Fecal Microbiota Transplantation (FMT) Alleviates Ovalbumin-induced Allergic Airway Inflammation in Neonatal Mice via the PD-1/PD-L1 Axis

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Research Article

Keywords: fecal microbiota transplantation, neonate, asthma, microbiota, PD-1

Posted Date: June 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-643967/v1

Fecal microbiota transplantation (FMT) alleviates ovalbumin-induced allergic airway inflammation in neonatal mice via the PD-1/PD-L1 axis

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ABSTRACT

Asthma is the common respiratory disorder in children, which is associated with abnormal gut microbiota. Fecal microbiota transplantation (FMT) has successfully ameliorated the symptoms of several diseases and restored the balance of gut microbiota. However, there are few researches about the role of FMT in asthma. This study aimed at exploring whether FMT can alleviate allergic airway inflammation in neonatal mice and elucidating the probable underlying mechanism. A neonatal mouse model of ovalbumin (OVA)-induced allergic asthma was established and transplanted with fecal filtrates. Our results manifested that FMT could protect against the allergic airway inflammation through enhancing mesenteric $CD11c^+CD103^+$DCs and accumulating mucosal $Helios^+$Tregs. Besides, the programmed cell death protein 1/programmed cell death protein 1 ligand (PD-1/PD-L1) signal pathway was inhibited after FMT intervention. Furthermore, this beneficial role of FMT was also associated with the rebalanced gut microbiota, such as Akkermansia. Thus, our findings indicated that FMT intervention could exert a therapeutic effect in a neonatal mouse model of OVA-induced allergic airway inflammation through its remodeling on gut microbiota and regulation of Treg homeostasis via the PD-1/PD-L1 axis, which might be used as an alternative therapy for allergic asthma.

Introduction

As a common public health issue, asthma affects 300 million individuals worldwide, which probably originates from the reduced exposure to microbial antigens and changing environment during infancy[1, 2]. Allergic asthma is considered as a T helper 2 (Th2) cell-mediated disease. Pro-inflammatory and Th2 cytokines drive the accumulation of eosinophils in the lungs of asthmatic patients, which are closely related to the severity of asthma[3]. In addition, compared to the adults and elders, children are at higher risk of allergic asthma which can affect their growth and development[4, 5].

Generally, high diversity of microbiota is thought to be a marker of healthy status and could exert effects on host immune system with dominant species[6, 7]. Microbiota alteration can contribute to the development of allergy asthma[8, 9]. Changed microbiota could affect the downstream immune response and immune development to eventually result in asthma, while immune system deficiencies can also influence the microbiota composition, distal mucosal sites and the development of atopic conditions[10]. Our previous studies have shown that probiotics can effectively relieve asthma symptoms by rebalancing gut microbiota and modulating regulatory T cells (Tregs)[11]. Moreover, the gut-intestinal microbiota in infancy is easily affected by various factors, which will cause imbalanced microbiota and threaten health status. Recently, fecal microbiota transplantation (FMT) has attracted more interest for its modulation on host microbiota, and it could clinically improve symptoms in several system disorders, such as clostridium difficile infection[12, 13], ulcerative colitis[14], Crohn's disease[15], hepatic

encephalopathy[16, 17] and autism spectrum disorder[18]. As a microbiota-targeted intervention, FMT could introduce gut microbiota from a healthy donor into recipient as a conventional therapeutic approach to re-establish a healthy gut microbial community[18, 19]. Therefore, FMT might be a potential alternative option for asthma treatment. However, the effect of FMT on allergic airway inflammation is rarely reported. Here, our research aimed to explore whether FMT can protect against the allergic asthma in neonatal mice and propose the underlying mechanism.

Results

- **FMT alleviated OVA-induced allergic airway inflammation**

Compared to OVA group, FMT intervention could improve the lung resistance (Fig. 1A, at 50 and 100mg/ml MCh, both $P<0.001$). Neonatal mice in OVA + FMT group displayed less rupture of alveolar septum and formation of pulmonary bullae, infiltration of inflammatory cells, mucus hypersecretion and the degree of pulmonary fibrosis (Fig. 1B). In line with the histopathology changes, FMT intervention could decrease total number of white blood cells in BALF, and especially inhibit the infiltration of eosinophils and neutrophils (Fig. 1C, both $P<0.05$). The percentage of lymphocytes in OVA + FMT group showed significant increase (Fig. 1C, $P<0.05$) while the percentage of macrophages showed no significant change. Similarly, after FMT treatment, the level of IL-4, IL-5 and IL-13 as well as OVA-specific IgE and OVA-specific IgG1 were dramatically decreased ($P<0.001$) whereas IL-10 increased (Fig. 1D-E, $P<0.05$).

