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Fecal microbiota transplantation ameliorates type 2 diabetes via metabolic remodeling of the gut microbiota in db/db mice

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ABSTRACT

Introduction Gut microbiome (GM) deregulation has been implicated in major conditions such as obesity and type 2 diabetes (T2DM). Our previous prospective study indicated that fecal microbiota transplantation (FMT) successfully improved patients with T2DM. We hypothesized that FMT may be a potential therapeutic method for T2DM, but its precise mechanisms in T2DM remains to be elucidated.

Research design and methods Eight db/m mice were FMT donors and control mice, and 16 genetically diabetic db/db mice were equally divided into two groups (db/db+phosphate-buffered saline (PBS) group, db/db+FMT group). The db/db+FMT group was administered fresh fecal suspension (0.2 mL/mice) daily for 4 weeks. Analysis of the GM and serum metabolome was carried out by 16S ribosomal RNA sequencing and liquid chromatogram-mass spectrometry, respectively. Effects of FMT on the gut barrier and pancreas were assessed using protein assays, messenger RNA, immunohistology and clinical indicators testing.

Results Our results showed that FMT treatment of db/db mice relieves a series of clinical indicators, including fasting plasma glucose, serum insulin and oral glucose tolerance test among others. Compared with non-diabetic control mice, db/db+PBS mice exhibited decreased abundance of Ruminococcaceae, Porphyromonadaceae and increased abundance of Rikenellaceae and Lactobacillaceae. FMT treatment reversed this effect on the microbiome. Eleven metabolites were changed between the db/db+PBS and db/db+FMT groups. Correlation analysis showed that the structural changes of the GM were correlated with host metabolite levels. We further showed that FMT treatment of db/db mice improved intestinal barrier function, reduced inflammation and caused an alteration in the number of circulating immune cells.

Conclusions FMT-mediated changes in the GM, serum metabolites, intestinal epithelial barrier, inflammation and circulating immune cells play an important role in the efficacy of FMT on T2DM disease progression.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Gut microbiome (GM) dysbiosis contributes to the development of type 2 diabetes mellitus (T2DM).

⇒ A previous prospective study suggested that fecal microbiota transplantation (FMT) could ameliorate glucose metabolism and insulin sensitivity in patients with T2DM.

WHAT THIS STUDY ADDS

⇒ This study reveals that FMT could significantly improve the glucose metabolism and insulin sensitivity in db/db mice.

⇒ Changes of GM, serum metabolites, intestinal epithelial barrier and circulating immune cells were identified as potential mechanisms of the beneficial effects of FMT on glucose metabolism and insulin sensitivity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The study provides potential mechanisms for the beneficial effect of FMT in T2DM, and provides a robust theoretical basis for prevention and treatment of T2DM using FMT.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is the most common chronic metabolic disease, which is characterized by abnormal glucose homeostasis. According to the International Diabetes Federation, the worldwide prevalence of diabetes is 9.7% (463 million people) among adults in 2021, which is predicted to increase to 10.9% (700 million) in 2045.[1] The global epidemic of T2DM has been shown to be closely associated with dietary structure, lifestyle, the use of drugs and environmental pollution.[2 3]

Growing evidence suggests that the gut microbiome (GM) is a critical mediator in the development of T2DM. The GM is a complex and dynamic microbial ecosystem and the number of gut microbes in the intestineisestimated to exceed 10^{14}.[4] Importantly, the GM

plays a critical role in many human physiological and pathological processes, including metabolism, immunity, tumor metastasis and inflammation.[5 6] Disruption of the GM balance frequently initiates a pathological cascade increasing risk for the development of various diseases, such as diabetes, obesity, food allergies and malignancies.[7 8] Specifically, dysbiosis of the GM significantly affects glucose metabolism. The diversity of dominant microbiota, and the relative abundance of microbiota were found to be different in patients with T2DM compared with controls.[9 10] Thus, an appropriate balance between the host and GM appears to be essential for maintaining glucose metabolism.

Experimental studies and human clinical trials have shown positive effects of various prebiotics and probiotic strains on metabolic control of patients with T2DM.[11 12] These compounds appear to reverse dysbiosis and restore the gut functional integrity. Fecal microbiota transplantation (FMT) has been shown to be safe and well-tolerated,[13] can significantly alter the recipient GM composition (eg, by increasing butyrate-producing bacterial strains) and can affect glucose control in subjects with metabolic syndrome based on baseline microbiota.[14] Notably, in our recent prospective study we found that transplantation of the fecal microbiota from healthy donors is sufficient to improve glucose metabolism and insulin sensitivity of patients with T2DM.[15] Importantly, recent research reports that FMT stabilized residual beta cell function in subjects with new-onset type 1 diabetes mellitus, further supporting the potential benefits of FMT therapy in patients with diabetes.[16] However, the pathophysiological mechanisms underlying the effects of FMT on T2DM remain not well understood.

In the present study, we investigate whether FMT exerts antidiabetic effects by modulating the composition of the GM and metabolites, intestinal barrier dysfunction, immune composition and the inflammatory response. These findings provide a basis for translational research on GM modulation and testing its therapeutic potential in T2DM.

MATERIALS AND METHODS

Animals and ethics approval

Jiangsu Jicui Yaokang Biotechnology provided db/m mice (20–25 g, 6–8 weeks, male) and db/db mice (30–35 g, 6–8 weeks, male).

Mice were housed in a specific pathogen-free (SPF) environment at 21±2°C and 45%±5% humidity (4 mice/cage, 12-hour day/night cycle). A total of 8 db/m mice and 16 db/db mice were used in the study. All mice were divided into three groups: the FMT donor group consisted of 8 db/m mice, whereas 16 db/db mice were randomly and evenly divided into two groups: db/db+phosphate-buffered saline (PBS) group and db/db+FMT group. Mice were treated with either 0.2 mL of PBS solution or 0.2 mL of fresh fecal suspension daily by oral gavage for 4 weeks.

Antibiotics pretreatment

After 1 week of adaptive feeding, all db/db mice were treated with antibiotic cocktail to eradicate the gut microbiota. Antibiotics were placed in autoclaved water supplemented with 1 mg/mL ampicillin, 1 mg/mL neomycin, 1 mg/mL metronidazole and 0.5 mg/mL vancomycin (Aladdin, Shanghai, China). Water was replaced every 3 days.

Preparation of fresh fecal suspension

Eight db/m mice were placed in sterile cages and approximately 200 mg of fresh fecal pellets were collected using sterile forceps after which fecal pellets were homogenized in 1.2 mL precooled sterile PBS solution as described previously.[17]

Fasting plasma glucose, weight and oral glucose tolerance test

The FPG and body weights were measured weekly. After fasting for 12–14 hours, all mice were fed 20% D-glucose at a dose of 1 g/kg, and the blood glucose was measured at 0, 30, 60, 90 and 120 min. Blood glucose was measured by an ACCU CHEK Advantage glucometer (Roche Diagnostics, Laval, Quebec, Canada).

Fasting serum insulin and homeostatic model assessment for insulin resistance

At the end of experiment, all mice were euthanized and blood, pancreas and small intestines were collected. Serum insulin was detected by ELISA kits (catalog# EMINS, Thermo-Fisher, USA) and followed the kit instructions. The homeostatic model assessment for insulin resistance index was calculated by the following formula: (Fasting serum insulin (FINS)×FPG)/22.5.

Flow cytometry

Anticoagulated blood (50 mL) was collected from the mouse ophthalmic vein and cells were stained with a combination of directly conjugated antibodies described in online supplemental table 1.

RT-quantitative PCR of tissue messenger RNA

Total RNA from intestinal tissues was isolated using TRIZOL (Vazyme BioTech, Nanjing, China) according to manufacturer's specifications. Complementary DNA was generated from 2 µg of total RNA with reverse transcription kit (Vazyme BioTech). Gene expression levels were evaluated by real-time PCR system (Thermo-Fisher) and normalized to β-actin gene. The primer sequences are collected in online supplemental table 2.

