



Influences of non-IgE-mediated cow's milk protein allergy-associated gut microbial dysbiosis on regulatory T cell-mediated intestinal immune tolerance and homeostasis

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ABSTRACT

Gut microbial dysbiosis is closely associated with cow's milk protein allergy (CMPA) during infancy. Recent research has highlighted the crucial role of the commensal microbiota-induced intestinal regulatory T (Treg) cell response in the development of oral tolerance and protection against IgE-mediated food allergies. However, the influences of CMPA (particularly non-IgE-mediated CMPA)-associated microbial dysbiosis on Treg cell-mediated intestinal immune tolerance and homeostasis remain poorly characterized. To investigate this issue, fecal microbiota from infant donors with food protein-induced allergic proctocolitis (FPIAP) associated with cow's milk, which is the most frequent clinical type of non-IgE-mediated gastrointestinal CMPA, and from age-matched healthy controls were transplanted into germ-free mice in this study. Two weeks post fecal microbiota transplantation, the gut microbiome of the recipient mice was analyzed by 16S rRNA gene sequencing, and the intestinal immunological alterations associated with the Treg cell compartment and intestinal immune homeostasis were detected. The specific gut microbial phylotypes that were potentially responsible for the disruption of intestinal immune homeostasis were also analyzed. We observed that the main characteristics of the gut microbiome in infant donors could be stably maintained in recipient mice. We also found that mice colonized with the gut microbiome from infants with cow's milk-induced FPIAP showed significant deficiencies in the accumulation and function of intestinal Treg cells. Furthermore, these mice showed disrupted intestinal immune homeostasis, which was characterized by an overactivated Th2 biased immune response. We further identified two potentially pathogenic genera that contribute to this disruption. Overall, our results highlight a destructive effect of non-IgE-mediated CMPA-associated microbial dysbiosis on intestinal immune tolerance and homeostasis. We believe these findings will help improve our understanding of the gut microbiota-mediated pathogenesis of non-IgE-mediated CMPA in the future.

1. Introduction

Food allergies (FAs) are particularly common diseases in infants and young children that result from the breakdown of immune tolerance toward harmless food antigens [1]. Cow's milk protein allergy (CMPA) is one of the earliest and most common types of FAs and seriously affects the physical health of infants. According to their different pathogenesises, FAs can be classified into two general categories: IgE-mediated and

non-IgE-mediated FAs [2]. IgE-mediated FAs are caused by type 1 hypersensitivity reactions resulting from the activation of T helper type 2 (Th2)-predominant immune responses [2–4]. In contrast, although accounting for up to 40–50% of CMPA cases [5], the pathogenesis of non-IgE-mediated reactions is markedly more complicated and remains poorly characterized. It is generally believed that this type of FAs predominately depends on cellular mechanisms that induce inflammation after exposure to food allergens. Patients with non-IgE-mediated FAs

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predominantly exhibit gastrointestinal tract symptoms. The main entities of non-IgE-mediated gastrointestinal food allergies (non-IgE-GI-FAs) include food protein-induced enterocolitis syndrome (FPIES), food protein-induced allergic proctocolitis (FPIAP), and food protein-induced enteropathy (FPE) [5].

Numerous investigations have reported the associations between gut microbial dysbiosis and various types of FAs, including CMPA. The important roles of gut microbial dysbiosis in promoting the occurrence and development of CMPA have begun to be revealed. For example, researchers have observed that the fecal microbiota from infants with CMPA promotes the clinical and immune characteristics of allergies in germ-free (GF) mice after fecal microbiota transplantation (FMT) [6]. However, the mechanisms underlying these observations remain unclear.

CD4⁺CD25⁺ regulatory T (Treg) cells expressing the transcription factor forkhead box P3 (Foxp3) are a unique subset of CD4⁺ T cells characterized by their function in suppressing both innate and adaptive immune responses [7,8]. Based on their strong suppressive activities, these cells play central roles in the establishment and maintenance of peripheral immune tolerance and immune homeostasis [9–11]. Based on different developmental patterns, two types of Treg cells have been identified: thymus-derived Treg cells (tTreg cells), which differentiate into Foxp3⁺ Treg cells in the thymus, and peripherally derived Treg cells (pTreg cells), which differentiate from naïve CD4⁺ T cells and gain Foxp3 expression in the periphery after recognition of their cognate antigen [12,13]. Expressions of the transcription factor Helios and the membrane protein neuropilin-1 are generally proposed as important markers that can be used to distinguish tTreg cells from pTreg cells [14, 15]. Although both tTreg and pTreg cells have been demonstrated to be essential in the maintenance of tissue-specific immune tolerance, recent investigations have suggested the predominant role of pTreg cells in mediating tolerance toward commensal microbiota and harmless food antigens (i.e., oral tolerance) in the intestine [16,17]. Furthermore, mice with a substantial deficiency in pTreg cells have been observed to spontaneously develop allergic and asthmatic symptoms in the intestine and respiratory tract, respectively, which highlights the central function of pTreg cells in the regulation of allergic-type pathologies at mucosal interfaces [18]. A subset of intestinal pTreg cells characterized by the expression of the T_H17 master transcription factor ROR-γt was recently identified, these cells have been proven to play a crucial role in the regulation of various types of immune responses and the maintenance of intestinal homeostasis [19–21].

In addition, based on the expression pattern of activation- and homing-associated molecules, including CD44 and CD62L [22,23], Treg cells in the periphery can be classified into distinct functional subsets. CD44^{hi}CD62L^{lo} effector Treg cells, which differentiate from CD44^{lo}CD62L^{hi} central Treg cells in response to antigen stimulation and predominantly reside in nonlymphoid tissues, display strong suppressive effects on the development and function of effector T cells [22]. Mechanistically, the expression of cytotoxic T-lymphocyte associated protein 4 (CTLA4) [19,24,25] and inducible T cell costimulator (ICOS) [26], as well as the production of anti-inflammatory cytokines such as IL-10 [27,28], mediate the suppressive function of intestinal Treg cells.

More importantly, recent studies have unveiled the dominant influence of microbiota on the induction and maintenance of Treg cells, particularly pTreg cells, in the intestine [14,29]. For example, the abundance of intestinal Treg cells in GF mice or mice administered broad-spectrum antibiotics has been observed to be significantly decreased [17,20,30]. Moreover, various commensal microbial phylogenotypes are able to induce pTreg cells in the intestine [19,20,26,31]. These investigations have highlighted the complex interactions between local environmental factors, particularly microbiota, and Treg cell responses in the intestine. In addition, a recent study demonstrated that the protective effects of *Clostridiales*- and *Bacteroidales*-based microbiota therapy against IgE-mediated FAs are mainly dependent on ROR-γt⁺ Treg cells [32], which highlights the crucial role of the commensal

microbiota-induced Treg cell subset in the development of oral tolerance and protection against IgE-mediated FAs. However, the influence of gut microbial dysbiosis on Treg cell-mediated intestinal immune tolerance and homeostasis in CMPA, particularly non-IgE-mediated CMPA, and the potentially pathogenic phylogenotypes capable of damaging intestinal Treg cell responses remain poorly characterized.

To investigate this issue, we collected stool samples from infants with cow's milk-induced FPIAP (CM-FPIAP), which is the most frequent clinical type of non-IgE-mediated gastrointestinal CMPA, and subjected GF mice to FMT. The immunological alterations of the recipient mice were then analyzed two weeks post FMT. We characterized the substantial influence of microbial dysbiosis on intestinal Treg cell compartments and immune homeostasis in detail and identified the specific gut microbial phylogenotypes that potentially contribute to these adverse influences. We believe these findings will help improve our understanding of the gut microbiota-mediated pathogenesis of non-IgE-mediated CMPA in the future.

2. Material and methods

2.1. Subject recruitment

Infants diagnosed with CM-FPIAP at the gastroenterology clinic at the Children's Hospital of Chongqing Medical University were recruited alongside age-matched healthy control (HC) infants from our children's healthcare clinic. All infants were less than 6 months of age, born at term after a normal pregnancy by spontaneous vaginal delivery, and completely fed formula without any supplementary food. The baseline characteristics of the enrolled infants are summarized in Table 1. CM-FPIAP was diagnosed based on the clinical manifestations (bloody stools, mild diarrhea) caused by ingestion of the infant formula and the responses to trial elimination diets (i.e., resolution of the symptoms within 2 weeks after switching to a hypoallergenic formula and the recurrence of symptoms when the normal infant formula was reintroduced) [5,33]. Cow's milk-specific IgE, as measured by skin prick tests (SPT) [33], was negative for all the patients. The diagnoses of all the patients were confirmed by oral food challenges [5,33]. Healthy infants with no history of allergic reactions were recruited as HC subjects. The exclusion criteria were as follows: (a) administration of antibiotics or probiotics in the previous month prior to the study; (b) infection with viral, bacterial, parasitic, and fungal pathogens in the previous month prior to the study; (c) history of asthma, allergic rhinitis, atopic eczema, or other allergic/atopic diseases; (d) administration of immunosuppressive agents; (e) history of other common digestive system diseases in

Table 1
The baseline characteristics of the enrolled infants.