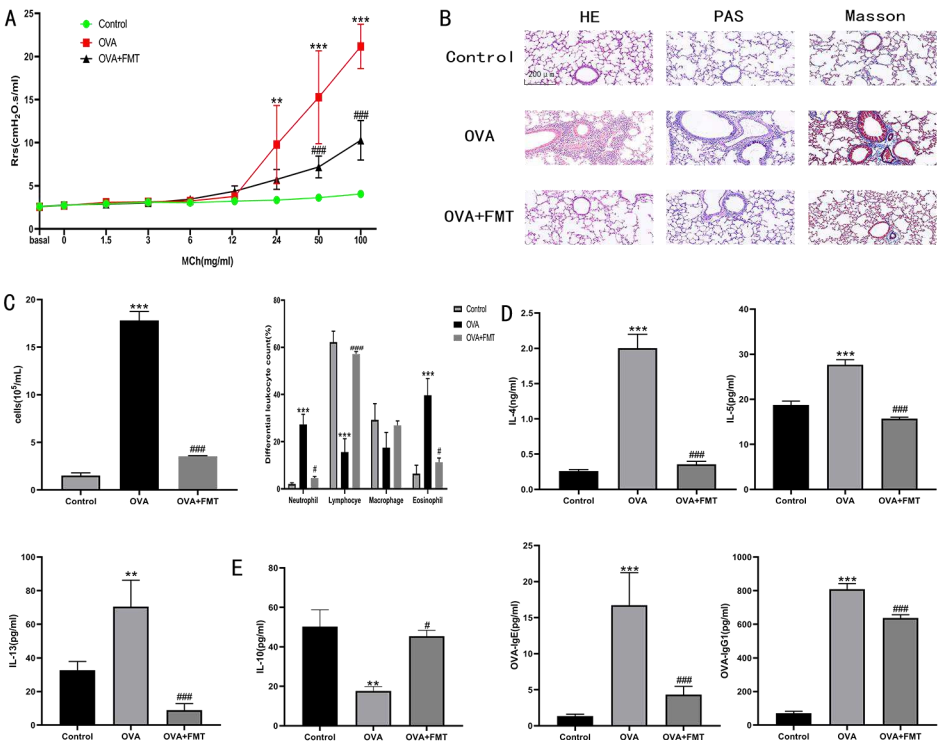

Figure 1 FMT reduced allergic airway inflammation. A. AHR was assessed by measuring airway response to increasing doses of MCh after the last challenge. B. Lung tissue sections (×200) were stained with HE, PAS and Masson. C. Infiltration of inflammatory cells in BALF. D. Concentrations of IL-4, IL-5, and IL-13 in BALF. E. Serum levels of IL-10 and OVA-specific Igs. Data are shown as the Means ± SEMs (n = 10 mice in Control group, 9 mice in OVA group, 9 mice in

OVA + FMT group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to Control group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to OVA group.

- **FMT triggered the accumulation of Tregs in lung and MLNs, and inhibited the expression of PD-1**

Adaptive immune cell is a main contributor of inflammatory cytokines and highly involved in the regulation of inflammation[20]. Since Tregs could exert inhibitory effect in the development of allergic asthma[21], the proportion of $CD4^+CD25^+Foxp3^+$ T lymphocytes and the expression of Helios were analyzed. After FMT intervention, Tregs and Helios expression were upregulated (both $P<0.05$) and PD-1 expression was inhibited both in lung ($P<0.05$, Fig. 2) and MLNs ($P<0.05$, Fig. 3).

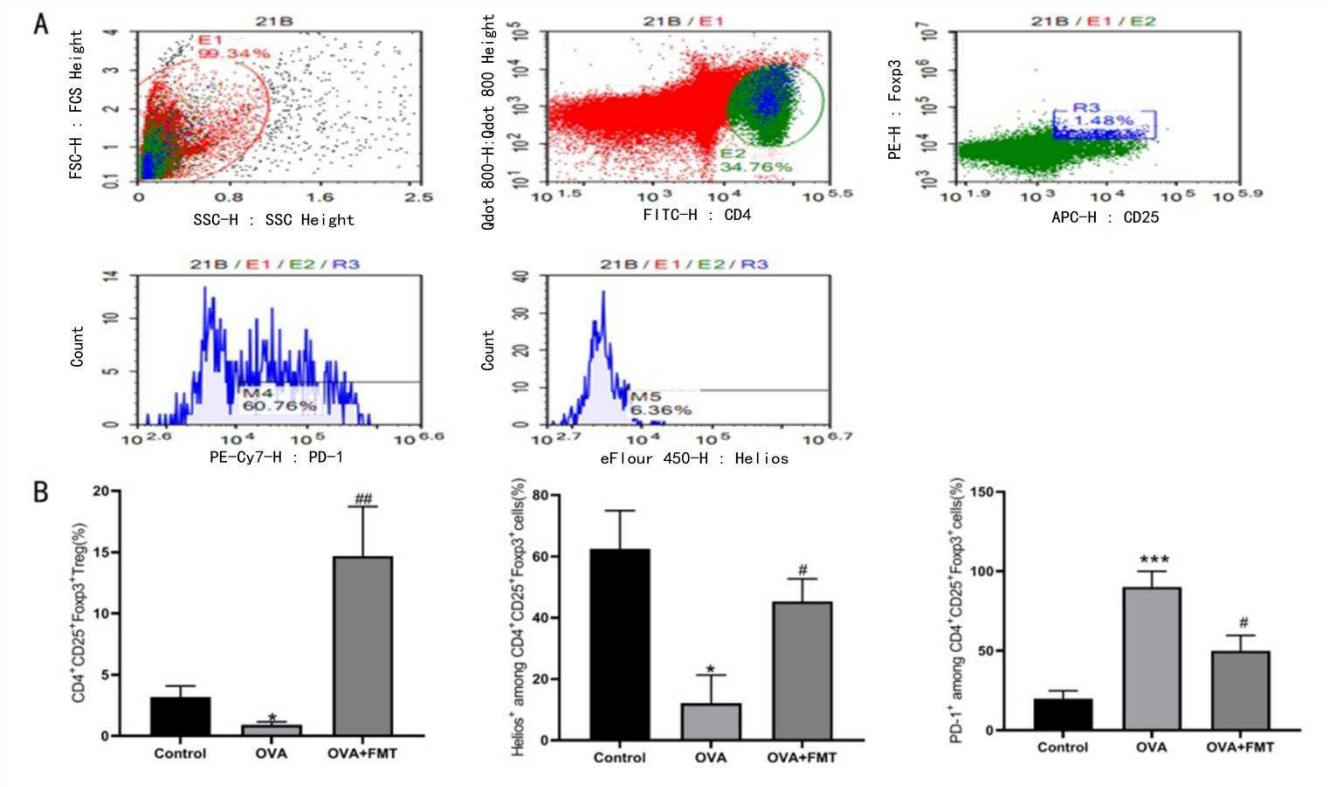

Figure 2 FMT triggered the accumulation of Tregs in lung. A. The representative images of flow cytometry analysis were shown. B. Frequency of $CD4^+CD25^+Foxp3^+$Tregs among $CD4^+$T cells and frequency of Helios- and PD-1-expressing cells among the $CD4^+CD25^+Foxp3^+$Treg population in lung. Data are shown as the Means ± SEMs (n = 10 mice in Control group, 9 mice in OVA group, 9 mice in OVA + FMT group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the Control group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the OVA group.