Western blot analysis

The small intestinal tissue extracts the protein by RIPA lysate (YWB001, Yi Fei Xue Biotechnology, Jiangsu, China) and determines the protein concentration by BCA kits (KGp902, Jiangsu KeyGen Bioech, China), then separated by electrophoresis using 10% sodium dodecyl sulfate polyacrylamide gels. The protein is transferred to a polyvinylidene fluoride (PVDF) membrane, 5% milk

is blocked for 1 hour, then Zonula occludens protein 1 (ZO-1) (1:1000, ab216880, abcam, USA), occludin (1:1000, ab224562, abcam), β-actin (1:5000, 66031-1-lg, Proteintech, USA) primary antibodies were incubated at 4°C overnight, Tris-Buffered Saline Tween-20 (TBST) washed the membrane three times and incubated with the secondary antibody for 1 hour at room temperature. The images were captured by Gel imaging system (Tanon, Shanghai, China), relative band densities were quantified using ImageJ software with β-actin as an internal control protein.

Histopathological analysis and immunohistochemistry

Samples of pancreas and small intestinal tissue were sent to Nanjing Free Thinking for H&E staining. The tissues were immune-stained and analyzed by Olympus microscope (Olympus, Tokyo, Japan). Six islets were used to count the score for each mouse. More than six different locations on each sample were used to count the score for each mouse. Pathology scoring is carried out by two professional pathologists using the pancreas and intestinal injury score, which are shown in online supplemental table 3-1/2, respectively.

Microbiota identification

Fecal samples from two db/m mice were discarded due to not meeting the collecting criteria, and all fecal samples were collected at the same time period. 16S recombinant DNA gene sequencing and data analysis Genomic DNA was extracted from fecal samples using the E.Z.N.A. soil DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA) following manufacturer's recommendations. The final DNA concentration and purification were determined by NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S ribosomal RNA (rRNA) gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR reactions were conducted using the program mention previously (3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C and 45 s for elongation at 72°C, and a final extension at 72°C for 10 min) and performed in triplicate 20 µL mixture containing 4 µL of 5×FastPfu Buffer, 2 µL of 2.5 mM Deoxy-ribonucleoside triphosphates (dNTPs), 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase and 10 ng of template DNA. The resulted PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, California, USA) and quantified using QuantiFluor-ST (Promega, USA) following manufacturer's protocol.

Metabolite analysis

In this project, a metabolomics study of 21 mouse serum samples (two mice were excluded since not enough blood was collected) was conducted using the liquid chromatogram-mass spectrometry (LC-MS) analysis platform. The sample is first pretreated to remove impurities and extract metabolites, and then the LC-MS-positive and LC-MS-negative modes are detected and collected, and the MS and MS/MS information of the metabolites are obtained. Metabolite analysis is performed using Progenesis QI software (Waters, Milford, Massachusetts, USA). The metabolite list and data matrix were analyzed using T-test and VIP (Orthogonal Projections to Latent Structures discriminant analysis (OPLS-DA)) to identify metabolites present at different levels between groups. Pathway analysis, association analysis, cluster analysis and other advanced analysis was used to mine the biological information of differential metabolism.

Statistical analysis

Data were analyzed using GraphPad Prism V.9.0.0 and R software V.4.3. Statistical differences among three groups were evaluated using one-way analysis of variance for normally distributed variables and the Kruskal-Wallis H test for non-normally distributed variables. Linear discriminant analysis (LDA) effect size analysis was used for the quantitative analysis of biomarkers within different cohorts. Correlation between variables was analyzed by Spearman's R coefficient. All data were expressed as the mean±SEM.

RESULT

FMT prevents the progression of diabetes in db/db mice

Alterations in the GM have been widely linked to changes in glucose metabolism, including improvement in insulin function. We examined whether FMT therapy can ameliorate disease progression in the db/db mouse model of type 2 diabetes. The GM was depleted in db/db mice (n=16) for 5 days followed by a 2-day washout after which mice were randomly divided into two groups (n=8 each) for FMT or control (PBS) treatment (figure 1A). Fecal samples were collected from control db/m mice and homogenized in PBS solution. FMT-treated db/db mice were gavaged daily for 28 consecutive days, whereas a control db/db cohort was gavaged with PBS alone. As expected, FMT therapy decreased plasma FPG levels in db/db mice starting in week 2, which became more pronounced in the fourth week of FMT ($p<0.01$; figure 1B). Interestingly, the improvement in FPG levels in the FMT cohort occurred in the absence of a reduction in body weight, even though there was a downward trend in db/db+FMT cohort compared with the control db/db+PBS cohort (figure 1C). Meanwhile, the oral glucose tolerance test (OGTT) was used to evaluate glucose tolerability. The areas under the curve (AUC) of glucose were calculated for each mouse after oral glucose administration. In comparison with PBS-treated db/db mice, FMT treatment of db/db mice significantly reduced the AUC (figure 1D). Together these results suggest that transplantation of a microbiota from db/m mice donors reversed