Characteristics	Healthy infants (n = 3)	CM-FPIAP infants (n = 3)
Age at study entry (mo)	3.33 ± 1.15	3.67 ± 1.15 ^a
Sex	Female	Female
Weight at study entry (kg)	5.97 ± 0.67	6.08 ± 0.63 ^a
Mode of delivery	Vaginal delivery	Vaginal delivery
Feeding mode after birth	Formula	Formula
Supplementary food intake	No	No
Antibiotics	No	No
Probiotics	No	No
Family history of allergic diseases	Negative	Positive
Stool characteristics	Normal	Watery + Bloody
EOS in peripheral blood	0.02 ± 0.01	0.10 ± 0.01 ^a
WBC in stool	Negative	0-1/HP
SPT	Negative	Negative

EOS: proportional of eosinophil; WBC: white blood cell; SPT: skin prick test.

^a *P* > 0.05 vs healthy infants, **P* < 0.05 vs healthy infants (Independent-Samples T Test).

infancy (e.g., lactose intolerance, necrotizing enterocolitis, celiac disease, digestive tract malformation, intestinal failure, intestinal polyps); and (f) history of other diseases, such as primary immunodeficiency, autoimmune diseases, and genetic or metabolic diseases. Fecal samples from infants with suspected CM-FPIAP were collected at the time of symptom onset prior to treatment initiation, immediately frozen and stored at -80°C . Only when all diagnostic criteria were fulfilled, were the samples used for the subsequent experiment. The study was ethically approved by the Ethics Committee of Children's Hospital of Chongqing Medical University. The parents of all the participants were informed and signed informed consent prior to enrollment.

2.2. Experimental animals

All animal experiments were licensed by the Animal Ethics Committee of the Army Medical University (Chongqing, China). GF BALB/c mice (aged 6 weeks) were provided by the Department of Laboratory Animal Science of the Basic Medical College of the Army Medical University (Chongqing, China). The mice were housed in sterile, plastic, flexible-film, gnotobiotic isolators under a strict 12-h dark/light cycle (lights on at 06:00 a.m.) and fed sterile infant formula and autoclaved water. The feeding environment temperature was $20\text{--}23^{\circ}\text{C}$, and the relative humidity was 40–70%. All the mice were allowed food and water ad libitum.

2.3. Fecal microbiota transplantation

GF mice were colonized with fecal samples collected from CM-FPIAP infant donors ($n = 3$) and age-matched HCs ($n = 3$). Eleven GF mice were randomly divided into two groups. The mice belonging to one of the groups (female, $n = 6$) were transplanted with fecal microbiota from HCs (M-HC), and those in the other group (female, $n = 5$) were transplanted with fecal microbiota from CM-FPIAP infant donors (M-FPIAP). FMT was performed as previously described [34]. Briefly, an aliquot of the fecal sample (1 g) from each donor was suspended in 15 mL of sterile phosphate buffer (0.1 M, pH 7.4) and mixed by vortexing. Equal volumes of fecal suspensions derived from donors belonging to each group were mixed to obtain pooled suspensions. All of the abovementioned procedures were performed under anaerobic conditions. Each GF mouse was then administered 0.3 mL of pooled fecal suspension by oral gavage, and its hair was smeared with 0.2 mL of the same suspension. The whole fecal transplantation procedure was completed in the gnotobiotic isolator. After FMT, the two groups of recipient mice were separately housed in different isolators to avoid microbial normalization. Due to the strong effect of both microbiota and dietary factors on the shaping of intestinal immune responses [14,17,29,35,36], all recipient mice were fed the same infant formula as the donors throughout 2 weeks of the microbial colonization process, which allowed us to investigate the exclusive effect of the microbiota. Fourteen days post transplantation, the feces of the recipient mice were collected, immediately frozen and stored at -80°C prior to 16S rRNA gene sequencing.

2.4. 16S rRNA gene sequencing

Fresh fecal samples from infant donors and the FMT model were collected, immediately frozen and stored at -80°C prior to bacterial genomic DNA extraction. Three hundred milligrams of each fecal sample was subjected to bacterial genomic DNA extraction using a QIAamp Fast DNA Stool Mini kit (Qiagen, Hilden, Germany), and the procedure was strictly conducted in accordance with the manufacturer's instructions.

For PCR amplification, the hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with the primer pair 338F (5'-ACTCCTACGGGAGGAGCAG-3') and 806R (5'-GGAC-TACHVGGGTWCTAAT-3') using an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). PCR was performed in a 20- μL reaction system containing 4 μL of 5 $\times$ TransStart FastPfu buffer, 2 μL of 2.5 mM dNTPs,

0.8 μL of each primer (5 μM), 0.4 μL of TransStart FastPfu DNA Polymerase, 10 ng of template DNA, and ddH₂O. The reaction procedure was conducted as follows: initial denaturation at 95°C for 3 min; 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s; a single extension at 72°C for 10 min; and a hold at 4°C . All PCRs were conducted in triplicate. We then purified the PCR products using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. For sequencing, the purified amplicons were pooled in equimolar amounts and subjected to paired-end sequencing on an Illumina MiSeq PE300 platform (San Diego, CA, USA).

2.5. Bioinformatics analysis

The raw reads obtained by Illumina MiSeq sequencing were demultiplexed, quality-filtered using fastp version 0.20.0 [37] and merged using FLASH version 1.2.7 [38]. The remaining sequences were assigned to operational taxonomic units (OTUs) according to 97% identity using UPARSE version 7.1 [39]. The chimeric sequences were discarded. Taxonomic assignment analysis was conducted with the RDP reference database of 16S rRNA gene sequences [40]. The alpha diversity analysis used for the assessment of microbial community richness (sobs) and diversity (Shannon, Simpson) was performed using mothur (version v1.30.1 http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity). Heatmaps showing the differential relative abundances of families and genera and the correlation heatmap were prepared using the R package pheatmap (version 1.0.12). For beta diversity analysis, hierarchical clustering was performed based on the Bray-Curtis distance matrix using the QIIME program. Principal component analysis (PCA) was performed using R (version 2.15 <http://www.R-project.org>). To evaluate the scale of the influence of each differentially abundant phylotype, a linear discriminant analysis effect size (LEfSe) analysis was performed (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload).

2.6. Flow cytometry

Lamina propria (LP) lymphocytes from the colon were isolated as previously described [32]. Briefly, after excising the colon from the mice, the luminal contents were cleaned with phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS). The colons were then cut into small pieces (~1 cm) with scissors and treated with RPMI containing 1.5 mM DTT, 10 mM EDTA and 2% FCS at 37°C for 30 min to remove the epithelial cells. The remaining tissues were then minced and dissociated using collagenase solution (2 mg/mL collagenase II; Gibco, 100 $\mu\text{g}/\text{mL}$ DNase I; Sigma, and 10% FCS in RPMI) under constant stirring (37°C , 40 min). The lymphocytes were purified by Ficoll gradient centrifugation and washed twice with PBS containing 2% FCS, at which point they were ready for further use.

For the detection of surface markers, the cells were stained in PBS containing 2% (wt/vol) BSA with anti-CD4 (RM4-5), anti-CD8 α (53–6.7), anti-TCR β (H57-597), anti-CD25 (PC61), anti-neuropilin-1 (3E12), anti-ICOS (15F9), anti-CD44 (IM7), and anti-CD62L (MEL-14) antibodies (BioLegend). Intracellular staining was performed after fixation and permeabilization with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) using the following antibodies: anti-Foxp3 (FJK-16s) and anti-ROR- γ t (AFKJS-9) (eBioscience); anti-Ki-67 (Ki-67), anti-CTLA4 (UC10-4B9), anti-IL-4 (11B11), anti-IFN- γ (XMG1.2), and anti-IL-17A (TC11-18H10.1) (BioLegend); and anti-IL-10 (JES5-16E3) (BD Biosciences). Caspase-3 activity was detected with an active caspase-3 apoptosis kit (BD Biosciences) according to the manufacturer's instructions. For cytokine measurements, we stimulated the cells with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Cayman) in the presence of GolgiStop (BD Biosciences) for 4 h. Flow cytometry data were then acquired with a FACSCanto II flow cytometer (BD Biosciences) and analyzed using

FlowJo software.

2.7. Treg suppression assay

The isolation of LP lymphocytes from the colon was performed as described above. The resulting cells were incubated with anti-CD4