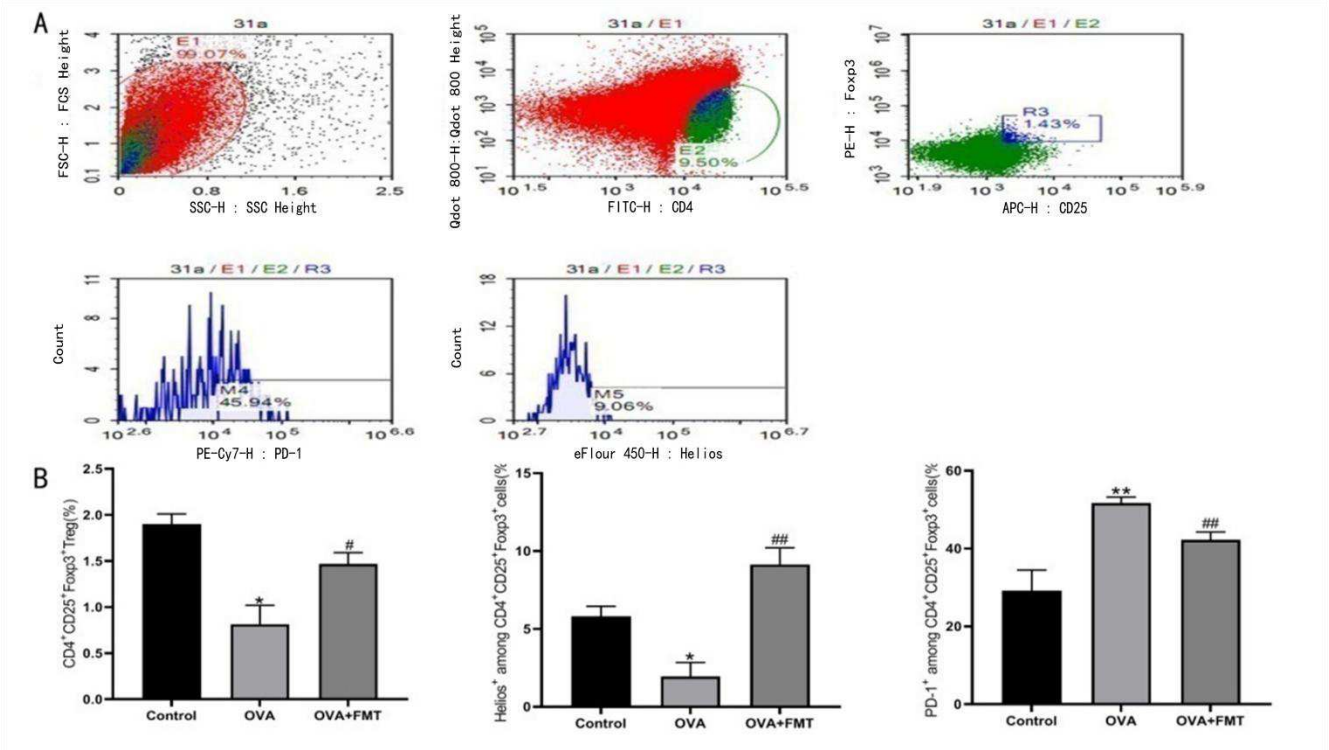

Figure 3 FMT triggered the accumulation of Tregs in MLN. A. The representative images of flow cytometry analysis were shown. B. Frequency of $CD4^+CD25^+Foxp3^+$Tregs among $CD4^+$T cells and frequency of Helios- and PD-1-expressing cells among the $CD4^+CD25^+Foxp3^+$Treg population in MLN. Data are shown as the Means ± SEMs (n = 10 mice in Control group, 9 mice in OVA group, 9 mice in OVA + FMT group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the Control group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the OVA group.

- **FMT promoted DC expression in MLN and inhibited the expression of PD-L1**

It has been widely described that $CD103^+$DCs are capable of inducing and maintaining immune tolerance through induction of Tregs[22, 23], and possess the specific tolerogenic function in mesenteries[24]. Therefore, we further examined the expression of $CD11c^+CD103^+$DC in MLNs. The results showed that FMT caused remarkable increases in the expression of $CD11c^+CD103^+$DCs and decreases in the expression of CD40, CD80 (both $P<0.05$), meaning that DCs in the MLNs displayed a less activated phenotype. Moreover, the expression of PD-L1 was also decreased by FMT treatment (Fig. 4, $P<0.05$).

