A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis

Magali Noval Rivas, PhD,[a]* Oliver T. Burton, PhD,[b] Petra Wise, PhD,[a] Yu-qian Zhang, PhD,[a] Suejy A. Hobson, MD,[a] Maria Garcia Lloret, MD,[a] Christel Chehoud, BA,[c] Justin Kuczynski, PhD,[c] Todd DeSantis, MSc,[c] Janet Warrington, PhD,[c] Embriette R. Hyde, BSc,[d,e] Joseph F. Petrosino, PhD,[d,f] Georg K. Gerber, MD, PhD,[g] Lynn Bry, MD, PhD,[g] Hans C. Oettgen, MD, PhD,[b] Sarkis K. Mazmanian, PhD,[h] and Talal A. Chatila, MD, MSc[a]* *Los Angeles, San Bruno, and Pasadena, Calif, Boston, Mass, and Houston, Tex*

Background: Commensal microbiota play a critical role in maintaining oral tolerance. The effect of food allergy on the gut microbial ecology remains unknown.

Objective: We sought to establish the composition of the gut microbiota in experimental food allergy and its role in disease pathogenesis.

Methods: Food allergy–prone mice with a gain-of-function mutation in the IL-4 receptor α chain (*Il4raF709*) and wild-type (WT) control animals were subjected to oral sensitization with chicken egg ovalbumin (OVA). Enforced tolerance was achieved by using allergen-specific regulatory T (Treg) cells. Community structure analysis of gut microbiota was performed by using a high-density 16S rDNA oligonucleotide microarrays (PhyloChip) and massively parallel pyrosequencing of 16S rDNA amplicons.

Results: OVA-sensitized *Il4raF709* mice exhibited a specific microbiota signature characterized by coordinate changes in the abundance of taxa of several bacterial families, including the Lachnospiraceae, Lactobacillaceae, Rikenellaceae, and Porphyromonadaceae. This signature was not shared by similarly sensitized WT mice, which did not exhibit an OVA-induced allergic response. Treatment of OVA-sensitized *Il4raF709* mice with OVA-specific Treg cells led to a distinct tolerance-associated signature coincident with the suppression of the allergic response. The microbiota of allergen-sensitized *Il4raF709* mice differentially promoted OVA-specific IgE responses and anaphylaxis when reconstituted in WT germ-free mice.

Conclusion: Mice with food allergy exhibit a specific gut microbiota signature capable of transmitting disease susceptibility and subject to reprogramming by enforced tolerance. Disease-associated microbiota may thus play a pathogenic role in food allergy. (J Allergy Clin Immunol 2013;131:201-12.)

Key words: *Food allergy, microbiome, microbiota, regulatory T cells, tolerance, anaphylaxis, IgE, 16S rDNA, IL-4 receptor*

It is unquestionable that food allergy has become a major health problem in developed countries, where the prevalence reaches up to 6% among children and 3% among adults.[1,2] Like other atopic diseases, food allergies have a strong genetic component.[3] However, the incidence of food allergy has increased dramatically in the last decades, particularly in affluent societies, pointing to lifestyle-associated environmental factors acting on genetically susceptible hosts to promote disease.[4] Evidence suggests that the microbial flora are a key environmental influence in programming oral tolerance.[5] Their lack in germ-free (GF) mice is associated with the development of T_H2 and IgE responses to dietary antigens.[6,7] Microbial signals, such as those delivered by polysaccharide A of the commensal bacterium *Bacteroides fragilis* or by a mix of clostridial species, induce mucosal tolerance by promoting the formation of induced regulatory T (iTreg) cells from naive $CD4^+$ T-cell precursors.[8,9] Polymorphisms in or deficiency of genetic elements encoding microbial sensors, such as CD14, a high-affinity receptor for bacterial LPSs, and Toll-like receptor 4, which mediates responses to LPS, are associated with food allergy.[10,11]