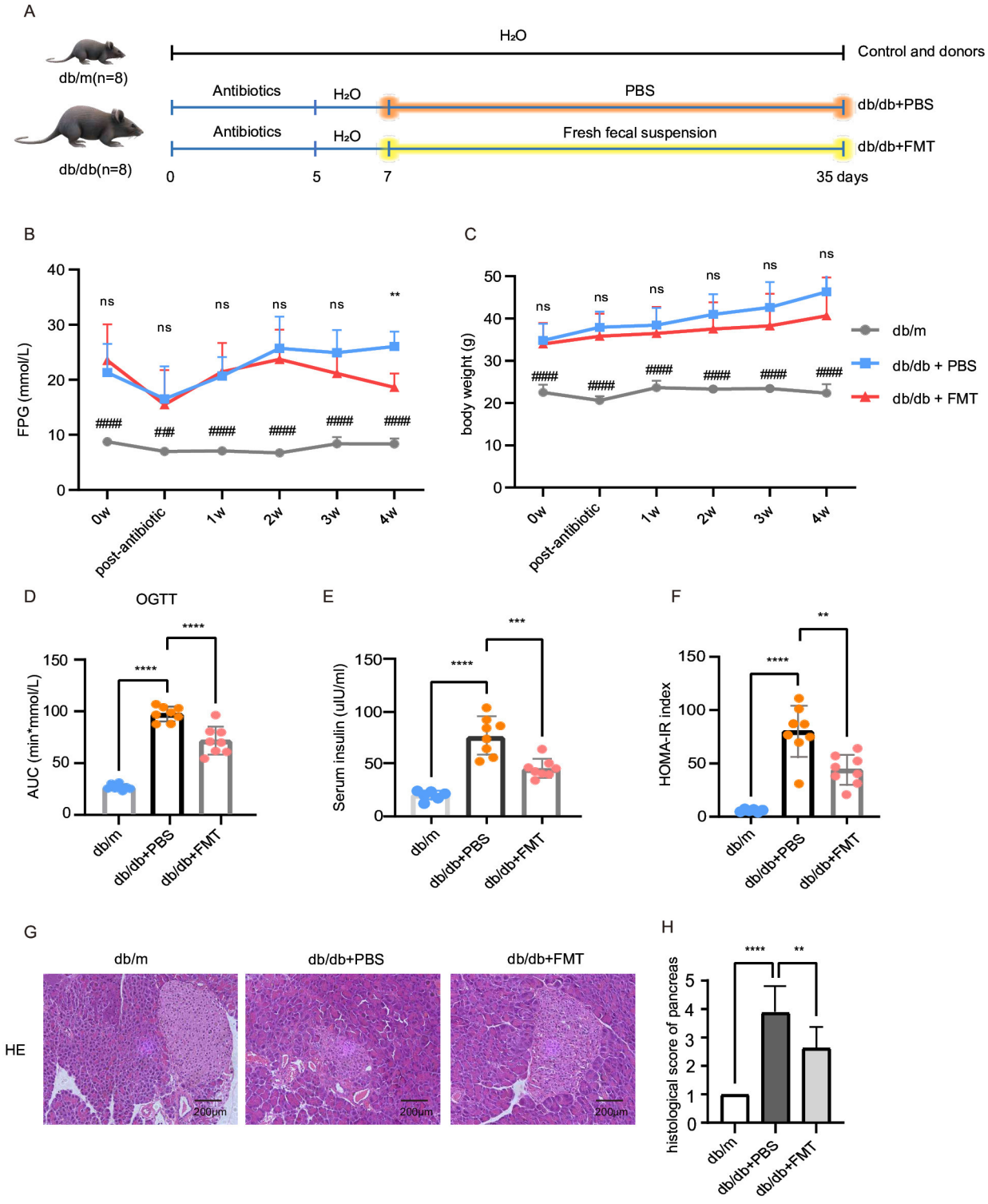

Figure 1 Phenotype and histological change of pancreas. (A) A simple graphic illustration of the study design (db/m: n=8, db/db+PBS: n=8, db/db+FMT: n=8). (B–D) Quantitative analysis of FBG, body weight, the AUC of OGTT in PBS-treated or FMT-treated db/db mice and db/m mice. (E) After 4 weeks of FMT treatment, serum insulin levels were measured between the three groups. (F) HOMA-IR index between the three groups. (G) Representative photomicrograph of H&E-stained pancreas section, magnification: 200×. (H) Histological score of pancreatic section; data were representative of multiple mice per group ('*' represents a comparison of db/db+PBS vs db/db+FMT group mice and '#' representss a comparison of db/db+PBS vs db/m group mice). Data were presented as mean±SEM (* or #: p<0.05; ** or ##: p<0.01; *** or ###: p<0.001; **** or ####: p<0.0001 and ns, not significant). AUC, area under the curve; FMT, fecal microbiota transplantation; HOMA-IR, homeostatic model assessment for insulin resistance; OGTT, oral glucose tolerance test; PBS, phosphate-buffered saline.

aberrant glucose metabolism characteristic of db/db mice, which is consistent with our previous study on FMT in patients with T2DM.[15]

Next, we examined whether FMT influenced the structure and function of islets in db/db mice. In addition, several related indicators, including fasting serum insulin and pancreas histology were assessed. Compared with db/m control mice, the level of fasting serum insulin was significantly increased in db/db mice ($p<0.0001$). However, after 4 weeks of FMT treatment, we observed a significant reduction in insulin levels ($p<0.0001$) (figure 1E). We then calculated HOMA-IR index, a measure for insulin resistance. The HOMA-IR index was significantly higher in db/db mice compared with db/m control mice ($p<0.0001$). However, the HOMA-IR index was significantly lower in the db/db+FMT cohort compared with the db/db+PBS cohort ($p<0.001$) (figure 1F). The histopathology of pancreas was evaluated by H&E staining using a scoring system that takes into account reduced islet area, hyperplasia of islet cells and nuclear size. The histological score was significantly higher in the db/db mouse cohort compared with the db/m mouse cohort (figure 1G). A significant improvement in the score was observed in the db/db+FMT cohort compared with db/db+PBS (figure 1H). These results indicate that db/db mice significantly benefit from FMT treatment, and FMT may improve islet function.

Effects of FMT on microbiota communication and diversity

To investigate the impact of the diabetic phenotype on GM, we analyzed the fecal microbiome of db/m and db/db mice using 16S rRNA amplicon sequencing. We also assessed composition and diversity after 4 weeks of FMT to examine engraftment and colonization dynamics of the FMT. A total of 662 operational taxonomic units (OTUs) were detected by 16S rRNA sequencing, and 420 OTUs were detected in the three groups. The composition of phylum-level flora showed a reduction in the Bacteroidetes/Firmicutes ratio in db/db mice compared with control db/m mice (figure 2A). After FMT treatment, this ratio was improved in db/db+FMT mice compared with db/db+PBS mice. The two α-diversity indexes, Shannon

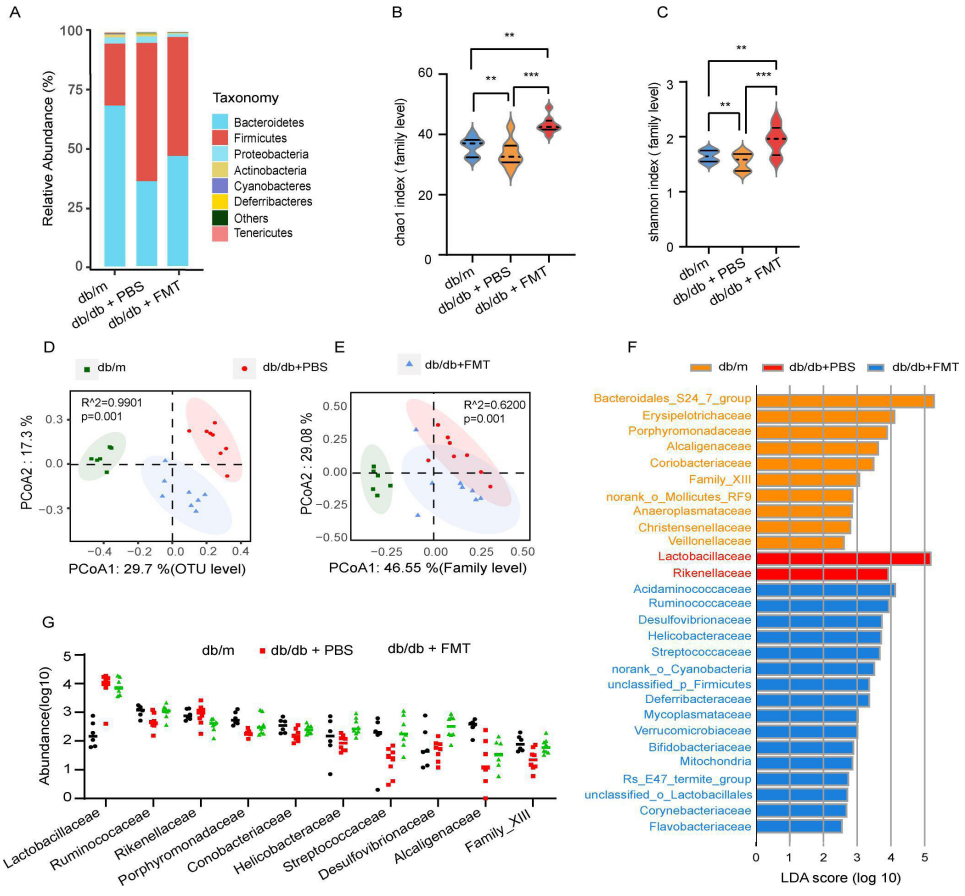

Figure 2 Effects of FMT on microbiota communication and diversity. (A) Species composition of db/m, db/db+PBS, db/db+FMT group at the phylum level. (B–C) Richness and uniformity of intestinal microbiota. Shannon index and Chao1 index at the family level (**: $0.001<p\leq0.01$; ***: $p\leq0.001$). (D–E) PCoA based on Bray-Curtis distance metric was analyzed on the OTU and family levels. (F) LEfSe was used to identify the biomarkers with significant differences between the three groups: db/m, db/db+PBS and db/db+FMT. (G) Relative abundance of family level in db/m (black), db/db+PBS (red) and db/db+FMT (green). Data significant difference was assessed through Kruskal-Wallis H test. FMT, fecal microbiota transplantation; LDA, linear discriminant analysis; LEfSe, LDA effect size; OTU, operational taxonomic unit; PBS, phosphate-buffered saline; PCoA, principal coordinate analysis.

and Chao1, were used to estimate OTU and family richness. Notably, we detected no differences among the three cohorts at the OTU level (online supplemental figure 1A,B). However, at the family level, we observed a significant increase in both Chao1 and Shannon indexes in fecal microbiota of db/db+FMT mice compared with db/m and db/db+PBS mice (p<0.01) (figure 2B and C). At the OTU and family levels, principal coordinate analysis based on Bray-Curtis dissimilarity showed a significant difference in the microbial community between the three mouse cohorts (R^2=0.9901, p=0.001) (figure 2D and E, The Principal Component Analysis (PCA) results were shown in online supplemental figure 1C). These results suggested that the relative abundance and diversity were significantly different between the three mouse cohorts, and FMT may restore these changes. LDA was performed to identify differences in abundance levels of bacterial clades in fecal samples across the three cohorts. Microbes with an LDA score >2 at the family level are shown in figure 2F. The abundance of *Lactobacillaceae* and *Rikenellaceae* were significantly enriched in db/db mice, and 16 family were changed following FMT, including *Ruminococcaceae*, *Desulfovibrionaceae*, *Helicobacteraceae* and *Streptococcaceae* (figure 2F). We further applied the Kruskal-Wallis H test to detect statistically significant differences in the relative abundances of microbial families between the three cohorts, 30 families were significantly changed. The db/db mice microbiota post-FMT tended to shift towards the donor microbiota, specifically through proportional increases of *Ruminococcaceae*, *Porphyromonadaceae*, *Conobacteriaceae*, *Helicobacteraceae*, *Streptococcaceae*, *Desulfovibrionaceae* and *Family_XIII*, and proportional decreases of *Rikenellaceae*, *Lactobacillaceae* and *Alcaligenaceae* (figure 2G).