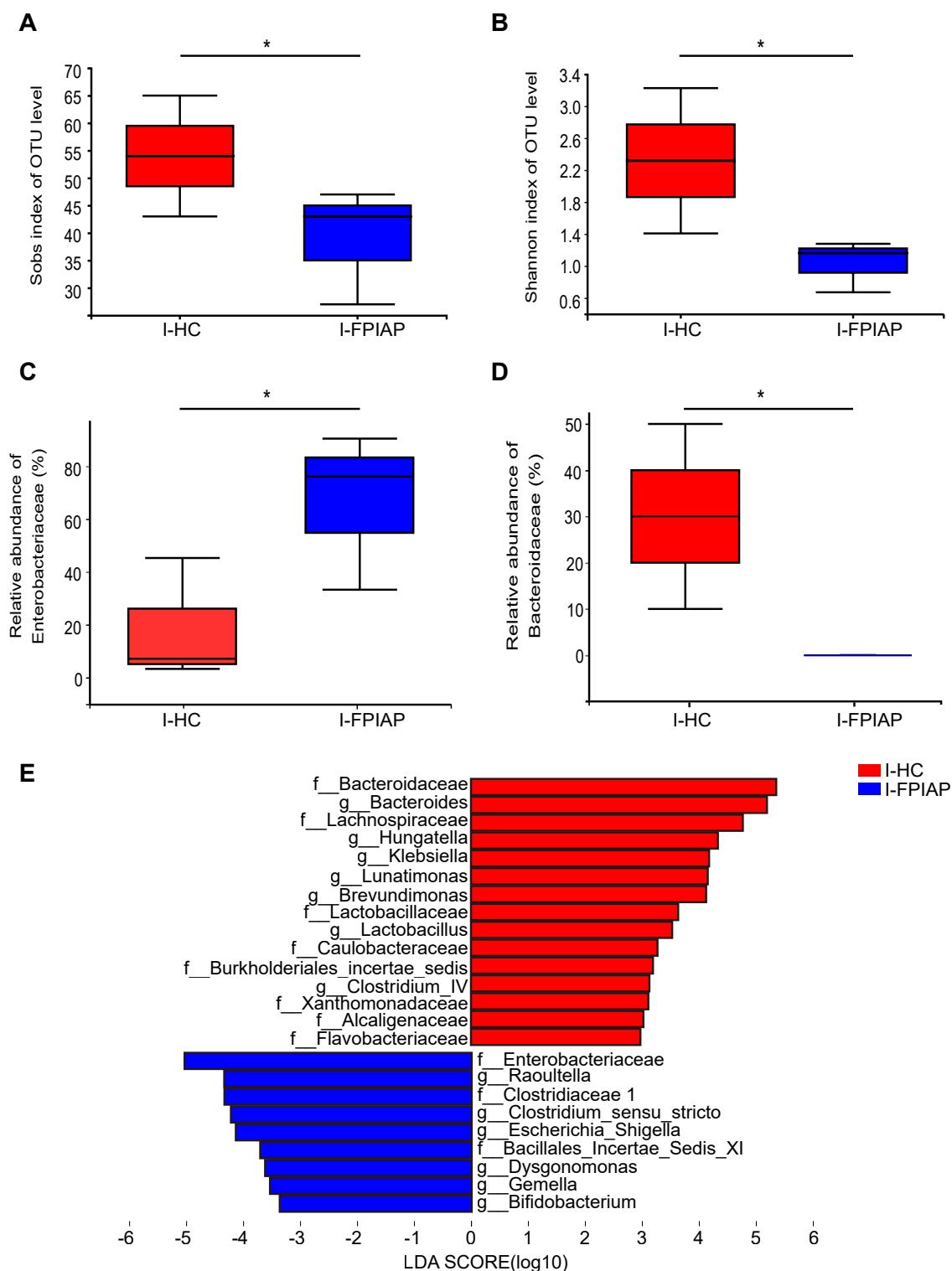


Fig. 1. Analysis of the gut microbiome of I-FPIAP and I-HC. (A, B) Alpha diversity based on the Sobs (A) and Shannon indexes (B) at the OTU level. The results are shown as box-and-whisker plots. (C, D) The relative abundance of *Enterobacteriaceae* (C) and *Bacteroidaceae* (D) in each group is shown as a box-and-whisker plot. (E) The most differentially abundant taxa at the genus and family levels were identified by LEfSe analysis. Taxa enriched in I-HC are indicated with positive scores, and taxa enriched in I-FPIAP are indicated with negative scores. Only taxa reaching an effect-size threshold of >3 (on a log10 scale) are shown. * $P < 0.05$ (A,B), * corrected P value < 0.05 (C,D). The data in A-D are presented as box-and-whisker plots showing the minimum, first quartile, median, third quartile, and maximum values.

(RM4-5) and anti-CD25 (PC61) antibodies (BioLegend) for 30 min and then used for the flow sorting of CD4⁺CD25⁺ T cells (sorted as Treg cells) and CD4⁺CD25⁻ T cells (sorted as responder T cells) with a FACS Aria II flow cytometer (BD). The purity of the sorted cells in each fraction was >95%. For the detection of Treg suppressive capacities, an in vitro Treg suppression assay was conducted using the sorted cells as previously described [41]. Briefly, after labeling the responder T cells with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's recommended protocols (Invitrogen), the labeled responder T cells (2.5 × 10⁴ cells/well) were cocultured with Treg cells (at a ratio of 2:1) in 96-well round-bottomed plates coated with anti-CD3 (145-2C11; eBioscience) and anti-CD28 (37.51; eBioscience). All the cells were cultured in RPMI-1640 supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin. After 72 h,

the cells were harvested, and the CFSE dilution revealing the proliferation of responder T cells was assessed by flow cytometry.

2.8. Statistical analysis

To analyze the differences in alpha diversity, we calculated the Sobs, Shannon, and Simpson indexes for each sample and performed statistical analyses using the Wilcoxon rank-sum test. A *P* value less than 0.05 was considered to indicate significance. Analyses of the differential relative abundances at multiple levels (e.g., family and genus) between groups were performed with the R package STATS. *P* values were calculated using the Wilcoxon rank-sum test, the false discovery rate (FDR) was used for multiple-test correction, and a corrected *P* value less than 0.05 was considered to indicate significance. For the LefSe analysis, the

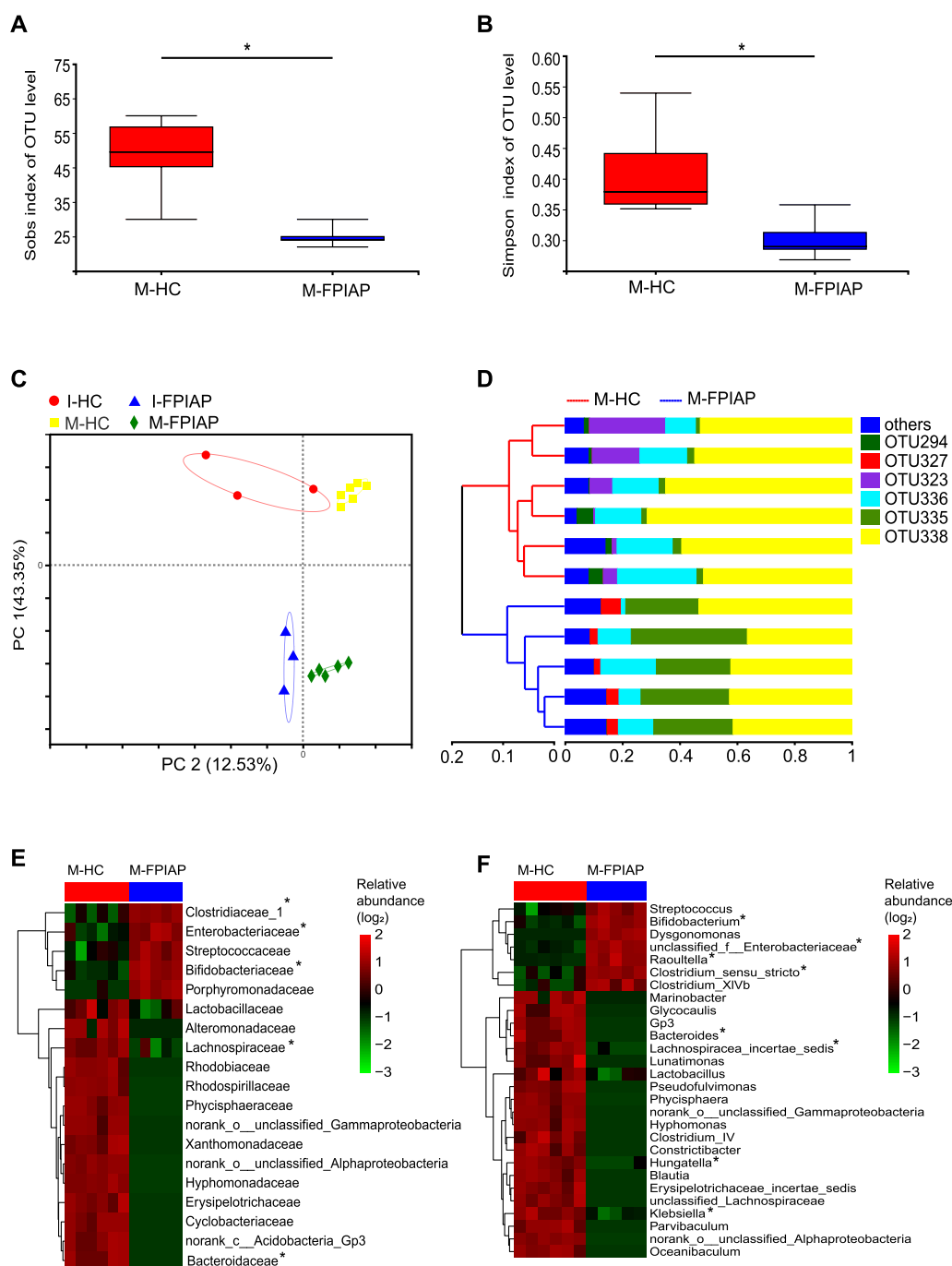


Fig. 2. Analysis of the gut microbiome of recipient mice. (A, B) The alpha diversity of the gut microbiota from recipient mice was analyzed based on the Sobs (A) and Simpson indexes (B) at the OTU level. The results are shown as box-and-whisker plots. (C) PCA of the gut microbiome compositions in donors and recipient mice at the genus level. (D) Hierarchical clustering analysis (OTU level) of the gut microbiome compositions of the recipient mice. Right panel, community bar plot analysis showing the relative abundances of OTUs present in samples from the recipient mice. (E) Heatmap of the 19 families showing differential abundances between the two groups of recipient mice. The predominant differential families with a relative abundance greater than 1% in either group are marked with an asterisk. (F) Heatmap of the 28 genera showing differential abundances between the two groups of recipient mice. The predominant differential genera with a relative abundance greater than 1% in either group are marked with an asterisk. **P* < 0.05 (A, B). The data in A and B are presented as box-and-whisker plots showing the minimum, first quartile, median, third quartile, and maximum values.

