial. *BMJ Open.* (2012) 2:e000637. doi: 10.1136/bmjopen-2011-000637

78. Vandenplas Y, Steenhout P, Planoudis Y, Grathwohl D. Althera Study G. Treating cow's milk protein allergy: a double-blind randomized trial comparing two extensively hydrolysed formulas with probiotics. *Acta Paediatr.* (2013) 102:990–8. doi: 10.1111/apa.12349

79. Chassard C, Wouters TD, Lacroix C. Probiotics tailored to the infant: A window of opportunity. *Curr Opin Biotechnol.* (2014) 26C:141–147. doi: 10.1016/j.copbio.2013.12.012

80. Candy DC, Van Ampting MT, Oude Nijhuis MM, Wopereis H, Butt AM, Peroni DG, et al. A synbiotic-containing amino-acid-based formula improves gut microbiota in non-IgE-mediated allergic infants. *Pediatr Res.* (2018) 83:677–86. doi: 10.1038/pr.2017.270

81. Canani RB, Di Costanzo M, Bedogni G, Amoroso A, Cosenza L, Di Scala C, et al. Extensively hydrolyzed casein formula containing *Lactobacillus rhamnosus* GG reduces the occurrence of other allergic manifestations in children with cow's milk allergy: 3-year randomized controlled trial. *J Aller Clin Immunol.* (2017) 139:1906. doi: 10.1016/j.jaci.2016.10.050

82. Yanru. *Characterization of fecal microbiota short-chain fatty acids concentrations in children with cow milk protein allergy*. Northeast Agricultural University (2018).

83. Rabe H, Lundell AC, Sjöberg F, Ljung A, Strömbeck A, Gio-Batta M, et al. Neonatal gut colonization by Bifidobacterium is associated with higher childhood cytokine responses. *Gut Microbes.* (2020) 12:1847628. doi: 10.1080/19490976.2020.1847628

84. Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev.* (2004) 28:405–40. doi: 10.1016/j.femsre.2004.01.003

85. Song J, Li Y, Li J, Wang H, Zhang Y, Suo H. *Lactobacillus rhamnosus* 2016SWU050601 regulates immune balance in ovalbumin-sensitized mice by modulating expression of the immune-related transcription factors and gut microbiota. *J Sci Food Agr.* (2020) 100:4930–9. doi: 10.1002/jsfa.10554

86. Zhang LL, Chen X, Zheng PY, Luo Y, Lu GF, Liu ZQ, et al. Oral Bifidobacterium modulates intestinal immune inflammation in mice with food allergy. *J Gastroenterol Hepatol.* (2010) 25:928–34. doi: 10.1111/j.1440-1746.2009.06193.x

87. Inoue Y, Iwabuchi N, Xiao JZ, Yaeshima T, Iwatsuki K. Suppressive effects of bifidobacterium breve strain M-16V on T-helper type 2 immune responses in a murine model. *Biol Pharmaceut Bull.* (2009) 32:760–3. doi: 10.1248/bpb.32.760

88. Shida K, Takahashi R, Iwadate E, Takamizawa K, Yasui H, Sato T, et al. Lactobacillus casei strain Shirota suppresses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. *Clin Exper Aller.* (2002) 32:563–70. doi: 10.1046/j.0954-7894.2002.01354.x

89. Murosaki S, Yamamoto Y, Ito K, Inokuchi T, Kusaka H, Ikeda H, et al. Heat-killed Lactobacillus plantarum L-137 suppresses naturally fed antigen-specific IgE production by stimulation of IL-12 production in mice. *J Aller Clin Immunol.* (1998) 102:57–64. doi: 10.1016/S0091-6749(98)70055-7

90. Muraro A, Werfel T, Hoffmann-Sommergruber K, Roberts G, Beyer K, Bindslev-Jensen C, et al. EAACI food allergy and anaphylaxis guidelines: diagnosis and management of food allergy. *Allergy.* (2014) 69:1008–1025. doi: 10.1111/all.12429

91. Aitoro R, Simeoli R, Amoroso A, Paparo L, Nocerino R, Pirozzi C, et al. Extensively hydrolyzed casein formula alone or with *L. rhamnosus* GG reduces β-lactoglobulin sensitization in mice. *Pediatr Aller Immunol.* (2017) 28:230–7. doi: 10.1111/pai.12687