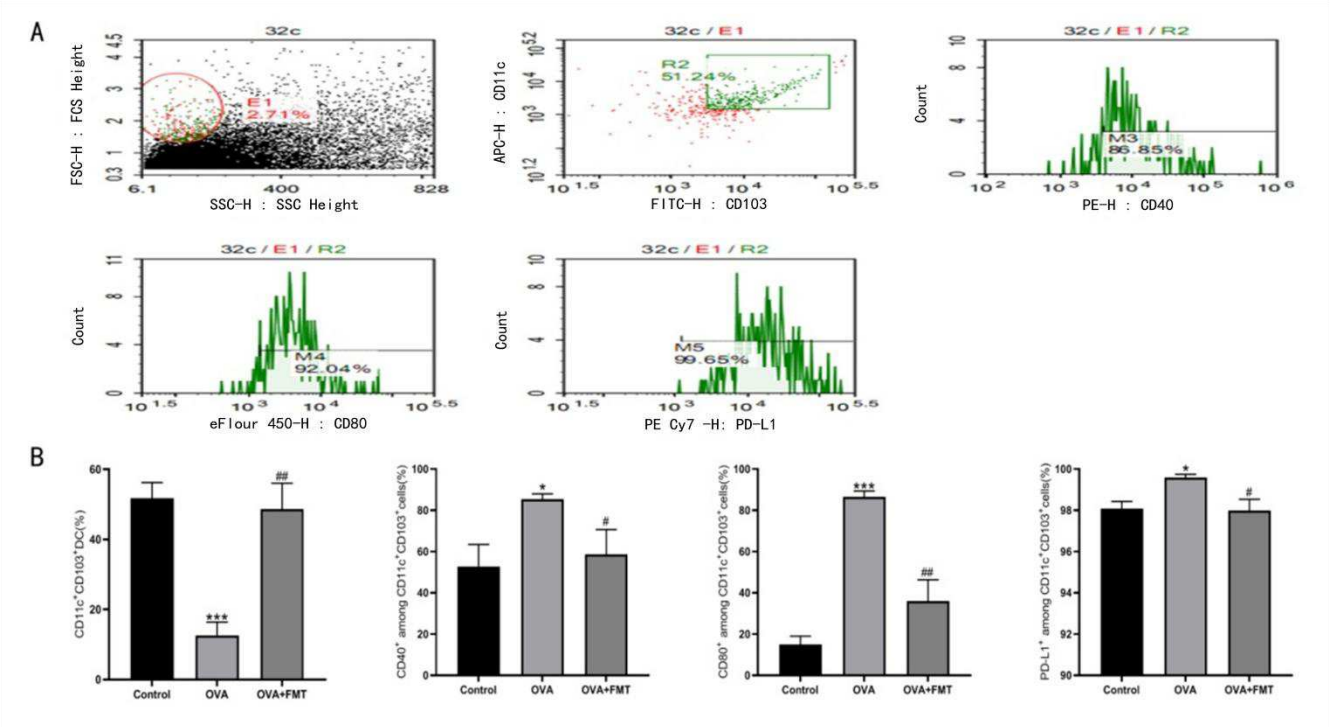

Figure 4 FMT promoted DC expression in MLN. A. Frequency of $CD11c^{+}CD103^{+}$DCs among lymphocytes in MLN. B-D. Frequency of CD40-(B), CD80-(C) and PD-L1(D)-expressing cells among the MLN $CD103^{+}CD11c^{+}$DCs. Data are shown as the Means ± SEMs (n = 10 mice in Control group, 9 mice in OVA group, 9 mice in OVA + FMT group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the Control group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the OVA group.

- **FMT improved gut microbial dysbiosis**

Microbiota has been proved to effect the susceptibility to allergic inflammation by influencing immune cell homeostasis[25-29], so we next analyzed FMT-induced effect on gut microbiota. Shannon diversity index was calculated to show the taxonomic diversity and bacterial diversity was compared among different subgroups. We found that bacterial diversity was significantly different between Control group and OVA group, and FMT-treatment could reverse this change especially in the morning subgroup (Fig. 5A). After finding differences in intestinal microbiota in each group, further detection was conducted at phylum level (Fig. 5B) and genus level (Fig. 5C). According to the bar chart, there were significant differences in the proportion of intestinal microbiota in different groups at different time points, especially in the morning subgroup, and there were significant differences in different phyla and genus levels among each groups. To compare the relative contribution of different taxa, linear discriminant analysis Effect Size (LEfSe) was used to detect the main taxa which was responsible for this discrepancy (Fig. 5D). The length of LDA value was proportional to the degree of influence of the species. As we can see, compared with Control group, the significantly increased relative abundance of intestinal bacteria in OVA group included Alistipes, Rikenellaceae, Porphyromonadaceae, Bacteroidales, Bacteroidia and Bacteroidetes, and their influence degree increased successively (P<0.05). The significantly decreased relative abundance of intestinal bacteria in OVA group included Acidobacteria, Ruminococcaceae, Corynebacteriaceae, Corynebacterium, Actinomycetales, Saccharibacteria genera incertaesedis and Candidatus Saccharibacteria, and their influence degree increased successively (P<0.05). On the other hand, compared with OVA group, the significantly increased relative abundance of intestinal bacteria in OVA + FMT group included Verrucomicrobiae, Verrucomicrobiales, Verrucomicrobiaceae, Akkermansia, Corynebacteriaceae, Corynebacterium, Verrucomicrobia, Parabacteroides, Bacteroides, Blautia, Porphyromonadaceae,

Bacteroidia and Bacteroidales, and their influence degrees increased successively (P<0.05). The significantly decreased relative abundance of intestinal bacteria in OVA + FMT group included Ruminococcaceae, Coriobacteriales, Coriobacteriaceae, Alistipes, Rikenellaceae, Candidatus Saccharibacteria and Saccharibacteria genera incertaesedis, and their influence degrees increased successively (P<0.05). In the end, we analyzed the differential abundance of these discriminative microbiota. At the phylum level, Verrucomicrobia were significantly decreased but Candidatus Saccharibacteria increased in asthmatic mice, while FMT-treatment reversed this difference (Fig. 5E). At the genus level, significant decrease in Akkermansia and Helicobacte and increase in Saccharibacteria genera incertae sedis and Corynebacterium were detected in asthmatic mice, while only Akkermansia and Saccharibacteria genera incertae sedis could remarkably change after FMT intervention (Fig. 5F).