Kruskal-Wallis rank sum test was used to determine the phylotypes with significantly different abundances, and the effect size of each differentially abundant phylotype was evaluated by linear discriminant analysis (LDA). The effect-size threshold was set to 3 or 2.5 (on a log10 scale) for the donor or recipient mouse analysis, respectively. The immune results were statistically analyzed by unpaired Student's t-test (two-tailed) using GraphPad Prism software version 8.00. A *P* value less than 0.05 was considered to indicate significance. This software program was also used for the linear regression analysis to measure the correlations between two variables. An *R*² value greater than 0.7 indicates a high correlation, a value in the range of 0.5–0.7 indicates a moderate correlation, and a value less than 0.5 indicates a weak correlation. A *P* value less than 0.05 was considered to indicate significance.

3. Results

3.1. Donors with CM-FPIAP exhibit significant alterations in their gut microbiome

First, we analyzed the gut microbiota of infant donors with CM-FPIAP (*n* = 3, I-FPIAP) and age-matched HCs (*n* = 3, I-HC) and found no significant differences in their demographic characteristics (Table 1). PCA analysis showed that the gut microbiota from the two groups could be separated into different clusters (Fig. 2C), revealing an overall structural difference in their gut microbiome compositions. Alpha diversity analysis showed that both the Sobs and Shannon indexes were significantly reduced in the I-FPIAP group (Fig. 1A and B), representing

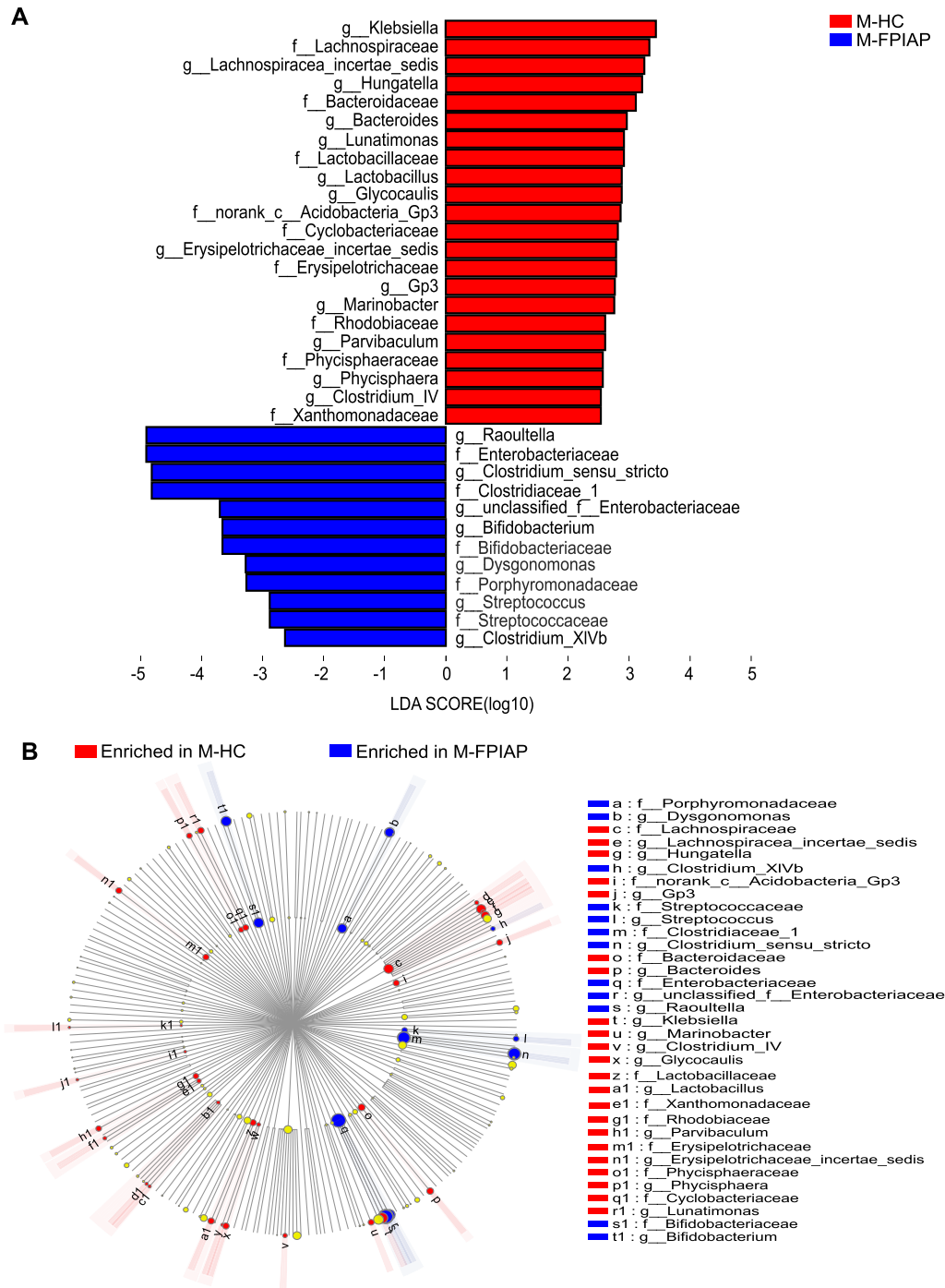


Fig. 3. LEfSe analysis showing the distinct gut microbiome compositions in the recipient mice. The most differentially abundant taxa between the two groups of recipient mice at the genus and family levels were identified by LEfSe analysis. (A) The LDA scores of differentially abundant taxa were calculated. The taxa enriched in M-HC mice are indicated with positive scores, and the taxa enriched in M-FPIAP mice are indicated with negative scores. Only taxa with an effect-size threshold of >2.5 (on a log10 scale) are shown. (B) The differentially abundant taxa are represented in a LEfSe cladogram. Red, taxa enriched in M-HC mice; blue, taxa enriched in M-FPIAP mice. The size of each dot is proportional to its effect size. Only taxa with an effect-size threshold of >2.5 (on a log10 scale) are shown in the right panel.

a significant decrease in the microbial richness and diversity. Moreover, we found that the I-FPIAP group presented a significant increase in the relative abundance of *Enterobacteriaceae* (Fig. 1C) and a decrease in the relative abundance of *Bacteroidaceae* (Fig. 1D), which resulted in an elevated *Enterobacteriaceae/Bacteroidaceae* ratio. The specific differential phylotypes between the two groups were further investigated by LEfSe, and the most differentially abundant families and genera are shown in Fig. 1E.

3.2. Analysis of gut microbiome changes in recipient mice

At 2 weeks post FMT, stool samples from the recipient mice were collected and subjected to 16S rRNA gene sequencing to analyze the gut microbiome. Despite adaptation to the murine host, the main microbial signatures of I-FPIAP and I-HC group were maintained in their recipient mice (Figs. 2C, 3A and 3B and 1E). Alpha diversity analysis showed a marked reduction of the microbial richness and diversity in the M-FPIAP group (Fig. 2A and B). Beta diversity analysis was performed to analyze the extent to which the gut microbiome of M-FPIAP mice differed from that of M-HC mice. We found that the gut microbiome from the two groups could be significantly separated based on the community composition by PCA (Fig. 2C) and could also be divided into different clusters using the Bray-Curtis distance matrix (Fig. 2D).

A taxon-dependent analysis using the RDP classifier was performed to identify specific phylotypes responsible for discriminating the gut microbiomes of recipient mice. At the family level, 19 families showed differential relative abundances between the two groups. Five predominant families with a relative abundance greater than 1% in either group were included. Among the five predominant families, *Bacteroidaceae* and *Lachnospiraceae* were significantly enriched in the M-HC group, whereas *Clostridiaceae* 1, *Enterobacteriaceae*, and *Bifidobacteriaceae* were enriched in the M-FPIAP group (Fig. 2E). At the genus level, 28 genera, including eight predominant genera, showed different abundances between the two groups. Among the eight predominant genera, *Lachnospiraceae incertae sedis* (family *Lachnospiraceae*), *Hungatella* (family *Lachnospiraceae*), *Klebsiella* (family *Enterobacteriaceae*), and *Bacteroides* (family *Bacteroidaceae*) were significantly enriched in the M-HC group, whereas *Bifidobacterium* (family *Bifidobacteriaceae*), *unclassified family Enterobacteriaceae* (family *Enterobacteriaceae*), *Raoultella* (family *Enterobacteriaceae*), and *Clostridium sensu stricto* (family *Clostridiaceae* 1) were significantly enriched in the M-FPIAP group (Fig. 2F). LEfSe analysis showed that *Raoultella* and *Clostridium sensu stricto* exhibited the highest LDA enrichment scores among all the phylotypes with differential abundances (increased by at least one order of magnitude), revealing that the two predominant genera were the most characteristic phylotypes responding to the M-FPIAP mice (Fig. 3A and B).