Changes in the microbial flora have been implicated in the pathogenesis of several disorders associated with the more affluent lifestyle common in developed countries. Obesity in both human subjects and experimental mouse models is associated with alterations in the intestinal microbiota that appear to be pathogenic, given that the microbiota of obese subjects promote weight gain when transferred into GF mice.[12-14] More limited data have been accrued in the study of allergic diseases. In suboptimally controlled asthmatic subjects, both bacterial burden and bacterial diversity were significantly higher compared with those seen in control subjects and correlated with bronchial hyperresponsiveness.[15] The development of atopy and atopic dermatitis is associated with altered early postnatal gut flora.[16,17] In the

From [a]the Division of Immunology, Allergy and Rheumatology, Department of Pediatrics, David Geffen School of Medicine at the University of California at Los Angeles; [b]the Division of Immunology, Boston Children's Hospital, Department of Pediatrics, Harvard Medical School, Boston; [c]Second Genome, Inc, San Bruno; [d]the Alkek Center for Metagenomics and Microbiome Research, [e]the Interdepartmental Program in Cell and Molecular Biology, and [f]the Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston; [g]the Center for Clinical and Translational Metagenomics, Department of Pathology, Brigham & Women's Hospital, Harvard Medical School, Boston; and [h]the Division of Biology, California Institute of Technology, Pasadena.

*Current affiliation: Division of Immunology, Boston Children's Hospital, Department of Pediatrics, Harvard Medical School, Boston, Mass.

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Corresponding author: Talal A. Chatila, MD, MSc, Division of Immunology, Boston Children's Hospital, Karp Family Building, 1 Blackfan Circle, Boston, MA 02115. E-mail: talal.chatila@childrens.harvard.edu.

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Abbreviations used
Foxp3: Forkhead box protein 3
GF: Germ free
HC-AN: Hierarchical clustering-average-neighbor
IL-4R: IL-4 receptor
iTreg: Induced regulatory T
KW: Kruskal-Wallis
mMCP-1: Murine mast cell protease 1
NMDS: Nonmetric multidimensional scaling
OTU: Operational taxonomic unit
OVA: Ovalbumin
PAM: Prediction Analysis for Microarrays
rDNA: Ribosomal DNA
SEB: Staphylococcal enterotoxin B
STAT6: Signal transducer and activator of transcription 6
TCR: T-cell receptor
Treg: Regulatory T
WT: Wild-type

case of food allergy and despite the well-known role of the commensal flora in oral tolerance induction, there is meager information on the status of the intestinal microbiota on disease onset and after tolerance establishment. One study found alterations in the microbiota of infants with milk allergy on diagnosis and again after treatment.[18] Another study in which atopic dermatitis cohorts were examined for changes in the microbiota in association with allergic food sensitization found no such relationship.[19] Limitations to the latter set of studies include reliance on bacterial culture methods, limited 16S RNA genotyping approaches, or both for microbiota analysis.[18,19]

In this study we employed a phylotyping approach using high-density 16S ribosomal DNA (rDNA) oligonucleotide microarrays (PhyloChip assay; Second Genome, San Bruno, Calif) and massively parallel pyrosequencing of 16S rDNA amplicons to investigate whether oral allergic sensitization to a dietary antigen is associated with a distinct intestinal microbiota signature. These studies were enabled by the use of a novel mouse model of food allergy that well replicates many of the features of the human disease. Using the same model, we also examined whether therapy with allergen-specific regulatory T (Treg) cells imparts a tolerance-associated signature on the host microbiota.

METHODS

Animals

BALB/cTac mice (wild-type [WT] and *Il4raY709* mice) were originally from Taconic Farms (Germantown, NY) and maintained as a separate line in the investigator's colony from which mice were used for the current studies. *C.129.Il4ra$^{F709/F709}$ (Il4raF709)* mice were bred onto the investigator's BALB/cTac line for 11 generations.[20] Foxp3^{EGFP} and DO11.10^{+}Foxp3^{EGFP} mice, both on a BALB/c background, were previously described.[20-22] All mice were kept on an ovalbumin (OVA)–free diet (Harlan 2018SX, Indianapolis, Ind). They were housed together in the same colony in a specific pathogen-free environment and were 8 to 12 weeks old when used for studies. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee policies and procedures of the University of California–Los Angeles and the Boston Children's Hospital.