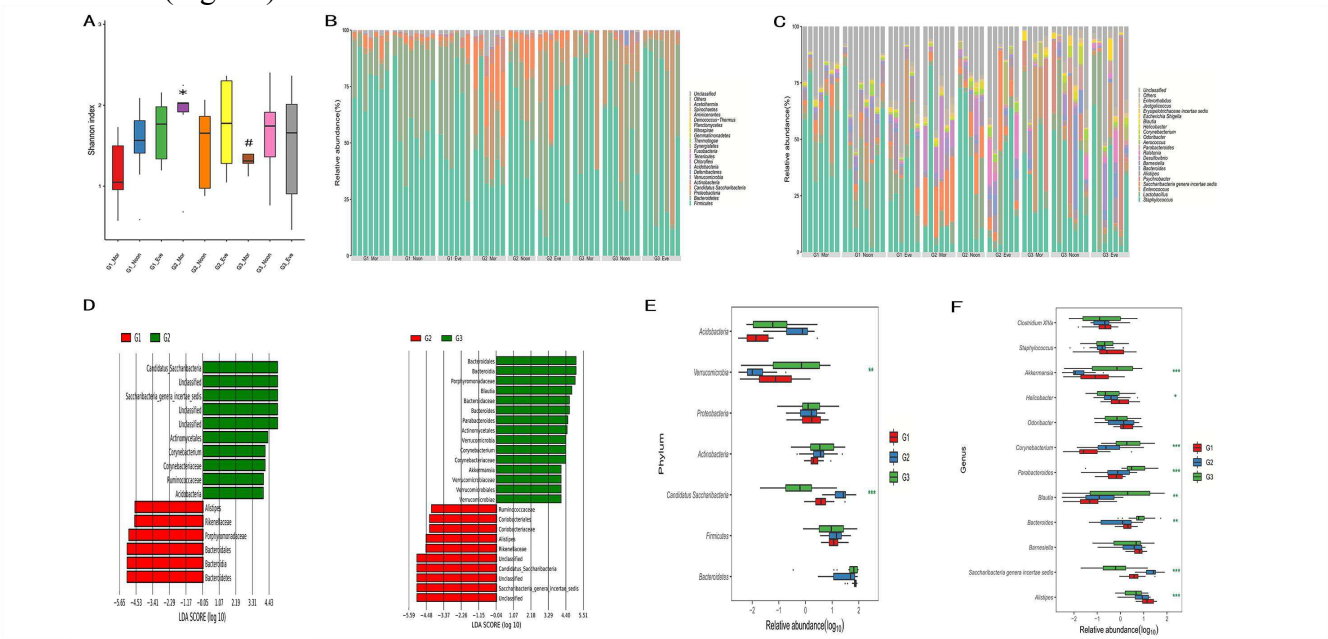

Figure 5 FMT improved gut microbial dysbiosis. A. Alpha-diversity was calculated with Shannon index. B-C. Bacterial composition of fecal samples at phylum(B) and genus(C) level. D. Differential taxa identified by LEfSe with linear discriminant analysis(LDA) values. E-F. Taxa enriched in different groups at phylum(E) and genus(F) level were identified by LEfSe. Data are shown as the Means ± SEMs (n = 10 mice in Control group, 9 mice in OVA group, 9 mice in OVA + FMT group). G1 = Control group, G2 = OVA group, G3 = OVA + FMT group, Mor = morning subgroup, Noon = noon subgroup, Eve = evening subgroup. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the Control group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the OVA group.

Discussion

Asthma is characterized by marked acute asthma symptoms, enhanced airway hyperresponsiveness, increased level of eosinophils and neutrophils in lungs, and significant increase in inflammatory cytokins. Studies have shown that in animal experiments, adult mice are mainly used for asthmatic modeling, while it is relatively difficult to establish the model of asthma in neonatal mice[30]. FMT is an effective intervention to improve intestinal microbiota, rebuild bacterial homeostasis and treat diseases by extracting and processing faeces from healthy donors and then transferring it to the gastrointestinal tracts of the recipients. There are a lot of experimental studies and clinical applications of FMT in the treatment of different diseases, and FMT has already achieved a relatively ideal curative effect. However, there are a variety of specific operational methods, and no unified standard is made at present. The transfer routes include nasogastric tube, nasojejunal tube, esophagogastroduodenoscopy, colonoscopy and retention enema[31]. In view of the obvious differences of intestinal microbiota

between the upper and lower digestive tract[32], different ways of FMT may affect the colonization of beneficial bacteria in intestine and result in different outcomes, so the best transfer method needs to be selected according to the anatomical location of the recipients and pathological characteristics of different diseases. Before using colonoscopy for FMT intervention, bowel cleaning preparation is often required, which may change the original intestinal microbiota and increase uncontrollable factors[33]. Studies have proved that gavage is highly operable and feasible, and the gastrointestinal microecological environment after gavage will gradually resemble that of the donor[34]. Moreover, the microbiota in transplantation fluid can be dispersed and colonized in the whole gastrointestinal tract, and the specific colonization site will vary according to the characteristics of different bacteria (such as acid resistance and adsorption capacity), but it does not affect the effect of the transplantation fluid on the immune environment. Therefore, intragastric gavage was ultimately selected as the transfer method for experimental operation in this study. Studies have shown that FMT can alleviate digestive tract inflammation by increasing the level of IL-10 and TGF-β, upregulating aromatic hydrocarbon receptor (AHRs)[35]. The shortcoming of the experiment is that the operation process of faecal bacteria transplantation is relatively simple, and no other operation methods have been tried, such as different fecal bacteria extraction methods, fecal filtrate concentration, fecal filtrate volume and transfer routes. In this study, neonatal mouse model of OVA-induced asthma was successfully established. Moreover, FMT was performed to examine its effect in allergic airway inflammation. Compared to Control group, the relative percentage of lymphocytes in BALF decreased in OVA group because of the change trend of eosinophils and neutrophils, while FMT could also reverse this change. This change trend of lymphocytes was consistent with other changed indicators ,which showed that the low percentage of lymphocytes in BALF among our healthy experimental mice is a normal phenomenon. We found that FMT intervention inhibited AHR, reduced lung histopathological changes and decreased the levels of inflammatory cells and cytokines, which indicated that FMT treatment could ameliorate airway allergic inflammation in asthmatic neonatal mice.

Gut microbiota plays a crucial role in the induction of regulatory immune responses by affecting antigen presentation and regulating the function of T lymphocytes[6, 36]. Under homeostatic conditions, Tregs can maintain immune tolerance to gut microbiota, and $Foxp3^{+}Helios^{+}$Tregs have a greater suppressive ability than either Helios or Foxp3 expressing Tregs[37-39]. The increase in Tregs after FMT intervention might contribute to specific induction by certain bacteria in the feces[40] and can modulate gut immune balance. Furthermore, gut microbiome and their metabolites (such as short-chain fatty acids) could influence lung function through gut-lung axis, which is pivotal for maintaining lung homeostasis. In our study, gut microbiota of asthmatic neonatal mice was distinguishable from that of the normal mice, while FMT intervention was able to correct the disorder of gut microbial communities. Science[41] reported that after the intestinal microbiota of cancer patients was disturbed by antibiotic treatment, the efficacy of PD-1-targeted drugs would be affected . Researchers found that patients with high levels of Akkermansia in intestine received better immunotherapy effect, suggesting that the difference in efficacy of PD-1-targeted drugs was related to the level of Akkermansia in the intestinal tract. In order to further verify this conclusion, they transplanted feces of patients with favorable immune responses into germ-free mice of cancer model, and discovered that the immune response of transplanted mice was better than that of untransplanted mice. Moreover, after the oral administration of Akkermansia, the immune function of germ free mice was improved and the immune efficacy against tumors was also significantly enhanced, which proved that Akkermansia played an important role in the immune response in vivo, and its relative abundance could promote the therapeutic effect of PD-1-targeted tumor drugs. Other studies[42] have shown that Akkermansia can affect the expression of $CD4^{+}$ central memory T cells (TCMs) in tumor tissues and MLNs, participate in the chemotaxis migration of T lymphocytes through CCR9/CCL25 pathway, and then regulate $CD4^{+}$ T cells and $CD8^{+}$ T cells to restore the anti-cancer effect of PD-1 treatment. In conclusion, intestinal microbiota can not only regulate local intestinal microecological balance, nutrient metabolism and immune response, but also may be a novel assistance for anti-immune checkpoint therapy of PD-1. After PD-1 interacts with