FMT alter serum metabolites in db/db mice

Comparisons of metabolite profiles in serum of germ-free rodents with those of conventionally housed demonstrated that the GM significantly influences blood metabolite concentrations.[18] Also, recent metabolomic studies revealed strong associations between gut microbial-derived and host endogenous metabolites and various diseases.[19 20] We used LC-MS to explore changes in blood metabolites between the three mouse cohorts, and examined the correlation between serum metabolites and the GM. We detected a total of 12 247 metabolites in db/m, db/db+PBS and db/db+FMT cohorts, of which 420 were identified. Abundance levels of 109, 101 and 11 metabolites were significantly different between db/m versus db/db+PBS, db/m versus db/db+FMT and db/db+PBS versus db/db+FMT, respectively (online supplemental figure 2A); p<0.05 and Fold change (FC) >2). Partial least squares DA clearly showed differences in the metabolite composition between db/db+PBS and db/db+FMT groups (figure 3A). Similar results were also shown in OPLS-DA (online supplemental figure 2B). As shown in online supplemental figure 2C, there were 109 changed metabolites between normal mice and db/db mice. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to elucidate the alterations in metabolic pathways. In total, 21 metabolic pathways containing significantly discriminate metabolites between the normal and db/db mice groups were identified (online supplemental figure 2D). In the FMT-treated cohort, a total of 11 metabolites changed significantly, when compared with the db/db cohort. Specifically, six metabolites increased, including dehydroabietic acid (DAA), 12(R)-hydroxyeicosatetraenoic acid (12(R)-HETE), (±)10-hydroxy-4Z,7Z,11E,13Z,16Z,19Z-docosahexaenoic acid ((±)10-HDoHE), eicosapentaenoic acid, 12(S)-hydroxyeicosatetraenoic acid (12(S)-HEPE) and allysine, while five metabolites were significantly downregulated, including melibiose, methionyl-glutamate, (R)-(+)-2-pyrrolidone-5-carboxylic acid, allantoin and D-glutamine (figure 3B). FMT treatment at least partially restored levels of nine metabolites to levels observed in db/m mice (figure 3B). These results further indicate that FMT could improve the intestinal microflora disorder-triggered serum metabolite disorders in db/db mice. Furthermore, KEGG functional analysis found that glycerophospholipid metabolism, glycosylphosphatidylinositol-anchor biosynthesis, linoleic acid metabolism, α-linolenic acid metabolism, galactose metabolism, arachidonic acid metabolism were enriched (figure 3C).

Spearman's correlation analysis was applied to integrate and visualize the complex relationships between GM and serum metabolites between db/db+PBS and db/db+FMT mice. We included only bacterial taxa (at the family level) and metabolites that were significantly different between the db/db+PBS and db/db+FMT mice. The result showed that Rikenellaceae, enriched in db/db+PBS mice, was significantly negatively correlated with 12(S)-HEPE, (±)10-HDoHE and DAA (p<0.01). A positive correlation between Rikenellaceae and D-glutamine was observed (p<0.001). Moreover, Ruminococcaceae, which was enriched in db/db+FMT mice, was positively correlated with 12(S)-HEPE, (±)10-HDoHE (p<0.05) and DAA (p<0.01) and was negatively correlated with D-glutamine (p<0.001), melibiose and (R)-(+)-2-pyrrolidone-5-carboxylic acid (p<0.05) (figure 3D). Spearman's correlation at the genus level is present in (online supplemental figure 2E). Our result suggested that FMT could restore host metabolic dysfunction mediated by potential bacteria.

FMT improves the intestinal barrier, and inflammatory response in db/db mice

Chronic intestinal inflammation in patients with diabetes is considered an important factor in damage of the intestinal mucosa. Decreased intestinal mucosal permeability is associated with disease progression in patients with diabetes.[21] Therefore, we examined the effect of FMT on intestinal barrier function and inflammation in db/db mice. H&E staining is shown in figure 4A and a scoring system was used to evaluate the tissue damage (online supplemental table 3). The histological score