3.3. Reduced colonic population of Treg cells in M-FPIAP mice

We sampled colon LP lymphocytes at 2 weeks post FMT, to determine the influences of gut microbial dysbiosis on Treg cell-mediated colonic immune tolerance and homeostasis. M-FPIAP mice showed a significant decrease in the frequency of Foxp3⁺ Treg cells in the colonic LP (Fig. 4A). No difference was observed in peripheral lymphoid organs, such as the spleen (Supplementary Fig. 1). Moreover, the frequency of pTreg cells, which were characterized by negative expression of the marker neuropilin-1 [14,15], was also significantly reduced in the total Foxp3⁺ Treg and CD4⁺TCRβ⁺ T cell populations from the colonic LP of M-FPIAP mice (Fig. 4B). In contrast, the frequency of tTreg cells, which were marked by positive expression of neuropilin-1, showed a compensatory increase in the total Foxp3⁺ Treg cell population and a minor decrease in the CD4⁺TCRβ⁺ T cell population (Fig. 4B). These data illustrated that the deficiency of Treg cells observed in M-FPIAP mice was predominantly caused by the significant reduction in pTreg cells but not tTreg cells. The frequency of ROR-γt⁺ Treg cells was also found to be significantly decreased in the M-FPIAP group (Fig. 4C). To

determine whether the deficiency of the Treg cell population was caused by abnormal proliferation and apoptosis, the frequencies of Ki67⁺ and caspase-3⁺ Treg cells were detected, and no differences were observed between the two groups of recipient mice (Fig. 4D and E). Overall, these results demonstrated that M-FPIAP mice exhibited obvious defects in the colonic accumulation of Treg cells.

3.4. Defective suppressive function of colonic Treg cells in M-FPIAP mice

By analyzing the expression of CD44 and CD62L in the total Treg cell population, we observed a substantial reduction of CD44^{hi}CD62L^{lo} effector Treg cells in the colonic LP of M-FPIAP mice (Fig. 5A), revealing a significant deficiency of this suppressive T cell subset. In addition, we found that the single-cell-level expression of both CTLA4 and ICOS showed a marked decrease in colonic LP Treg cells from M-FPIAP mice (Fig. 5B). The capacity of these cells to produce IL-10 also exhibited a significant deficiency (Fig. 5C). To directly examine the suppressive function of colonic Treg cells, an in vitro Treg suppression assay was conducted. Colonic LP Treg cells from the M-FPIAP group showed a substantial reduction in their ability to suppress the proliferation of responder T cells compared with those from the M-HC group (Fig. 5D). Overall, these results revealed that colonic Treg cells from M-FPIAP mice exhibited substantial impairment in their suppressive capacities, which was accompanied by a series of deficiencies in their major suppressive mechanisms.

3.5. M-FPIAP mice show disrupted colonic immune homeostasis due to defects in the Treg cell compartment

The deficiency in the accumulation and function of Treg cells prompted us to examine whether colonic immune homeostasis was influenced in M-FPIAP mice. Although the frequencies of total CD4⁺ and CD8⁺ T cells in the colonic LP were largely comparable between the two groups (Fig. 6A), the M-FPIAP mice presented significantly increased frequencies of activated CD62L^{lo}CD44^{hi} effector/memory T cells in both the CD4⁺Foxp3⁻ and CD8⁺ T cell populations (Fig. 6B). Moreover, activated (CD44^{hi}) CD4⁺Foxp3⁻ colonic LP T cells from M-FPIAP mice showed significantly enhanced abilities to produce the proinflammatory cytokine IL-4 and, to a lesser extent, IFN-γ but a largely similar ability to produce IL-17 compared with those from the M-HC group (Fig. 6C), which reflected enhanced Th2-biased cytokine responses. Overall, these results demonstrated that M-FPIAP mice exhibited disruptions in colonic immune homeostasis, which was characterized by overactivated Th2 biased responses.

3.6. Specific gut phylotypes responsible for the disruption of colonic immune homeostasis

To identify the specific phylotypes that potentially contributed to the disruption of colonic immune homeostasis, we analyzed the correlations between genera with differential abundances and indicators measuring the frequency and function of Treg cells (Fig. 7). The relative abundances of *Bacteroides*, *Lactobacillus* and *Clostridium* IV showed moderately positive correlations ($0.7 > R^2 > 0.5$) with the frequencies of total Treg cells, pTreg cells, and ROR-γt⁺ Treg cells in the colonic LP. Moderately positive correlations between the relative abundances of *Klebsiella*, *Phycisphaera*, and the frequencies of pTreg cells were also observed. Moreover, all of these abovementioned phylotypes, which were reduced in M-FPIAP mice (Fig. 2F), exhibited moderately positive correlations with different types of indicators reflecting the function of colonic LP Treg cells. Interestingly, the abundances of *Raoultella* and *Clostridium sensu stricto*, which showed significant enrichment in M-FPIAP mice (Fig. 2F), presented highly negative correlations ($R^2 > 0.7$) with the frequencies of total Treg, pTreg, and ROR-γt⁺ Treg cells in colonic LP. Highly negative correlations of these two genera with the major suppressive factors (i.e., IL-10, CTLA4 and ICOS) and in vitro

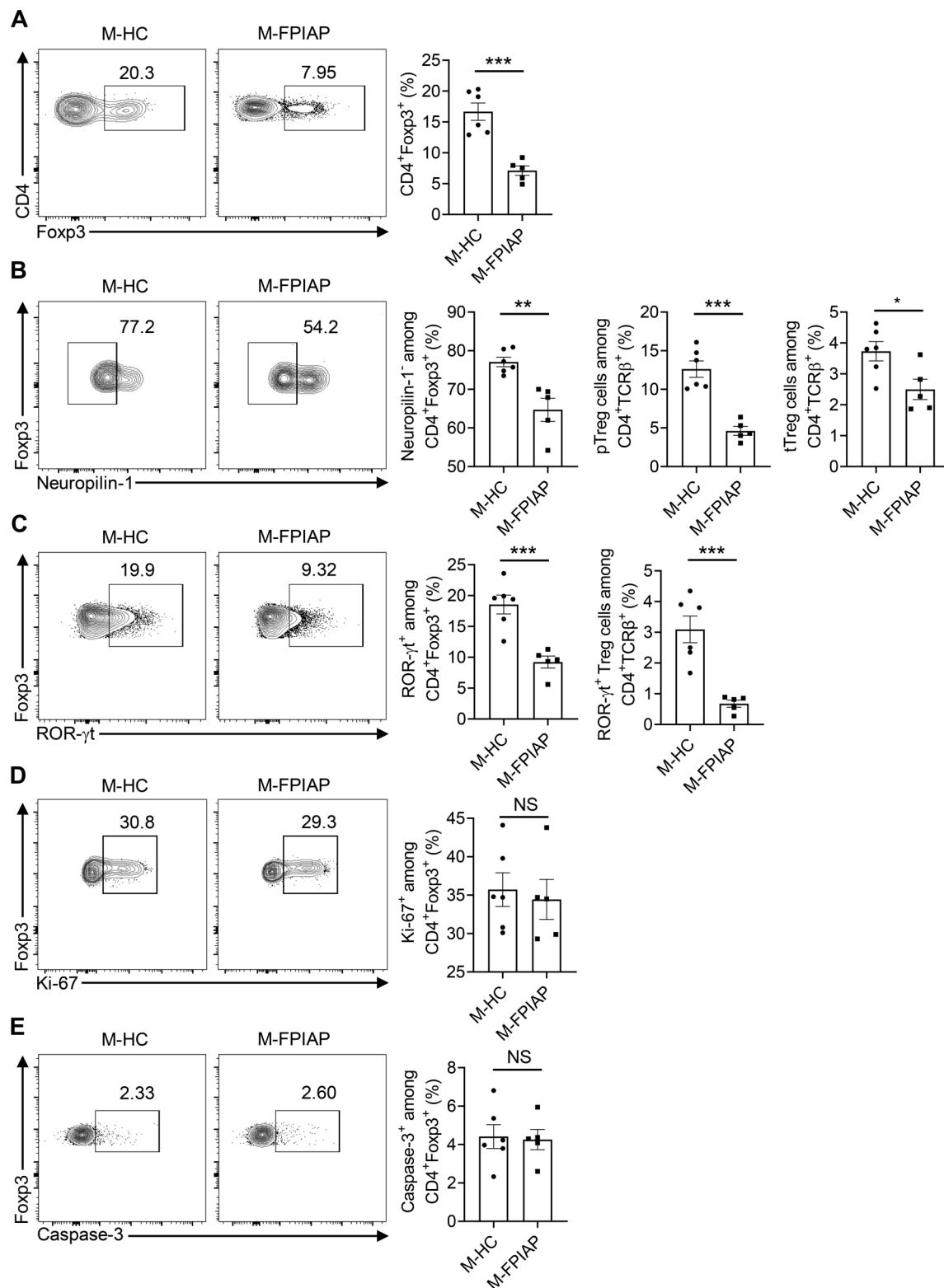


Fig. 4. Reduced colonic population of Treg cells in M-FPIAP mice. (A) Flow cytometry analysis of Treg (CD4⁺Foxp3⁺) cells (gated on CD4⁺TCRβ⁺ cells) in the colonic LP of M-HC and M-FPIAP mice. Right, frequency of Treg cells. (B) Flow cytometry analysis of pTreg (neuropilin-1⁺Foxp3⁺) cells in the colonic LP Treg cell populations of M-HC and M-FPIAP mice. Right panel, frequencies of pTreg cells among the total Treg and CD4⁺TCRβ⁺ cell populations and frequency of tTreg (neuropilin-1⁺Foxp3⁺) cells among the CD4⁺TCRβ⁺ cell population. (C) Flow cytometry analysis of ROR-γt⁺ cells in colonic LP Treg cells from M-HC and M-FPIAP mice. Right panel, frequencies of ROR-γt⁺ Treg cells among the total Treg and CD4⁺TCRβ⁺ cell populations. (D) Expression of Ki-67 in colonic LP Treg cells of M-HC and M-FPIAP mice. Right, quantification of Ki-67⁺ cells in the Treg cell population. (E) Caspase-3 activity in Treg cells from the colonic LP of M-HC and M-FPIAP mice. Right, quantification of Caspase-3⁺ cells in the Treg cell population. NS, not significant ($P > 0.05$); ** $P < 0.01$ and *** $P < 0.001$. The data are presented as the means $\pm$ s.e.m.s. Each symbol represents one mouse.