PD-L1, it could affect the functions of DCs, T cells and other immune cells, interfere with the Th1/Th2 immune response and then regulate the development of asthma. In this experiment, the analysis of fecal samples indicated that the intestinal microbiota of neonatal asthmatic mice was significantly different from that of normal mice, and FMT could rebalance this disturbed intestinal microbiota and restore intestinal homeostasis. These results were in accordance with previous studies to support the theory that the intestine microbiota could have influence on the development of allergopathology[11, 22]. We also found that Alistipes, Ruminococcaceae, Collinsella and Akkermansia were all significantly changed. Among them, the level of Akkermansia in the intestinal tract of neonatal asthmatic mice was low, but after the intervention of FMT, it could significantly increased. This phenomenon was consistent with previous reports, suggesting that after the intervention of FMT Akkermansia could recover the normal state of intestinal microbiota and regulate the immune response through PD-1/PD-L1 axis, thus reducing the allergic airway inflammation of asthma.

Tregs are important anti-inflammatory cells and play a vital role in multiple immune regulation[43, 44]. In the early stage of asthma, Tregs inhibit the activation of eosinophils by secreting cytokines such as IL-10, IL-35 and TGF- β , thereby inhibiting the inflammatory response mediated by Th2 cells[45]. It has been reported that the proportion of Tregs and IL-10 levels in peripheral blood, lung tissue, sputum and BALF were significantly reduced in children and adults with asthma[46, 47]. Tregs can also inhibit effector T cells through cell-to-cell contact, maintain immune tolerance and alleviate allergic airway inflammation. Therefore, Tregs can contact with DCs, downregulate the co-stimulatory molecules, inhibit the ability of maturation and immune activation and ultimately alleviate the inflammatory response. Studies have shown that after removal of Tregs in mice, the level of $CD80^{+}MHCII^{+}$ DCs in hilar lymph nodes is significantly increased, and the disease symptoms are significantly improved, suggesting that Tregs can inhibit the activity of DCs, reduce the function of effector T cells and thus inhibit the immune effect[48]. After the activation of DCs by allergens, the costimulatory molecules such as CD40 and CD80 on the surface of DCs are highly expressed[49, 50], combine with CD28 on the surface of T cells to promote the differentiation of T cells to Th2 type cells, cause the imbalance of Th1/Th2 immune status and then inducing asthma[51]. Besides, the balanced gut microbiota after FMT treatment could increase the number of inhibitory $Helios^{+}$Tregs and $CD11c^{+}CD103^{+}$DCs in MLNs, while the level of surface costimulatory molecules of CD40, CD80 was reduced. The activated DCs have been reported to have the ability to upregulate surface costimulatory molecular, secrete IL-2, IL-6, IL-1 and other cytokines, reduce the inhibitory function of Tregs and restore the proliferation of effector T cells, which can resist the inhibition effect of Tregs[52]. And the DCs and Tregs could then immigrate to lung through the gut-homing chemokine receptor CCR9, as we examined previously[11]. Therefore, we pointed out that the beneficial role of FMT could be also related to the increased level of $CD11c^{+}CD103^{+}$DCs and Tregs.

To further elucidate the cellular mechanism underlying the protective effect of FMT, we investigated the expression of PD-1 and PD-L1. As the negative regulatory costimulatory molecules, PD-1/PD-L1 signal can regulate T-cell immune response and T-cell-mediated peripheral immune tolerance, and subsequently effect the immune regulation of various diseases, including asthma[53-56]. In this study, the asthmatic neonatal mice were presented with elevated expression of PD-1 in Treg and PD-L1 in DC. This phenomenon might be partially due to the abnormity of PD-1/PD-L1 signaling pathway, which could lead to the deficiency of Treg quantity. The upregulated expression of PD-1 could interact with PD-L1 to attenuate the activity of Tregs toward blocking Tregs in the G0/G1 phase and then suppressing cell proliferation, cytokine secretion, and cytotoxic capacity[57-59]. Our results also showed that the expression of PD-1 and PD-L1 was decreased after FMT intervention while Treg percentages and Helios levels were increased. Besides, Helios-deficient Tregs had defective expression of Foxp3 and inhibited IL-2-driven STAT5 activation[60], while PD-1 on Tregs negatively regulated $CD4^{+}CD25^{+}Foxp3^{+}$Tregs quantity and function through inhibiting STAT-5 phosphorylation[61, 62]. Therefore, we hypothesized that FMT could upregulate Tregs to suppress the proliferation of hyperactivated effector T cells in asthma by inhibiting PD-1/PD-L1 signal and IL-2-driven STAT-5

phosphorylation, which needs to be further explored.

To our best knowledge, few studies have reported the therapeutic effects of FMT on allergic airway inflammation. Our study found that FMT was effective in suppressing OVA-induced allergic asthma through the regulation of Th1/Th2 and $CD4^+CD25^+$Tregs in a PD-1-dependent manner. Nevertheless, there are several limitations to be noted. First, we just found the phenomenon that FMT could play a protective role on allergic asthma, thus further studies, such as antibody intervention or gene knockout methods, are highly wanted to verify this discovery. Secondly, we missed the Control + FMT group in the experimental group to fully compare the actual effect of FMT. At last, since the gut microbiota could be a confounding variable, mice with depleted intestinal microbiota will be the subjects of further research. In further experiment, we will upgrade the whole research and make up for all these deficiencies to verify and expand our conclusions.