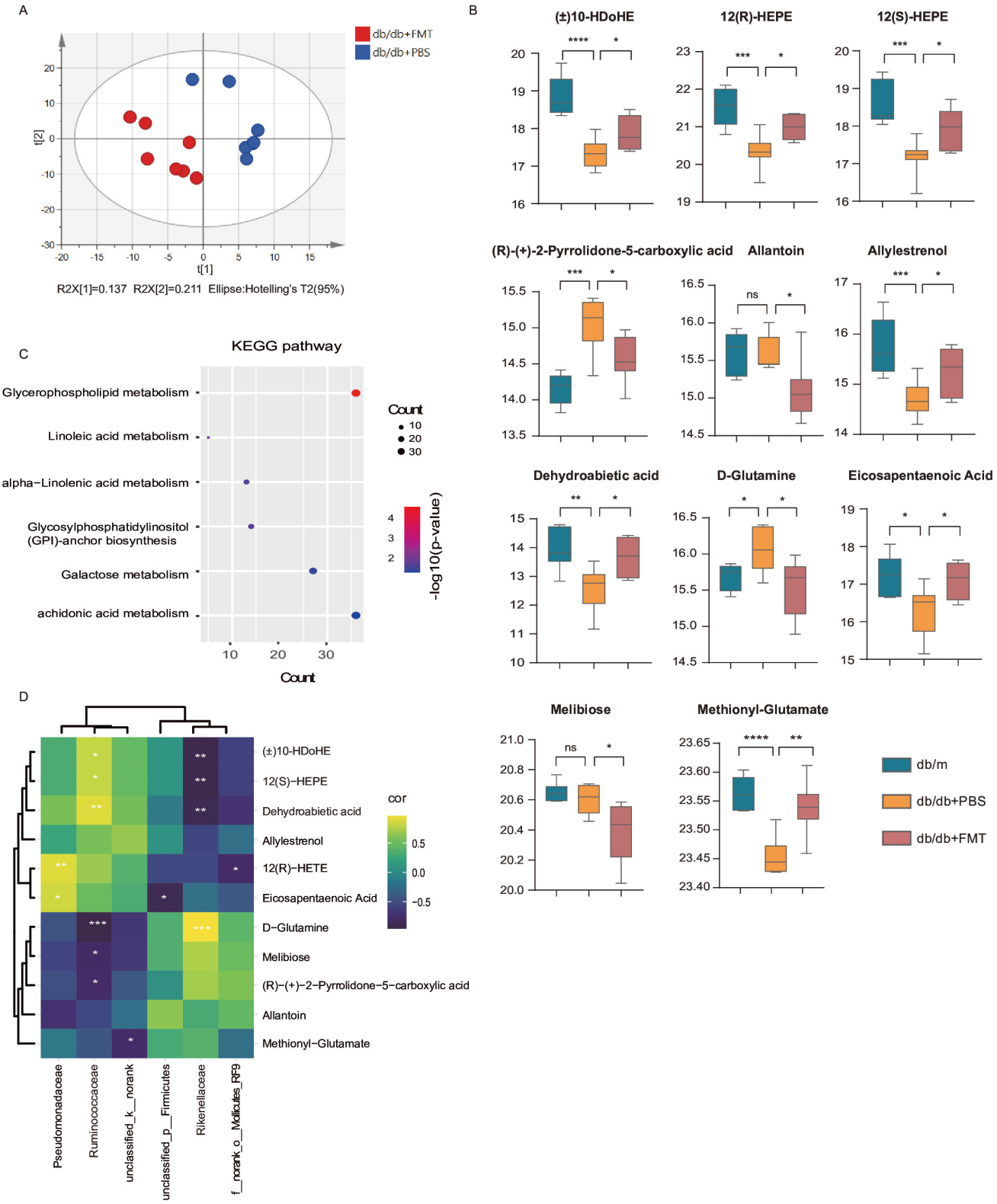

Figure 3 FMT alter serum metabolites in db/db mice. (A) PLS-DA score plot between db/db+PBS and db/db+FMT. The Bray-Curtis distance metric was used to assess the significance of differences between db/db+PBS (blue points) and db/db+FMT (red points); (B) box plot shows the log2 abundance of 11 changed metabolites between db/db+PBS and FMT in three mouse cohorts. (C) Metabo-Analyst V.5.0 (https://www.metaboanalyst.ca/) was used for pathway enrichment analysis. The scatter plot shows the KEGG pathway of differential metabolites between db/db mice and FMT-treated mice (*0.01<p<0.05; **p<0.01). (D) Heatmap of the Spearman's correlation between 11 discriminatory metabolites and key bacteria species at the family level (db/db+PBS vs db/db+FMT,*: 0.01<p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001). The yellow squares indicate positive correlations, whereas the dark blue squares indicate negative correlations. FMT, fecal microbiota transplantation; ns, not significant; PBS, phosphate-buffered saline; PLS-DA, partial least squares discriminant analysis.

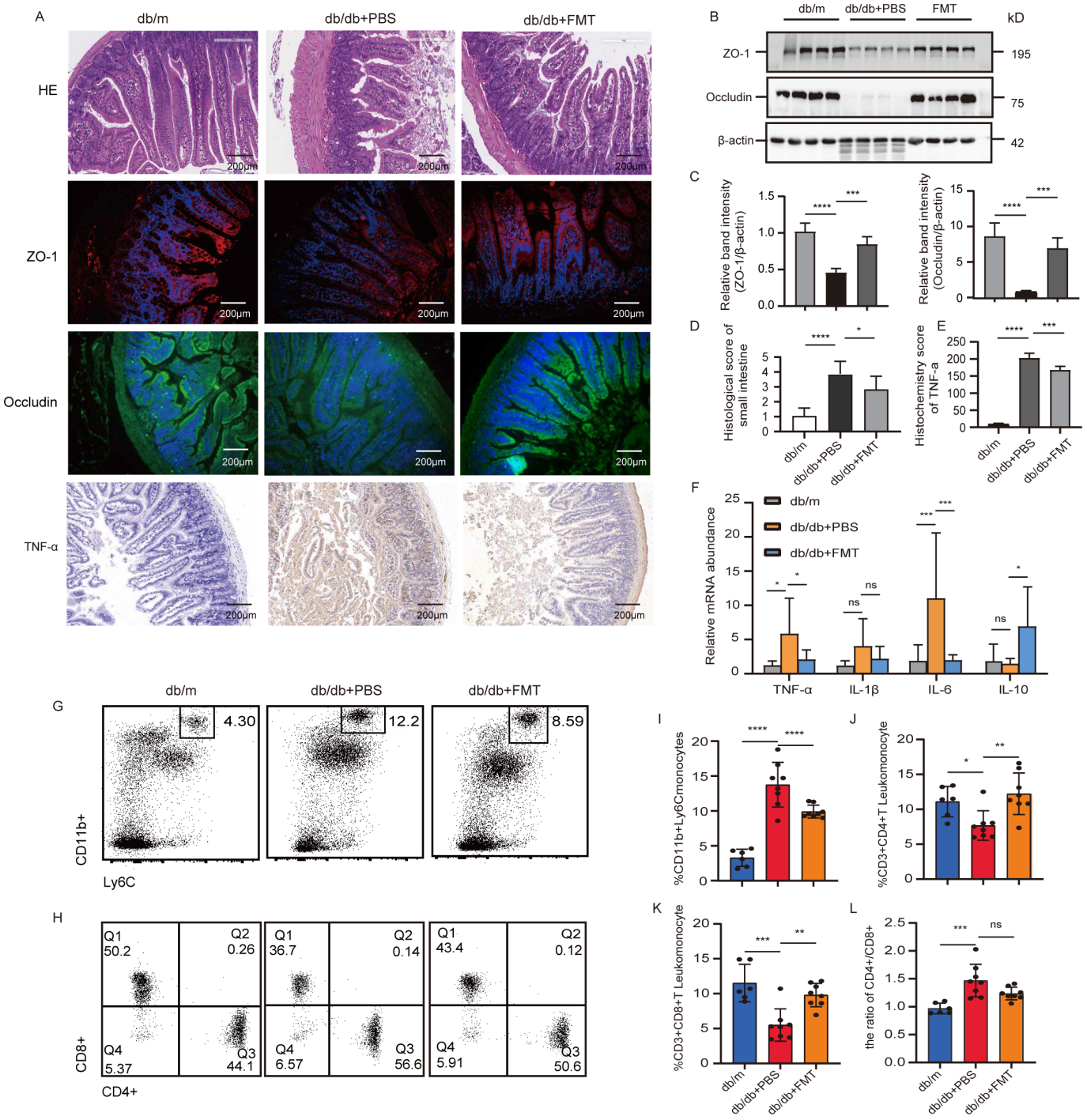

Figure 4 FMT modulate the intestinal barrier, immune composition, and the inflammatory response in db/db mice. (A) Representative photomicrographs of H&E-stained small intestine sections and photomicrographs of immunofluorescence for ZO-1, occludin and TNF-α in db/m, db/db+PBS and db/db+FMT mice (magnification: 200×). (B–C) Western blot analysis and densitometric quantification of ZO-1, occludin and β-actin. (D–E) Histological score of small intestine section and histochemistry score of TNF-α between three groups. (F) Relative mRNA abundance of TNF-α, IL-1β, IL-6, IL-10. (G) Representative flow cytometry plots of CD45+CD11b+Ly6C+ monocytes in db/m, db/db+PBS and db/db+FMT mice. (H) Representative flow cytometry plots of CD45+CD3+CD4+CD8+ lymphocytes in db/m, db/db+PBS and db/db+FMT mice. (I) The percentage of CD45+CD11b+Ly6C+ monocytes in db/m, db/db+PBS and db/db+FMT mice. (J) The percentage of CD4+ T lymphocytes in total CD45+ cells. (K) The percentage of CD8+ T lymphocytes in total CD45+ cells. (L) The ratio of CD4+ and CD8+ T lymphocytes in db/m, db/db+PBS and db/db+FMT mice. (*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001) FMT, fecal microbiota transplantation; IL, interleukin; mRNA, messenger RNA; ns, not significant; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

was significantly higher in db/db mice compared with db/m mice (p<0.001). FMT treatment partially rescued the intestinal phenotype and the histological score was significantly decreased in FMT-treated db/db mice compared with control-treated db/db mice (p<0.05) (figure 4D). However, mild damage was found in colonic tissues between three groups (online supplemental figure 3A–C).Furthermore, we confirmed the impaired gut barrier in db/db mice by immunofluorescence (figure 4A) and western blot analysis (figure 4B and C) for tight-junction proteins ZO-1 and occludin. Decreased expression of ZO-1 and occludin was detected in db/db mice compared with db/m mice. Importantly, FMT treatment substantially recovered ZO-1 and occludin protein expression in db/db mice (p<0.001). To confirm the beneficial effect of FMT on intestinal inflammation, immunostaining of tumor necrosis factor (TNF)-α (figure 4A) and RT-quantitative PCR of interleukin (IL)-1β, IL-6, IL-10 and TNF-α was performed. Immunostaining revealed significantly decreased levels of TNF-α after FMT treatment in the small intestine of db/db mice (figure 4E). Relative messenger RNA (mRNA) level of IL-6 and TNF-α were higher in db/db mice compared with db/m mice, and FMT treatment in db/db mice significantly decreased transcript levels of IL-6 (p<0.001). IL-10 transcript levels were significantly increase in FMT-treated db/db mice (p<0.05) (figure 4F). IL-10 is an anti-inflammatory factor and was confirmed to be involved in the recovery of intestinal inflammation in IBD mice after FMT treatment.[22] Together these results suggest that FMT can protect against gut barrier dysfunction, mucosal inflammation in db/db mice.