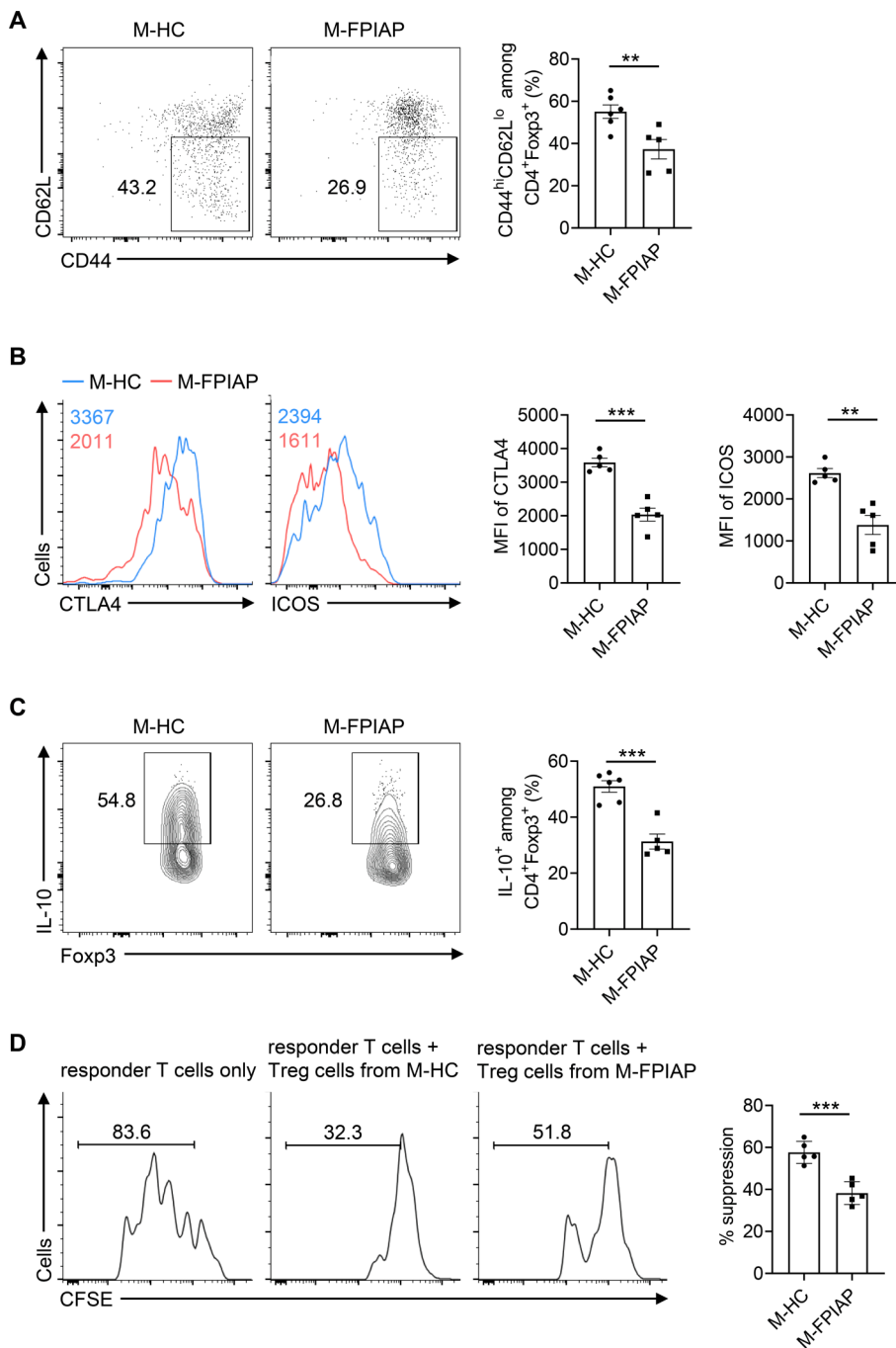


Fig. 5. Defective suppressive function of colonic Treg cells in M-FPIAP mice. (A) Flow cytometry analysis of CD44^{hi}CD62L^{lo} effector Treg cells in the total Treg cell population from the colonic LP of M-HC and M-FPIAP mice. Right panel, frequency of CD44^{hi}CD62L^{lo} effector Treg cells. (B) Expression of CTLA4 and ICOS in Treg cells from the colonic LP of M-HC and M-FPIAP mice. The mean fluorescent intensity (MFI) is presented in the plots. Right, quantification and statistical analyses of the MFI of CTLA4 and ICOS. (C) The colonic LP Treg cells of M-HC and M-FPIAP mice were sorted and stimulated with PMA and ionomycin in the presence of GolgiStop for 4 h. The release of IL-10 was measured by flow cytometry. Right panel, quantification of IL-10-producing Treg cells. (D) Representative suppression of responder T cell proliferation by Treg cells sorted from the colonic LP of M-HC and M-FPIAP mice. Responder T cells and Treg cells were cocultured (at a ratio of 2:1) and stimulated for 3 days with anti-CD3 and anti-CD28 antibodies. Right, percentage of suppression. ** $P < 0.01$ and *** $P < 0.001$. The data are presented as the means $\pm$ s.e.m.s. Each symbol represents one mouse.

suppressive capacities (i.e., % suppression) of Treg cells were also observed. Overall, these results revealed that significant enrichment of *Raoultella* and *Clostridium sensu stricto* was tightly associated with the defective accumulation and function of Treg cells, which further led to the disruption of immune homeostasis.

4. Discussion

A large number of studies have observed dynamic alterations in the gut microbiome compositions in infants with CMPA. Recent investigations have also begun to reveal the important roles of gut microbial dysbiosis in the development of CMPA [6]. However, the internal mechanisms remain unclear until now. In this study, we attempted to investigate whether non-IgE-mediated CMPA-associated gut microbial dysbiosis affects the establishment and maintenance of Treg

cell-mediated intestinal immune tolerance and immune homeostasis, given that disruption of these processes is considered one of the primary triggers for increased susceptibility to CMPA [1,3]. We found that due to dysbiosis of the gut microbiota, mice colonized with the gut microbiome from CM-FPIAP infants exhibited disruptions in intestinal Treg cell responses and immune homeostasis, as characterized by an overactivated Th2 biased immune response in their intestinal sites. Moreover, a correlation analysis identified two specific gut phylotypes, *Raoultella* and *Clostridium sensu stricto*, whose upregulation was closely associated with defects in the Treg cell compartment. Of concern, this phenomenon has not been observed in previous studies.

To guarantee the accuracy and effectiveness of our study, we first characterized the gut microbiome alterations in donors with CM-FPIAP and analyzed the consistency between our results and previous research findings. We found that donors with CM-FPIAP displayed an overall