In our study, FMT was confirmed to effectively attenuate allergic inflammation in the neonatal mouse model of allergic asthma induced by OVA. In addition, FMT can remodel gut microbiota and increase the DCs and Tregs in lung and MLN, with upregulated expression of Helios and downregulated expression of PD-1 and PD-L1. Collectively, we put forward hypothesis that the beneficial effect of FMT in allergic asthma is possibly achieved by restoring the steady state of gut microbiota and suppressing PD-1/PD-L1 signaling pathway to promote Tregs, which could immigrate to lung and then release anti-inflammatory cytokines to ameliorate allergic airway inflammation (Fig. 6). Hence, FMT might be an alternative therapeutic strategy for allergic airway inflammation.

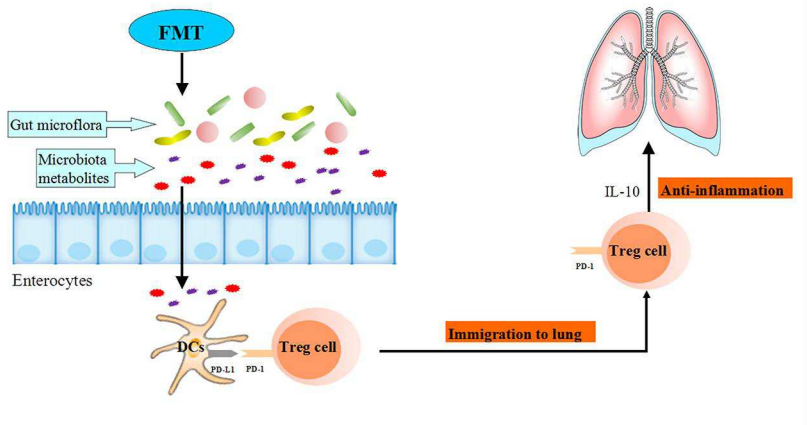

Figure 6 Schematic diagram of the effects of FMT on OVA-induced allergic asthma in neonatal mice through rebalancing the composition of intestinal microbiota and the PD-1/PD-L1 signaling pathways. Upon FMT intervention, homeostasis of the gut microbiota and metabolites will be rebalanced, which can inhibit the activation of PD-1/PD-L1 signaling pathway. And then Tregs could be promoted and immigrate to lung, along with the inducing expression of anti-inflammatory cytokines (such as IL-10).

Methods

- **Mice**

Pregnant BALB/c mice were obtained from the Laboratory Animal Center of the Fourth Military Medical University and acclimated to new conventional conditions until delivery. The animal room was maintained at 20 ± 2℃ and 50 ± 10% humidity with a 12h light-dark cycle. Standard mouse pellet diet and water were provided *ad libitum*. Experimental procedures were approved by the Ethics Committee for Animal Studies of the Fourth Military Medical University and performed in accordance with their guidelines of the Institutional Animal Care and Use Committee (IACUC). The study was carried out in compliance with ARRIVE guidelines.

- **Experimental design**

The pregnant BALB/c mice were randomly divided into three experimental groups: the normal control group (Control group), ovalbumin group (OVA group) and FMT intervention with OVA sensitization and challenge group (OVA + FMT group). After natural delivery of pregnant mice, there were 18 neonatal mice in Control group, 19 neonatal mice in OVA group and 15 neonatal mice in OVA + FMT group. Considering the different responses to OVA treatment in male and females, we decided to choose 10 male mice in Control group, 9 male mice in OVA group, and 9 male mice in OVA+FMT group to conduct this experiment.

OVA and OVA + FMT group intraperitoneally received 10 μg ovalbumin (OVA, grade V, Sigma, St Louis, MO, USA) plus 1 mg Alum in 20 μl of phosphate-buffered saline (PBS) on postnatal day (PND) 0, 7 and 14, and were then challenged by 1% OVA aerosols for 30 min every 2 days from PND 21 to 28 with an INQUA NEB plus (Omron Company limited, Dalian, Liaoning, China). Control group was sensitized and challenged with PBS instead.

For FMT intervention, feces were daily collected from the Control group at 8 o'clock in the morning from PND 21 to 28. Fresh feces were pooled, resuspended in sterile normal saline (W/V=1:5) and then passed through a 20mm pore nylon filter to remove large particulate and fibrous matter. Filtrate was centrifuged at 6000g for 5min at 4℃ using an Eppendorf 5804R centrifuge and dissolved in sterile normal saline to a concentration of 400mg/ml for transplantation. OVA + FMT group was intragastrically administered with 150μl fresh fecal solution daily from PND 21 to 28, while Control and OVA group were given same dose of sterile normal saline. All experimental protocols were performed as previously described with minor modifications[63, 64] (Fig. 7).

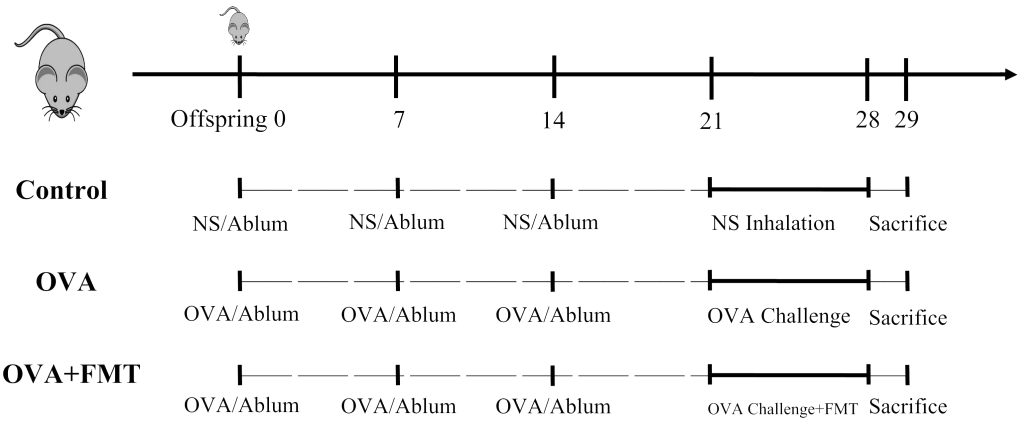

Figure 7 Scheme of experimental protocol. Mice were sensitized with three intraperitoneal injections of ovalbumin (OVA, 10 μg per mouse) at 7-day intervals (PND 0, 7, 14) and challenged with 1% OVA aerosols every two days from 21 to 28 to establish a neonatal mouse model of allergy asthma. Mice were orally received fecal filtrates in OVA + FMT group from PND 21 to 28 while Control and OVA group were given NS instead. Analyses were performed 24 hours after the last aerosol.