FMT regulates peripheral blood immune cells in db/db mice

The gut microbiota is a key regulator of host immunity especially in diabetes.[23] Moreover, the immune system influences chronic inflammatory-associated insulin resistance. Thus, the immune system may be an important link between alterations in the gut microbiota and potential effects on the pancreas in diabetes. To determine whether diabetes-associated immune alterations can be ameliorated by FMT in db/db mice, we explored whether FMT can affect abundance levels of circulating immune cells. Flow cytometric analysis of circulating blood revealed that the per cent monocytes (CD45+, CD11b+, Ly6C+) in CD45+ cells were significantly higher in db/db mice compared with the db/m mice (p<0.0001). However, 4weeks of FMT treatment in db/db mice partially reversed this increase (p<0.0001) (figure 4G and I). In addition, we observed an overall relative decrease in T lymphocytes in CD45+ cells in db/db mice compared with db/m mice (online supplemental figure 4A,B). Both the percentage of CD4+ T (figure 4J) and the CD8+ T (figure 4K) in CD45+ cells were decreased (p<0.05 and p<0.001, respectively) compared with db/m mice. FMT treatment rescued the number of circulating T lymphocytes in db/db mice (figure 4H and K). The CD4+/CD8+ ratio significantly increased in the db/db mice compared with db/m mice, however, FMT treatment only caused a modest decrease (figure 4L).

DISCUSSION

In addition to direct effects of genetic and environmental factors on diabetes, they can also mediate their effects on diabetes by affecting the composition of the GM. FMT has been recognized as an efficient way to alter the composition of GM, and has shown good efficacy in diabetes, but the underlying mechanisms remain unclear. Here, we show that transplantation of a microbiota from db/m mice could improve the symptoms of diabetes in db/db mice, and that remodeling of GM, changes of serum metabolites, inflammation and immunity may be the potential mechanisms.

A previous study showed that C57BL/6 mice have impaired glucose and insulin tolerance after receiving fecal microbiota from patients with T2DM.[24] In contrast, T2DM mice receiving fecal suspension by oral gavage from normal mice showed improved FPG and fasting insulin level.[25] Furthermore, GM modulation from young mice dramatically improved the glucose sensitivity, inflammation and intestinal barrier in the natural aging mice.[26] As expected, our study observed that FMT treatment successfully reduced FBG and improved the insulin tolerance in mice with diabetes. These results indicate that the fecal microbiome of normal mice is beneficial for improving glucose metabolism and insulin tolerance.

Compared with normal mice without diabetes, we observed decreased GM richness and diversity in db/db mice at the family level. 16S rRNA gene sequencing revealed that db/db mice have significantly higher levels of *Rikenellaceae*, *ParapParaprevotella* (family level) and *Alistipes* (genus level), *Alloprevotella* and lower levels of *Phascolarctobacterium*, *Prevotellaceae*, *Porphyromonadaceae* and *Ruminococcaceae* (family level). Our previous animal study, investigating the role of resveratrol on diabetic nephropathy through modulating the GM, and prospective cohort trial, investigating FMT effects on T2DM, also observed that *Rikenellaceae* and *Alistipes* were increased significantly, *Ruminococcaceae* and *Prevotellaceae* were decreased.[15 17] Similar studies also revealed that *Rikenellaceae* and *Alistipes* is significantly enriched in individuals with obesity and patients with T2DM,[27 28] which can modulate inflammatory responses and exert a pro-inflammatory effect, contributing ultimately to metabolic disorder.[28 29] *Rikenellaceae* was found to be positively correlated with FBG and AUC of OGTT, which was consistent with studies in mice on a high fat diet, which were characterized by increased relative abundance of *Rikenellaceae*.[30] In addition, in a Dextran Sulfate Sodium Salt (DSS)-induced enteritis mouse model, *Alistipes* exerted dominant effects and was involved in the process of inflammation.[31] Moreover, *Alistipes* has been shown to be positively correlated with FPG and glucose intolerance.[32]

In contrast, *Ruminococcaceae* and *Prevotellaceae* as the short-chain fatty acid (SCFA)-producing bacteria,

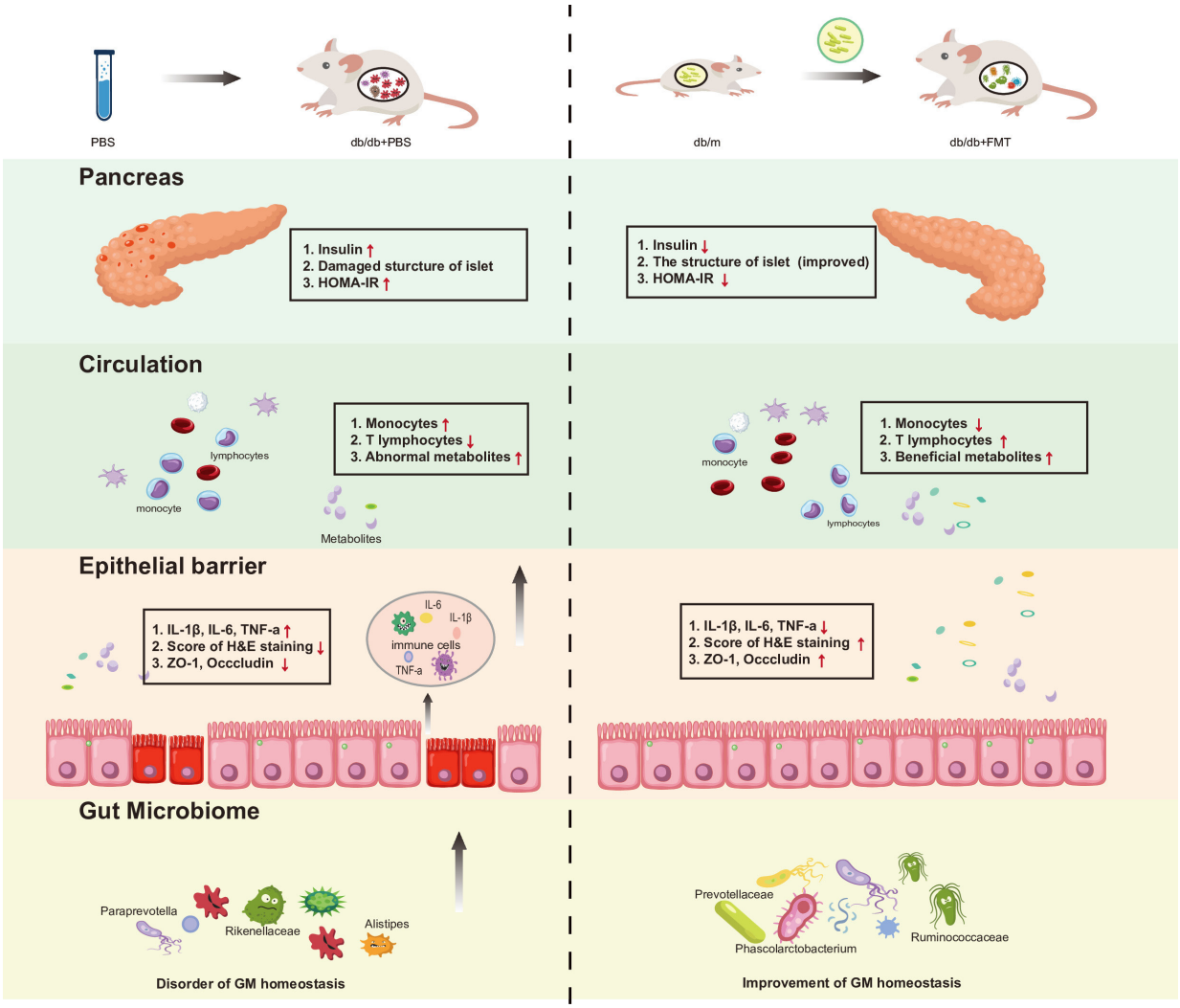

Figure 5 FMT treatment improved the T2DM. FMT, fecal microbiota transplantation; GM, gut microbiome; HOMA-IR, homeostatic model assessment for insulin resistance; IL, interleukin; PBS, phosphate-buffered saline; T2DM, type 2 diabetes mellitus; TNF, tumor necrosis factor.