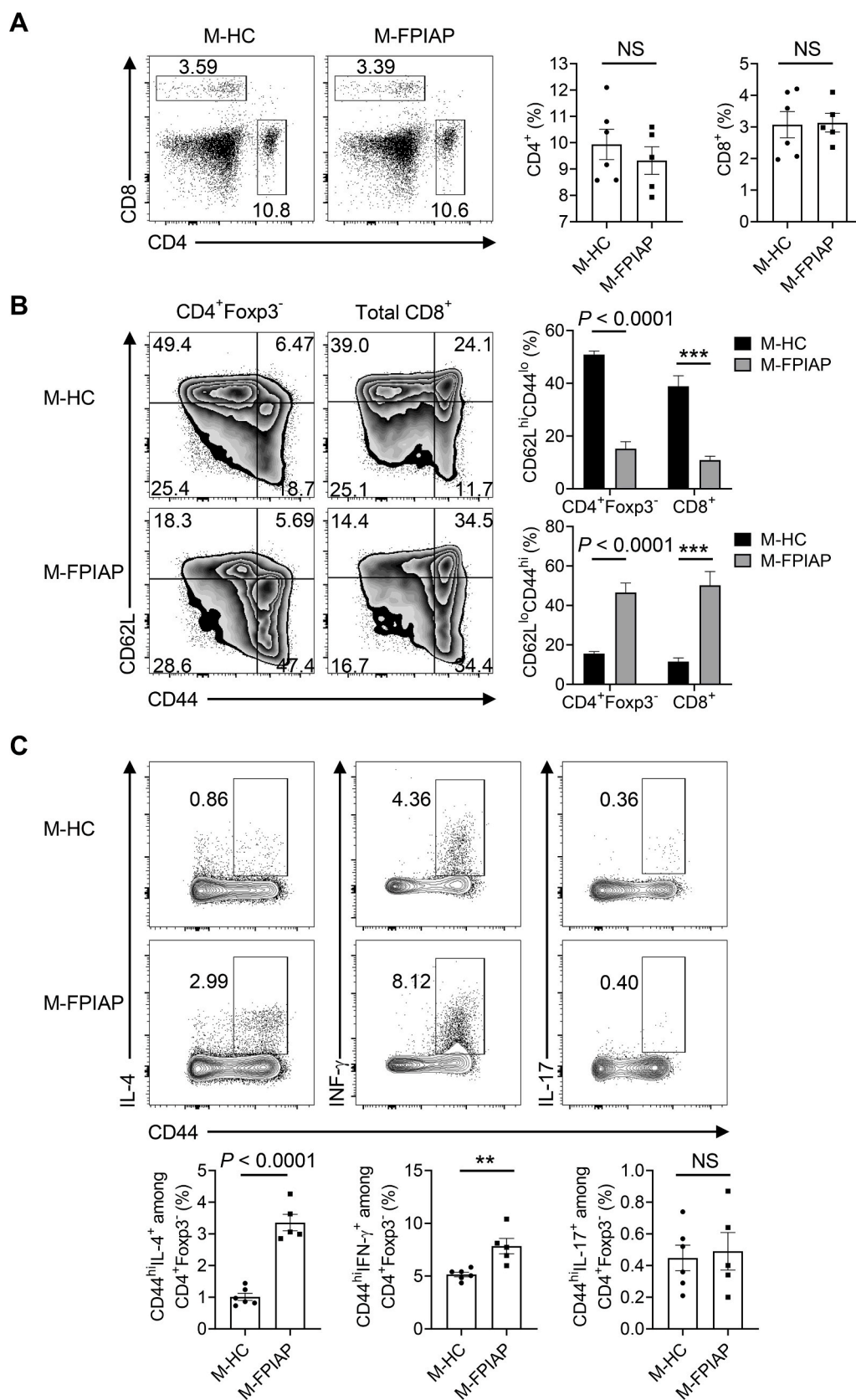


Fig. 6. Increased T cell activation and disrupted colonic immune homeostasis in M-FPIAP mice. (A) Flow cytometry analysis of CD4⁺ and CD8⁺ cells in the colonic LP lymphocyte populations of M-HC and M-FPIAP mice. Right, frequencies of CD4⁺ and CD8⁺ cells. (B) Flow cytometry analysis of effector/memory T cells (CD62L^{lo}CD44^{hi}) and naïve T cells (CD62L^{hi}CD44^{lo}) in the CD4⁺Foxp3⁻ and CD8⁺ T cell populations from the colonic LP of M-HC and M-FPIAP mice. Right panel, frequencies of effector/memory and naïve T cells. (C) Colonic LP lymphocytes of M-HC and M-FPIAP mice were stimulated with PMA and ionomycin in the presence of GolgiStop for 4 h. The release of IL-4, IFN-γ, and IL-17 from activated cells (CD44^{hi}) in CD4⁺Foxp3⁻ T cells was measured by flow cytometry. Bottom, quantification of IL-4⁺, IFN-γ⁺, and IL-17⁺ activated cells among CD4⁺Foxp3⁻ T cells. NS, not significant; **P < 0.01 and ***P < 0.001. The data are presented as the means ± s.e.m.s.

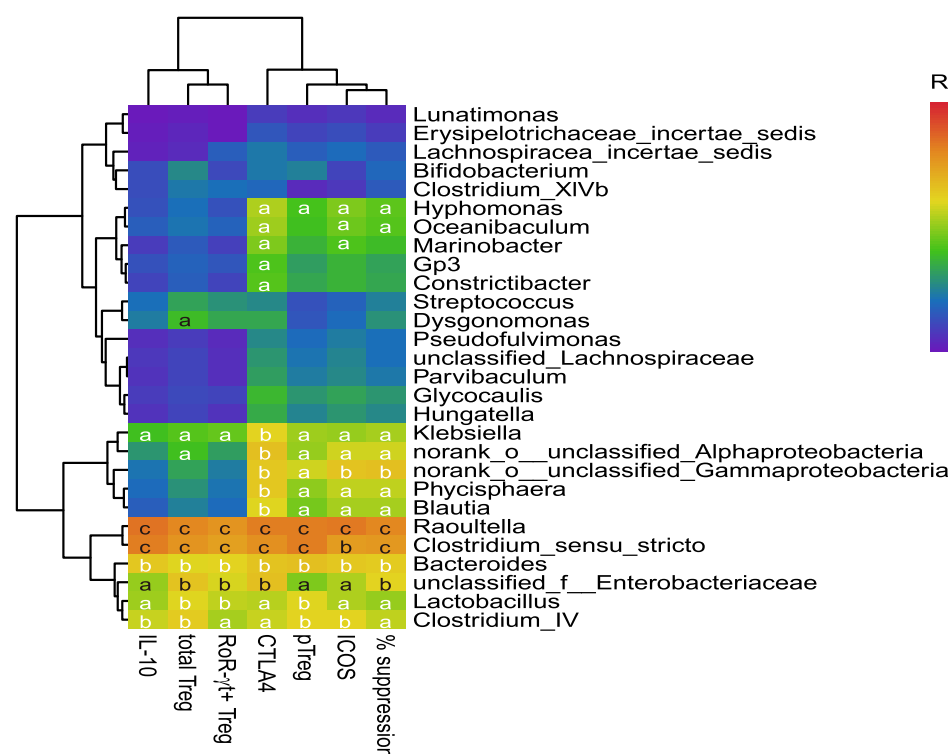


Fig. 7. Heatmap showing correlations between genera with differential abundances and indicators measuring the frequency and function of Treg cells. The value of the coefficient of determination (R^2) is represented in each grid of the heatmap. The grid with a white letter indicates a significant positive correlation between two variables, and the grid with a black letter indicates a significant negative correlation between two variables. The significance of the correlation is marked with ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$.

structural difference in their gut microbiome compositions (Fig. 2C) and observed a significant decrease in their gut microbial richness and diversity. We also observed a significantly elevated *Enterobacteriaceae/Bacteroidaceae* ratio in the gut microbiome of donors with CM-FPIAP. These findings are highly consistent with previous studies on infant CMPA and food sensitization [42,43]. Moreover, at the family level, *Clostridiaceae* 1 was observed to be enriched in CM-FPIAP donors, whereas *Alcaligenaceae*, *Xanthomonadaceae*, and *Flavobacteriaceae* were enriched in HCs (Fig. 1E). At the genus level, *Clostridium sensu stricto* and *Escherichia Shigella* were found to be enriched in CM-FPIAP donors, whereas *Bacteroides* and *Klebsiella* were enriched in HCs (Fig. 1E). These microbiota signatures showed consistency with previous studies on infant FAs [44]. In line with our expectations, *Lactobacillus*, a well-known probiotic, was significantly increased in HCs (Fig. 1E). Overall, these results demonstrated that the fecal microbiota samples that were collected in this study displayed a certain degree of accordance with the characteristics of the gut microbiome compositions in infant CMPA or FA that have been reported previously, which ensured the correctness and effectiveness of our subsequent investigations.

The fecal microbiota from the infant donors was transplanted into GF mice, and the gut microbiome changes in the recipient mice were analyzed at 2 weeks post FMT. Beta diversity analysis revealed a significant difference in the gut microbiome compositions between the two groups of recipient mice (Fig. 2C and D). Using the RDP classifier coupled with LEfSe, we identified a series of differentially abundant taxa between the two groups of recipient mice. These results demonstrated that M-FPIAP mice exhibited a large disturbance in their gut microbiome compositions. More importantly, we found that despite adaptation to the murine host, the main microbial signatures of CM-FPIAP donors and HCs were maintained in the recipient mice. Moreover, alpha diversity analysis revealed significant reductions in microbial richness and diversity in M-FPIAP mice, which also showed consistency with the trends found for the donors. These results revealed that the main characteristics of the gut microbiome in infant CM-FPIAP could be stably maintained in recipient mice, which was critical for our subsequent investigations.

The establishment of immune tolerance to commensal microbiota

and harmless food antigens and the maintenance of immune homeostasis are essential for the health of the intestine, which remains in constant contact with the outside world. Abundant evidence has demonstrated that Treg cells, by regulating the immune responses to microbiota and diet-related antigens, play a central role in achieving homeostasis [45–48]. Although both tTreg cells and pTreg cells exist in the intestine, growing evidence indicates that pTreg cells might play a predominant role in mediating tolerance toward microbiota and diet-related antigens [16,17]. Therefore, based on the significant reduction in intestinal pTreg cells observed in our mouse model, we speculate that non-IgE-mediated CMPA-associated microbial dysbiosis can substantially damage the establishment and maintenance of intestinal immune tolerance to food antigens, which is central in the pathogenesis of this type of FAs.

A large number of investigations have demonstrated that the gut microbiota has a significant impact on the induction and maintenance of intestinal Treg cells, particularly pTreg cells [14,29]. Both GF mice and mice administered broad-spectrum antibiotics showed significant deficiency in their intestinal pTreg cell compartment [17,20,30]. Moreover, a wide range of bacterial phylotypes reportedly have the capacity to promote Treg cell (particularly pTreg cell) accumulation in the intestine through multiple pathways [49–53]. Based on these studies, we speculate that the significant reductions in microbial richness and diversity contribute substantially to the marked reduction in intestinal Treg cells, particularly pTreg cells, observed in M-FPIAP mice. Furthermore, given that the proliferation and apoptosis of Treg cells were comparable between the two groups of recipient mice (Fig. 4D and E), we speculate that the impact of gut microbial dysbiosis in CM-FPIAP on the accumulation of intestinal Treg cells is mainly reflected in the obstruction of induction or development but not maintenance.