92. McGowan EC, Keet CA. Primary prevention of food allergy in children and adults: systematic review. *Pediatrics.* (2014) 134:S138. doi: 10.1542/peds.2014-1817J

93. Thang CL, Baurhoo B, Boye JI, Simpson BK, Zhao X. Effects of *Lactobacillus rhamnosus* GG supplementation on cow's milk allergy in a mouse model. *Aller Asthma Clin Immunol.* (2011) 7:1–9. doi: 10.1186/1710-1492-7-20

94. Rodovalho VDR, Luz SRD B, Rabah H, Carmo LRD F, Guédon E. Extracellular vesicles produced by the probiotic *Propionibacterium freudenreichii* CIRM-BIA 129 mitigate inflammation by modulating the NF-κB pathway. *Front Microbiol.* (2020) 11:1544. doi: 10.3389/fmicb.2020.01544

95. Dotterud CK, Avershina E, Sekelja M, Simpson MR, Ien T. Does maternal perinatal probiotic supplementation alter the intestinal microbiota of mother and child? *J Pediatr Gastroenterol Nutr.* (2015) 61:200–7. doi: 10.1097/MPG.0000000000000781

96. Nentwich I, Szepfalusi ZS, Kunz C, Spuergin P, Urbanek R. Antigenicity for humans of cow milk caseins, casein hydrolysate and casein hydrolysate fractions. *Acta Veterinaria Brno.* (2004) 73:291–8. doi: 10.2754/avb200473020291

97. Wang HT, Anvari S, Anagnostou K. The role of probiotics in preventing allergic disease. *Children.* (2019) 6:24. doi: 10.3390/children6020024

98. Strachan DP. Hay fever, hygiene, and household size. *BMJ.* (1989) 299:1259. doi: 10.1136/bmj.299.6710.1259

99. Nawaz M, Ma C, Basra MA, Wang J, Xu J. Amelioration of ovalbumin induced allergic symptoms in Balb/c mice by potentially probiotic strains of lactobacilli. *Benef Microbes.* (2015) 6:669–78. doi: 10.3920/BM2014.0141

100. Wang S, Cui J, Jiang S, Zheng C, Zhao J, Zhang H, et al. Early life gut microbiota: Consequences for health and opportunities for prevention. *Crit Rev Food Sci Nutr.* (2022) 13:1–25. doi: 10.1080/10408398.2022.2158451

101. DeMuri GP, Lehtoranta LM, Eickhoff JC, Lehtinen MJ, Wald ER. Ex vivo peripheral blood mononuclear cell response to R848 in children after supplementation with the probiotic Lactobacillus acidophilus NCFM/Bifidobacterium lactis Bi-07. *Benef Microbes.* (2021) 12:85–93. doi: 10.3920/BM2020.0068

102. Li N, Yu Y, Chen X, Gao S, Zhang Q, Xu C. Bifidobacterium breve M-16V alters the gut microbiota to alleviate OVA-induced food allergy through

IL-33/ST2 signal pathway. *J Cell Physiol.* (2020) 235:9464–73. doi: 10.1002/jcp.29751

103. Wang HF, Zhang QX, Niu YH, Zhang X, Lu RR. Surface-layer protein from Lactobacillus acidophilus NCFM attenuates tumor necrosis factor-alpha-induced intestinal barrier dysfunction and inflammation. *Int J Biol Macromol.* (2019) 136:27–34. doi: 10.1016/j.ijbiomac.2019.06.041

104. Chen X, Zhao X, Hu Y, Zhang B, Zhang Y, Wang S. *Lactobacillus rhamnosus* GG alleviates beta-conglycinin-induced allergy by regulating the T cell receptor signaling pathway. *Food Funct*. (2020) 11:10554–10567. doi: 10.1039/D0FO02124E

105. Capurso L. Thirty years of *Lactobacillus rhamnosus* GG a review. *J Clin Gastroenterol.* (2019) 53:S1–41. doi: 10.1097/MCG.0000000000001170