competitively inhibit the sodium-glucose symporters and regulate the level of blood glucose by increasing the level of SCFAs, especially butyric acid.[33 34] SCFAs have been reported to promote glucagon-like peptide-1 (GLP-1) and peptides YY (PYY) production through binding with free fatty acid receptors (FFAR2 or 3) on the L-cell membrane in the intestine.[35 36] GLP-1 and PYY can control appetite, regulate gastric emptying and improve the survival and proliferation of beta cells, which can strongly improve glucose metabolism. A prospective cohort study showed that high-abundance *Ruminococcaceae* was positively associated with low HOMA-IR index.[37] Another study confirmed that intestinal barrier injury was linked to *Ruminococcaceae* and *Prevotellaceae* genus depletion, gut microbiota dysbiosis promoted M1-like polarization of colonic macrophages and the production of pro-inflammatory factors TNF-α and IL-1β through downregulation of peroxisome proliferator-activated receptor (PPAR)α-CYP4×1 axis.[38] Other reports identified beneficial impacts of *Prevotella* species on glucose metabolism.[39] Besides, *Paraprevotella* is generally more abundant in the stool of patients with T2DM, and is positively correlated with the risk of diabetes and chronic kidney disease,[40 41] potentially by promoting the production of pro-inflammatory factors (IL-1β, IL-6, TNF-α).[42] From our study, the abundance levels of these bacteria were restored after 4 weeks of FMT treatment.

It was reported that changes of the GM can alter host metabolite levels, which could be the result of alterations in microbiota-derived and host-derived metabolites.[43] Our study has confirmed the association between serum metabolites and the GM. DAA, 12(S)-HEPE and (±)10-HDoHE, which were upregulated in db/db+FMT mice compared with db/db mice, were positively correlated with *Ruminococcaceae* and negatively related with *Rikenellaceae*. DAA is a naturally occurring diterpene resin acid derived from coniferous plants such as *Pinus* and *Picea*. Various bioactive effects of DAA have been studied including antibacterial, anti-inflammation and anti-oxidant activities. A previous study confirmed that DAA suppresses the inflammatory response potentially by modulating the nuclear factor kappa B and TGF-β activated kinase 1

(TAK1)-mediated pathways.[44] Takahashi *et al*[45] found that DAA could activate PPAR-γ to suppress chronic inflammation in obese adipose tissues and stimulates adipocyte differentiation, which can stimulate insulin-dependent glucose uptake into 3T3-L1 adipocytes. Their result suggested that DAA is a valuable food-derived compound for the treatment of diabetic conditions. Another study confirmed that 12(S)-HETE represents a potential molecular mediator that targets the ENS-duodenal contraction couple to improve glucose homeostasis.[46] 12-HEPE was also found to inhibit the foamy transformation of macrophages in a PPAR-γ-dependent manner to ameliorate the atherosclerosis.[47] Taken together, the inflammatory-related bacteria and metabolites, which characterized the chronic inflammatory state in the db/db mice, were significantly reconstituted by FMT treatment. Therefore, we propose that FMT reduces the abundance levels of inflammatory-related bacteria, resulting in the improvement of chronic inflammation and glucose homeostasis.

Intestinal inflammation promotes intestinal barrier dysfunction and increases intestinal permeability, resulting in chronic low-grade inflammation in the host.[21] We previously noted that the morphology of the small intestine together with relative expressions of ZO-1 and claudin-1 were decreased in db/db mice.[17] In the present study, we also observed that the abundance of ZO-1 and occludin were decreased in db/db mice. The production of pro-inflammatory cytokines were significantly increased in the intestine of a DSS-induced mouse model, and the gut barrier was severely damaged.[22] We also found that pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, etc) were increased in db/db+PBS mice, concomitant with decreased barrier dysfunction. FMT treatment restored inflammatory cytokine levels and tight junction structure. Circulating monocytes are an important component of innate immunity, which can acquire a pro-inflammatory phenotype after specific stimuli.[48] This persistent activation of circulating monocytes is characterized by an increased expression of pro-inflammatory cytokines and growth factors.[49] In our study, flow cytometry analysis showed that the number of T cells were increased significantly after FMT treatment, whereas monocyte levels were decreased. Overall, our data suggested that FMT can decrease the destruction of epithelial integrity and inflammatory responses in the db/db mice.

Our study has a few limitations. First, we only used the genetic db/db mouse model of diabetes in our research. Other mouse models of diabetes should be investigated. Second, we have assessed the peripheral immune state, but have not assessed the intestinal immune state. Additional studies are needed to confirm these and other possible effects and mechanisms. Future work might identify the functional mechanisms by which some bacterial species and metabolites are responsible for beneficial therapeutic T2DM conferred by FMT, and explore the applicability of FMT in T2DM.

This study provides further evidence that FMT is a potentially beneficial therapeutic intervention against T2DM by reconstructing the GM, changing the serum metabolites, regulating host immunological changes and reducing the inflammation response, which subsequently affect host glucose metabolic phenotypes (figure 5). Therefore, our research provides further evidence that FMT may establish a beneficial host-microbiota relationship, generating insights for FMT as an effective treatment of diabetes.

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REFERENCES

1 Saeedi P, Petersohn I, Salpea P, *et al*. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International diabetes Federation diabetes Atlas. *Diabetes Res Clin Pract* 2019;157:107843.
2 Zheng Y, Ley SH, Hu FB. Global Aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat Rev Endocrinol* 2018;14:88–98.