ROR- γ t⁺ Treg cells, which constitute a predominant component of intestinal pTreg cells, play an important role in the regulation of intestinal immune responses, including $T_H1/T_H2/T_H17$ inflammation [19–21]. Moreover, mice deficient in ROR- γ t⁺ Treg cells exhibited increased susceptibility to autoimmune colitis [21,54,55], indicating a pivotal role of these cells in maintaining intestinal immune tolerance

and homeostasis. As a subset of pTreg cells, the induction and maintenance of ROR- γ^+ Treg cells are also heavily influenced by gut microbiota. Moreover, many studies have indicated that these cells exhibit high sensitivity to gut microbial alterations [19,20,32]. Therefore, based on the significant reduction in intestinal ROR- γ^+ Treg cells observed in M-FPIAP mice, we speculate that the gut microbial dysbiosis-mediated disruption of immune tolerance and homeostasis under non-IgE-mediated CMPA conditions might partially depend on the impaired induction of ROR- γ^+ Treg cells.

In addition to their normal abundance, the intact suppressive function of Treg cells is also critical for the maintenance of immune tolerance and homeostasis. Growing evidence has indicated that the gut microbiota plays a central role in promoting the function of intestinal Treg cells [24,26,52]. Here, an in vitro Treg suppression assay demonstrated that gut microbial dysbiosis under non-IgE-mediated CMPA conditions leads to not only impaired accumulation but also substantial defects in the function of intestinal Treg cells (Fig. 5D). To achieve their suppressive function, intestinal Treg cells utilize a variety of suppressive mechanisms, such as the expression of CTLA4 [19,24,25] and ICOS [26] and the production of anti-inflammatory cytokines such as IL-10 [27,28]. Furthermore, some investigations suggest that the establishment of these suppressive mechanisms is dependent on the intestinal microbiota. For example, it has been reported that the production of IL-10 by intestinal Treg cells is significantly reduced in GF mice and mice treated with antibiotics [24,52]. Moreover, GF mice colonized with *Clostridium* species presented a substantial increase in CTLA4 expression in intestinal Treg cells [24]. Here, analysis of the possible mechanisms resulting in the defective function of intestinal Treg cells due to gut microbial dysbiosis revealed marked deficiencies in the expression of CTLA4 and ICOS and the production of IL-10 by intestinal Treg cells from M-FPIAP mice. Overall, these results demonstrate that the defects in the suppressive function of intestinal Treg cells caused by gut microbial dysbiosis under non-IgE-mediated CMPA conditions are mediated by a series of impairments in their major suppressive mechanisms, including CTLA4, ICOS and IL-10.

To directly investigate the influence of gut microbial dysbiosis under non-IgE-mediated CMPA conditions on intestinal immune homeostasis, the characteristics of intestinal T cell responses were analyzed in detail. Although the overall intestinal development and accumulation of the T cell compartment were not obviously affected (Fig. 6A), we found substantially increased frequencies of effector/memory T cells in both the CD4⁺Foxp3⁻ and CD8⁺ T cell populations, which showed that the conventional T cell compartment was generally overactivated due to the deficiency of Treg cells in M-FPIAP mice. Moreover, the identification of the dominant type of immune responses revealed that activated T cells of M-FPIAP mice developed enhanced cytokine responses, which were more biased toward a Th2-type response, in the intestinal sites (Fig. 6C). Overall, these results demonstrate that the disruption of intestinal immune homeostasis due to defects in Treg cell compartments, as characterized by an overactivated Th2 biased immune response, is a serious consequence of gut microbial dysbiosis. More importantly, we hypothesize that this “disruption” might play a fundamental role in triggering the initiation of non-IgE-mediated CMPA.

A variety of beneficial commensal microbial phylotypes, which are mainly assigned to the genera *Clostridium*, *Bacteroides*, *Bifidobacterium*, *Lactobacillus* and *Helicobacter*, promote the accumulation of intestinal Treg cells through both antigen-specific and nonspecific mechanisms [20,21,24,26,31,49,50,56]. However, the potentially pathogenic microbial phylotypes capable of damaging intestinal Treg cell responses and disrupting intestinal immune tolerance and homeostasis have not been fully characterized. Here, the identification of the possible gut phylotypes responsible for the disruption of intestinal immune tolerance and homeostasis showed that the abundances of a series of phylotypes, such as *Bacteroides*, *Lactobacillus*, *Clostridium* IV, *Klebsiella*, and *Phycisphaera*, which were substantially reduced in M-FPIAP mice, exhibited moderately positive correlations with the frequencies of Treg cell

compartments in the colon. These results were consistent with previous studies showing that some of these phylotypes can promote intestinal Treg cells [20,24,26,31] and indicated that the reduction of these phylotypes might have at least partially contributed to the defects of Treg cells and the disrupted intestinal immune homeostasis in M-FPIAP mice. More importantly, we identified two genera, *Raoultella* (belonging to the family *Enterobacteriaceae*) and *Clostridium sensu stricto* (belonging to the family *Clostridiaceae* 1), whose abundances presented highly negative correlations with a series of indicators measuring the accumulation and function of Treg cells. These results imply that the two genera could contain potentially pathogenic taxa capable of damaging intestinal Treg cell responses and subsequently disrupting intestinal immune tolerance and homeostasis under non-IgE-mediated CMPA conditions. However, we hypothesize that many future studies are needed to fully understand the mechanism underlying this phenomenon.

Compared with IgE-mediated FAs, the pathogenesis of non-IgE-mediated reactions is considered to be markedly more complicated and remains poorly characterized. Various types of adaptive immune (CD4⁺ and CD8⁺ T cells and B cells) and innate immune cells (monocytes, neutrophils, NK cells, and eosinophils) have been implicated in the pathology of this type of FAs [57–59]. Using transgenic mice with mutations in the IL-4R α chain, which are particularly prone to oral allergic sensitization, a recent study demonstrated the central role that the defective induction and function of mucosal allergen-specific Treg cells play in promoting the development of IgE-mediated reactions [60]. Moreover, the failure of gut microbiota from patients with IgE-mediated FAs to protect against the same type of allergic reactions in a mouse disease model has been reported [32]. More importantly, studies have also demonstrated the capacities of human beneficial commensal microbiota to promote Treg cell-mediated oral tolerance and protect against IgE-mediated reactions [32]. Overall, these results have highlighted the substantial interactions between gut microbiota and Treg cell responses, which are central to oral tolerance, under IgE-mediated FA conditions. In contrast, little is known about whether interactions would exist and what type of interactions could occur under non-IgE-mediated FA conditions. The results of our study suggested that the dynamic alterations in the gut microbiome compositions in non-IgE-mediated gastrointestinal CMPA can result in substantially adverse effects on Treg cell-mediated intestinal immune tolerance and homeostasis. Furthermore, we found that these adverse effects are established through multiple pathways, including defects in the induction of effector Treg cells, defective expression of CTLA4 and ICOS, and impaired production of IL-10 by intestinal Treg cells. These results will help improve our understanding of the pathogenesis of non-IgE-mediated FA in the future.

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Author statement

Jinzi Wang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Writing – review & editing. Shuang Zheng: Conceptualization, Methodology, Investigation, Data curation. Xin Yang: Investigation. Ben Huazeng: Conceptualization, Resources. Qian Cheng: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2021.105020>.

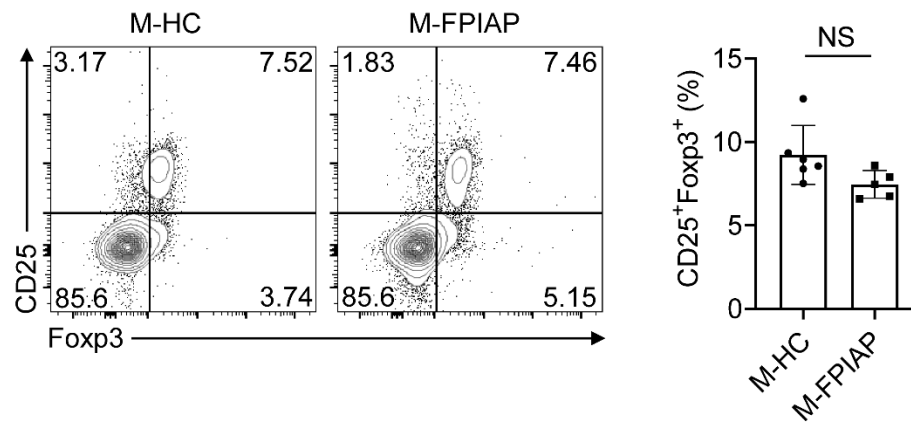
Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplementary Figure 1. Flow cytometry analysis of Treg (CD25⁺Foxp3⁺) cells (gated on CD4⁺TCRβ⁺ cells) in the spleen of M-HC and M-FPIAP mice. Right, frequency of Treg cells. NS, not significant (P > 0.05). The data are presented as the means ± s.e.m.s. Each symbol represents one mouse.