- **Airway hyperresponsiveness**

Airway hyperresponsiveness (AHR) was assessed 24h after last challenge by measuring the total lung resistance (Rrs, cm H_2O.s/mL) using the FlexiVent system (SCIREQ, Montreal, Canada) on PND 29, as described previously[65]. Mice were anaesthetized with pentobarbital sodium (90 mg/kg), tracheostomized with stainless steel cannula and then nebulized to increasing doses of acetyl-β-methylcholine chloride (1.5-100 mg/ml, MCh, Sigma). Values were averaged for each dose and analyzed to obtain dose-response curves.

- **Bronchoalveolar lavage**

Bronchoalveolar lavage fluid (BALF) was obtained on PND 29 after mice were sacrificed. The airways of mice were lavaged three times with 0.6mL of PBS using a tracheal cannula. The total number of white blood cell in BALF was determined, and the BALF were then centrifuged at 2500 r/min for 5 min at 4℃. The precipitations were stained with Wright Giemsa stain to detect the infiltration of inflammatory cells, and the supernatants were analyzed for inflammatory cytokines. One hundred cells on each slide were calculated to classify individual leucocyte populations.

- **Measurement of inflammatory cytokines and immunoglobulins**

Serum and BALF supernatants samples were collected to analyze the presence of inflammatory cytokines and immunoglobulins (Igs). Serum samples were collected 24h after last challenge and immediately separated by centrifugation. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the levels of IL-4, IL-5, IL-13 in BALF and IL-10, OVA-specific IgE and OVA-specific IgG1 in serum by ELISA Kits (Sabbiotech, MD, USA; Chondrex, Redmond, WA, USA) according to the instructions.

- **Histopathology**

The lungs were obtained in a sterile condition, fixed with 4% paraformaldehyde (PFA) for at least 24h and passed through graded ethanol series. Five-micrometre histological sections were cut and stained with hematoxylin and eosin (HE) for inflammation, Masson's trichrome for collagen fibers and periodic acid-schiff reagent (PAS) for goblet cell hyperplasia. Slides were all assessed blindly under light microscopy.

- **Flow cytometry analysis**

Single cell suspensions isolated from lungs and mesenteric lymph nodes (MLNs) were prepared and stained for fluorescence activated cell sorting (FACS) analyses, as described previously[11]. Flow cytometry analysis was conducted to detect the level of DCs and Tregs and the expression of their costimulatory molecules, programmed cell death protein 1 (PD-1) and programmed cell death protein 1 ligand (PD-L1). To detect dendritic cells (DCs), cells were stained with CD11c-APC, CD103-FITC, CD40-PE, CD80-eFluor 450 and PD-L1-PE-Cyanine7 (eBioscience, Carlsbad, CA, USA). To detect Tregs, cells were stained with CD4-FITC, CD25-APC and PD-1-PE-Cyanine7 (eBioscience, Carlsbad, CA, USA) for surface markers. And then, cells were incubated at room temperature for 30-60 min with fixation/permeabilization reagent under light-resistant conditions, washed at least twice using permeabilization reagent (eBioscience, Carlsbad, CA, USA) and stained with Foxp3-PE and Helios-eFlour 450 (eBioscience, Carlsbad, CA, USA) for intracellular markers. Data were obtained with BD FACSCanto system (BD Biosciences, San Jose, CA, USA) and then analyzed by FlowJo 10.7 software (Tree Star Inc., Ashland, OR).

- **Microbiome Analysis**

Mouse stools were collected at morning (08:00), afternoon (14:00) and evening (20:00), and then stored at -80 °C. The stools were sent to Shenzhen Wehealthgene for 16S rRNA sequencing by an Illumina miseq platform. The structure and quantity of the microbiota were analyzed as previously described with modifications[11].

- **Statistical analysis**

Data were shown as Means ± SEMs. All statistics were calculated with SPSS software (Version 25.0; IBM, Armonk, NY, USA) or GraphPad Prism Software (Version 8.0.2, Inc.La Jolla, CA, USA) by one-way analysis of variance (ANOVA) and Mann-Whitney U non-parametric tests. T test was used for parametric

tests, where appropriate. All reported p-values were two-sided and $P < 0.05$ was considered statistically significant.

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Acknowledgements

The authors would like to acknowledge and thank our funding source. This work was supported by the National Natural Science Foundation [grant numbers 31371151]; the research and development plan of natural science and technology of Shaanxi province [grant numbers 2017KW-045]; and Xi Jing Hospital Subject Promotion Program, Xi'an, China [grant numbers XJZT18MJ23].

Author contributions statement

Cheng Wu and Juan Zhang designed the experiments. Cheng Wu, Yuan-Yuan Jia, Xing-Zhi Wang and Qiu-Hong Li carried out experiments. Yuan-Yuan Jia and Qiu-Hong Li provided statistical expertise in experiments. Cheng Wu and Xing-Zhi Wang conducted the primary statistical analysis. Cheng Wu wrote the manuscript. Hui Su and Xin Sun supervised the whole experiments and supported the funding acquisition. All authors contributed to refinement of the study protocol and approved the final manuscript. [†]These authors have contributed equally to this work and share first authorship. [*]These authors have contributed equally to this work and share correspondencing authorship.

Competing interests statement

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