106. Aziz N, Bonavida B. Activation of natural killer cells by probiotics. *For Immunopathol Dis Therap*. (2016) 7:41–55. doi: 10.1615/ForumImmunDisTher.2016017095

107. Bu G, Luo Y, Zhang Y, Chen F. Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. *J Sci Food Agric.* (2010) 90:2015–20. doi: 10.1002/jsfa.4046

108. Fugl A, Berhe T, Kiran A, Hussain S, Hansen EB. Characterisation of lactic acid bacteria in spontaneously fermented camel milk and selection of strains for fermentation of camel milk. *Int Dairy J.* (2017) 73:19–24. doi: 10.1016/j.idairyj.2017.04.007

109. Azad MA, Sarker M, Li T, Yin J. Probiotic species in the modulation of gut microbiota: an overview. *Biomed Res Int*. (2018) 2018:9478630. doi: 10.1155/2018/9478630

110. Finamore A, Roselli M, Britti MS, Merendino N, Mengheri E. *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* MB5 induce intestinal but not systemic antigen-specific hyporesponsiveness in ovalbumin-immunized rats. *J Nutr.* (2012) 142:375–81. doi: 10.3945/jn.111.148924

111. Kieliszek M, Pobiega K, Piwowarek K, Kot AM. Characteristics of the proteolytic enzymes produced by lactic acid bacteria. *Molecules.* (2021) 26:1858. doi: 10.3390/molecules26071858

112. Huang M, Li X, Wu Y, Meng X, Tong P, Yuan J. Potential allergenicity and hydrolysis assessment of bovine casein and β-casein by treatment with lactic acid bacteria. *J Food Biochem.* (2022) 46:1–9. doi: 10.1111/jfbc.14424

113. Donato KA, Gareau MG, Wang YJJ, Sherman PM. *Lactobacillus rhamnosus* GG attenuates interferon-{gamma} and tumour necrosis factor-alpha-induced barrier dysfunction and pro-inflammatory signalling. *Microbiology.* (2010) 156:3288. doi: 10.1099/mic.0.040139-0

114. Yuping A, Changyu A, A. brief review of the development history of domestic infant formula from phase with an analysis on their intrisinc qualities. *China Dairy Ind.* (2004) 4:26–8. doi: 10.3969/j.issn.1001-2230.2004.04.008

115. Goh A, Muhardi L, Ali A, Liew WK, EstradaReyes E, Zepeda-Ortega B, et al. Differences between peptide profiles of extensive hydrolysates and their influence on functionality for the management of cow's milk allergy: a short review. *Front Allergy.* (2022) 3:950609. doi: 10.3389/falgy.2022.950609

116. dos Santos SC, Konstantyner T, Cocco RR. Effects of probiotics in the treatment of food hypersensitivity in children: a systematic review. *Allergol Immunopathol.* (2020) 48:95–104. doi: 10.1016/j.aller.2019.04.009

117. Guest JF, Fuller GW. Effectiveness of using an extensively hydrolyzed casein formula supplemented with *Lactobacillus rhamnosus* GG compared with an extensively hydrolysed whey formula in managing cow's milk protein allergic infants. *J Comp Eff Res.* (2019) 8:1317–26. doi: 10.2217/cer-2019-0088

118. Bertelsen RJ, Jensen ET, Ringel-Kulka T. Use of probiotics and prebiotics in infant feeding. *Best Pract Res Clin Gastroenterol.* (2016) 30:39–48. doi: 10.1016/j.bpg.2016.01.001

119. Nocerino R, Coppola S, Carucci L, de Giovanni di Santa Severina AF, Oglio F, Bedogni G, et al. The step-down approach in children with cow's milk allergy: results of a randomized controlled trial. *Allergy.* (2023) 78:2477–86. doi: 10.1111/all.15750

120. Nocerino R, Di Costanzo M, Bedogni G, Cosenza L, Maddalena Y, Di Scala C, et al. Dietary treatment with extensively hydrolyzed casein formula containing the probiotic *Lactobacillus rhamnosus* GG prevents the occurrence of functional gastrointestinal disorders in children with cow's milk allergy. *J Pediatr.* (2019) 213:137–42. doi: 10.1016/j.jpeds.2019.06.004

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