Sensitization and challenge protocols

Studies were conducted on female mice to eliminate confounding effects of sex on the results of the microbiota analyses. In separate experiments there were no significant differences between male and female *Il4raF709* mice in terms of sensitization and response to allergen challenge. For sensitization, female WT and *Il4raF709* mice were treated intragastrically with sterile PBS or 100 μg of OVA alone or together with 10 μg of staphylococcal enterotoxin B (SEB; Sigma-Aldrich, St Louis, Mo) in 100 μL of sterile PBS (saline) once weekly for 8 weeks. On the ninth week, mice were challenged intragastrically with 5 or 150 mg of OVA in 100 μL of PBS. Anaphylaxis was assessed in challenged mice by measuring changes in body temperature and recording symptom scores. Temperature changes were measured with a rectal temperature probe (RET3) coupled to the Physitemp Thermalert Model TH-5 (Physitemp, Clifton, NJ). After OVA challenge, temperatures were measured every 5 minutes. Symptom scores were determined according to previously detailed criteria.[22]

For studies on flora-reconstituted GF mice, fecal pellets of either OVA/SEB-sensitized WT or *Il4raF709* mice were collected on the eighth week of sensitization. The pellets were dissolved at 1 pellet (20-25 mg) in 100 μL of PBS and administered to GF mice at 200 μL per mouse. The mice were then sensitized with OVA/SEB for 8 weeks and challenged with OVA, as described above.

Tolerance induction

CD4^{+}DO11.10^{+}Foxp3^{EGFP+} T cells, representing thymus-derived (natural) Treg cells that express the DO11.10 T-cell receptor (TCR), which recognizes the OVA$_{323-339}$ peptide in the context of I-A^{d}, were isolated by means of cell sorting from *DO11.10^{+}Foxp3^{EGFP}* mice. On day 0 of the sensitization protocol, *Il4raF709* mice were given 5×10^{5} CD4^{+}DO11.10^{+}Foxp3^{EGFP+} T cells by means of retro-orbital transfer. The mice were then sensitized intragastrically with 100 μg of OVA in 100 μL of PBS once weekly for 8 weeks. They were challenged on week 9 with 5 mg of OVA in 100 μL of PBS administered intragastrically and monitored for their core body temperature and symptom scores, as described above.

ELISA for murine mast cell protease 1 and total and OVA-specific IgE

A murine mast cell protease 1 (mMCP-1) ELISA was performed on serum samples by using a kit (eBioscience, San Jose, Calif). Total and OVA-specific IgE concentrations were measured by using sandwich ELISAs. For total IgE, the capture and biotinylated detection antibodies (rat anti-mouse IgE clones R35-72 and R35-118, respectively) and purified mouse IgE isotype standard antibody (clone C38-2) were from BD Biosciences (San Jose, Calif). For OVA-specific IgE, the plates were coated with 100 μg/mL OVA. The detection antibody was as above, whereas the standard was a purified anti-OVA monoclonal IgE antibody (AbD Serotec, Oxford, United Kingdom).

Histologic analysis and enumeration of mast cells

Intestinal mast cells were enumerated by means of microscopic examination of sections of paraffin-embedded jejunal tissues stained with toluidine blue. Mast cells were counted and averaged across 10 high-power fields spanning the entire jejunum.

PhyloChip sample processing and hybridization

Fecal pellets were obtained from sham- and allergen-sensitized mice just before allergen challenge. DNA extraction was performed with the UltraClean Fecal extraction kit (Mo Bio, Carlsbad, Calif), according to the manufacturer's instructions. A total of 50 to 100 ng of extracted DNA per sample was used to amplify bacterial 16S rDNA gene sequences by using PCR with the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Amplified products were hybridized to G3 PhyloChip arrays (Second Genome), which display 1,016,064 16S rDNA oligonucleotide probes that track microbial population shifts across greater than 59,000 operational taxonomic units (OTUs), each mapped to the Greengenes taxonomy.[23-25] OTUs are defined as organisms that share sequence similarity of their 16S rDNA, which for most OTUs is greater than 99%. The hybridization procedures were as described in the aforementioned references.