3 Pillon NJ, Loos RJF, Marshall SM, *et al*. Metabolic consequences of obesity and type 2 diabetes: Balancing genes and environment for personalized care. *Cell* 2021;184:1530–44.
4 Thursby E, Juge N. Introduction to the human gut Microbiota. *Biochem J* 2017;474:1823–36.
5 Pickard JM, Zeng MY, Caruso R, *et al*. Gut Microbiota: Role in pathogen Colonization, immune responses, and inflammatory disease. *Immunol Rev* 2017;279:70–89.
6 Fan Y, Pedersen O. Gut Microbiota in human metabolic health and disease. *Nat Rev Microbiol* 2021;19:55–71.
7 Joseph CL, Sitarik AR, Kim H, *et al*. Infant gut bacterial community composition and food-related manifestation of Atopy in early childhood. Pediatr allergy Immunol. *Pediatr Allergy Immunol* 2022;33:e13704.
8 Boulangé CL, Neves AL, Chilloux J, *et al*. Impact of the gut Microbiota on inflammation, obesity, and metabolic disease. *Genome Med* 2016;8:42.
9 Wu H, Tremaroli V, Schmidt C, *et al*. The gut Microbiota in Prediabetes and diabetes: A population-based cross-sectional study. *Cell Metab* 2020;32:379–90.
10 Ghaemi F, Fateh A, Sepahy AA, *et al*. Intestinal Microbiota composition in Iranian diabetic, pre-diabetic and healthy individuals. *J Diabetes Metab Disord* 2020;19:1199–203.
11 Rittiphairoj T, Pongpirul K, Janchot K, *et al*. Probiotics contribute to Glycemic control in patients with type 2 diabetes mellitus: A systematic review and meta-analysis. *Adv Nutr* 2021;12:722–34.
12 Yang HJ, Kim MJ, Kwon DY, *et al*. Combination of Aronia, red Ginseng, Shiitake mushroom and Nattokinase potentiated insulin secretion and reduced insulin resistance with improving gut Microbiome Dysbiosis in insulin deficient type 2 diabetic rats. *Nutrients* 2018;10:948.
13 Marcella C, Cui B, Kelly CR, *et al*. Systematic review: The global incidence of Faecal Microbiota transplantation-related adverse events from 2000 to 2020. *Aliment Pharmacol Ther* 2021;53:33–42.
14 Geng S, Cheng S, Li Y, *et al*. Faecal Microbiota transplantation reduces susceptibility to epithelial injury and modulates Tryptophan metabolism of the microbial community in a Piglet model. *J Crohns Colitis* 2018;12:1359–74.
15 Ding D, Yong H, You N, *et al*. Prospective study reveals host microbial determinants of clinical response to fecal Microbiota transplant therapy in type 2 diabetes patients. *Front Cell Infect Microbiol* 2022;12:820367.
16 de Groot P, Nikolic T, Pellegrini S, *et al*. Faecal Microbiota transplantation HALTS progression of human new-onset type 1 diabetes in a randomised controlled trial. *Gut* 2021;70:92–105.
17 Cai T-T, Ye X-L, Li R-R, *et al*. Resveratrol modulates the gut Microbiota and inflammation to protect against diabetic nephropathy in mice. *Front Pharmacol* 2020;11:1249.
18 de Groot P, Scheithauer T, Bakker GJ, *et al*. Donor metabolic characteristics drive effects of Faecal Microbiota transplantation on recipient insulin sensitivity, energy expenditure and intestinal transit time. *Gut* 2020;69:502–12.
19 Plantamura E, Dzutsev A, Chamaillard M, *et al*. MAVS deficiency induces gut Dysbiotic Microbiota conferring a Proallergic phenotype. *Proc Natl Acad Sci U S A* 2018;115:10404–9.
20 Jenkins BJ, Seyssel K, Chiu S, *et al*. Odd chain fatty acids; new insights of the relationship between the gut Microbiota, dietary intake, biosynthesis and glucose intolerance. *Sci Rep* 2017;7:44845.
21 Riedel S, Pheiffer C, Johnson R, *et al*. Intestinal barrier function and immune homeostasis are missing links in obesity and type 2 diabetes development. *Front Endocrinol (Lausanne)* 2021;12:833544.
22 Burrello C, Garavaglia F, Cribiù FM, *et al*. Therapeutic Faecal Microbiota transplantation controls intestinal inflammation through Il10 secretion by immune cells. *Nat Commun* 2018;9:5184.
23 Pomié C, Blasco-Baque V, Klopp P, *et al*. Triggering the adaptive immune system with Commensal gut bacteria protects against insulin resistance and Dysglycemia. *Mol Metab* 2016;5:392–403.
24 Wang C, Wang Y, Yang H, *et al*. Uygur type 2 diabetes patient fecal Microbiota transplantation disrupts blood glucose and bile acid levels by changing the ability of the intestinal flora to Metabolize bile acids in C57Bl/6 mice. *BMC Endocr Disord* 2022;22:236.
25 Wang H, Lu Y, Yan Y, *et al*. Promising treatment for type 2 diabetes: fecal Microbiota transplantation reverses insulin resistance and impaired Islets. *Front Cell Infect Microbiol* 2019;9:455.
26 Ma J, Liu Z, Gao X, *et al*. Gut Microbiota remodeling improves natural aging-related disorders through Akkermansia Muciniphila and its derived acetic acid. *Pharmacol Res* 2023;189:106687.
27 Arnoriaga-Rodríguez M, Mayneris-Perxachs J, Burokas A, *et al*. Gut bacterial Clpb-like Gene function is associated with decreased body weight and a characteristic Microbiota profile. *Microbiome* 2020;8:59.
28 Borton MA, Sabag-Daigle A, Wu J, *et al*. Chemical and pathogen-induced inflammation disrupt the murine intestinal Microbiome. *Microbiome* 2017;5:47.
29 Alipour M, Zaidi D, Valcheva R, *et al*. Mucosal barrier depletion and loss of bacterial diversity are primary abnormalities in Paediatric ulcerative colitis. *J Crohns Colitis* 2016;10:462–71.
30 Daniel H, Gholami AM, Berry D, *et al*. High-fat diet alters gut Microbiota physiology in mice. *ISME J* 2014;8:295–308.
31 Forster SC, Clare S, Beresford-Jones BS, *et al*. Identification of gut microbial species linked with disease variability in a widely used mouse model of colitis. *Nat Microbiol* 2022;7:590–9.
32 Wei S, Han R, Zhao J, *et al*. Intermittent administration of a fasting-mimicking diet INTERVENES in diabetes progression, restores beta cells and reconstructs gut Microbiota in mice. *Nutr Metab (Lond)* 2018;15:80.
33 Wu F, Guo X, Zhang J, *et al*. Phascolarctobacterium Faecium abundant Colonization in human gastrointestinal tract. *Exp Ther Med* 2017;14:3122–6.
34 Li B-Y, Xu X-Y, Gan R-Y, *et al*. Targeting gut Microbiota for the prevention and management of diabetes mellitus by dietary natural products. *Foods* 2019;8:440.
35 Brooks L, Viardot A, Tsakmaki A, *et al*. Fermentable carbohydrate stimulates Ffar2-dependent Colonic PYY cell expansion to increase satiety. *Mol Metab* 2017;6:48–60.
36 Larraufie P, Martin-Gallausiaux C, Lapaque N, *et al*. Scfas strongly stimulate PYY production in human Enteroendocrine cells. *Sci Rep* 2018;8:74.
37 Chen Z, Radjabzadeh D, Chen L, *et al*. Association of insulin resistance and type 2Diabetes with gut microbial diversity.A Microbiome-wide analysis from population studies. *JAMA Netw Open* 2021;4:e2118811.
38 Chen Y, Liu Y, Wang Y, *et al*. Prevotellaceae produces butyrate to alleviate PD-1/PD-L1 inhibitor-related cardiotoxicity via Pparalpha-Cyp4X1 axis in Colonic Macrophages. *J Exp Clin Cancer Res* 2022;41:1.
39 Kovatcheva-Datchary P, Nilsson A, Akrami R, *et al*. Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of Prevotella. *Cell Metab* 2015;22:971–82.
40 Holle J, Bartolomaeus H, Löber U, *et al*. Inflammation in children with CKD linked to gut Dysbiosis and metabolite imbalance. *J Am Soc Nephrol* 2022;33:2259–75.
41 Camargo A, Vals-Delgado C, Alcala-Diaz JF, *et al*. A diet-dependent Microbiota profile associated with incident type 2 diabetes: from the CORDIOPREV study. *Mol Nutr Food Res* 2020;64:e2000730.
42 Pan G, Liu B, Li S, *et al*. Kuijieling, a Chinese medicine Alleviates DSS-induced colitis in C57Bl/6Jmouse by improving the diversity and function of gut Microbiota. *FEMS Microbiol Lett* 2020;367:fnaa082.
43 Zhang P-P, Li L-L, Han X, *et al*. Fecal Microbiota transplantation improves metabolism and gut Microbiome composition in dB/dB mice. *Acta Pharmacol Sin* 2020;41:678–85.
44 Kim E, Kang Y-G, Kim Y-J, *et al*. n.d. Dehydroabietic acid suppresses inflammatory response via suppression of Src-, Syk-, and Tak1-mediated pathways. *IJMS*;20:1593.
45 Takahashi N, Yao R, Kang M-S, *et al*. Dehydroabietic acid activates peroxisome Proliferator-activated receptor-gamma and stimulates insulin-dependent glucose uptake into 3T3-L1 Adipocytes. *Biofactors* 2011;37:309–14.
46 Abot A, Wemelle E, Laurens C, *et al*. Identification of new Enterosynes using Prebiotics: roles of bioactive lipids and mu-opioid receptor signalling in humans and mice. *Gut* 2021;70:1078–87.
47 Nagatake T, Shibata Y, Morimoto S, *et al*. 12-Hydroxyeicosapentaenoic acid inhibits foam cell formation and ameliorates high-fat diet-induced pathology of Atherosclerosis in mice. *Sci Rep* 2021;11:10426.
48 Kantari C, Pederzoli-Ribeil M, Witko-Sarsat V. The role of neutrophils and monocytes in innate immunity. *Contrib Microbiol* 2008;15:118–46.
49 Bekkering S, van den Munckhof I, Nielen T, *et al*. Innate immune cell activation and epigenetic remodeling in symptomatic and asymptomatic Atherosclerosis in humans in vivo. *Atherosclerosis* 2016;254:228–36.