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Mineralocorticoid receptor deficiency in Treg cells ameliorates DSS-induced colitis in a gut microbiota-dependent manner

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Abstract

Mineralocorticoid receptor (MR) is a classic nuclear receptor and an effective drug target in the cardiovascular system. The function of MR in immune cells such as macrophages and T cells has been increasingly appreciated. The aim of this study was to investigate the function of Treg MR in the process of inflammatory bowel disease (IBD). We treated Treg MR-deficient ($MR^{flox/flox}Foxp3^{YFP\text{-}Cre}$, KO) mice and control ($Foxp3^{YFP\text{-}Cre}$, WT) mice with dextran sodium sulphate (DSS) to induce colitis and found that the severity of DSS-induced colitis was markedly alleviated in Treg MR-deficient mice, accompanied by reduced production of inflammatory cytokines, and relieved infiltration of monocytes, neutrophils and interferon γ^+ T cells in colon lamina propria. Faecal microbiota of mice with colitis was analysed by 16S rRNA gene sequencing and the composition of gut microbiota was vastly changed in Treg MR-deficient mice. Furthermore, depletion of gut microbiota by antibiotics abolished the protective effects of Treg MR deficiency and resulted in similar severity of DSS-induced colitis in WT and KO mice. Faecal microbiota transplantation from KO mice attenuated DSS-induced colitis characterized by alleviated inflammatory infiltration compared to that from WT mice. Hence, our study demonstrates that Treg MR deficiency protects against DSS-induced colitis by attenuation of colonic inflammatory infiltration. Gut microbiota is both sufficient and necessary for Treg MR deficiency to exert the beneficial effects.

KEYWORDS
inflammatory bowel disease, microbiota, mineralocorticoid receptor, Treg cells

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, are chronic relapsing disorders resulting from excessive intestinal responses against

Abbreviations: ABX, antibiotics; FMT, faecal microbiota transplantation; IBD, inflammatory bowel disease; LP, lamina propria; MR, mineralocorticoid receptor.

Ting Liu and Yu-Lin Li contributed equally to this study.

environmental factors [1]. The incidence of IBD has risen greatly worldwide over the past few decades, causing profound financial and social burdens [2, 3]. It is in great needs to develop novel therapeutic strategies to treat IBD [4].

Dysregulated immune response is a major contributor to IBD [5]. It has been reported that infiltration of immune cells such as inflammatory monocytes and neutrophils, and the disbalance between regulatory T cells (Tregs) and pathogenic Th1 and Th17 cells play important roles in the development and progression of IBD [6–9]. Tregs are a subset of $CD4^+$ T cells that express transcription factor Foxp3 and are critical for the body to maintain self-tolerance in both lymphoid and non-lymphoid tissues [10, 11]. They exist in large numbers in the lamina propria (LP) of the intestine and numerous studies have demonstrated the protective role of Tregs in sustaining immune homeostasis and alleviating the severity of IBD [12, 13].

It is increasingly acknowledged that gut microbiota is a crucial environmental factor contributing to the development of IBD apart from host genetics [14, 15]. Transplantation of gut microbiota from healthy donors has been shown to be an effective treatment strategy for gastrointestinal disorders [16, 17]. Interestingly, intestinal commensal microbiota such as *Bacteroides fragilis* and *Clostridium* species are able to enhance the induction of Tregs [18, 19].

Mineralocorticoid receptor (MR), a member of nuclear receptor superfamily, is an important drug target in the cardiovascular system and MR antagonists have been widely used to treat hypertension and heart failure [20, 21]. In recent years, the contribution of immune cell MR in cardiovascular diseases and metabolic disorders has been increasingly appreciated [22]. Deficiency of MR in myeloid cells has been reported to protect mice from pathological cardiac hypertrophy, myocardial infarction, atherosclerosis, neointimal hyperplasia and nonalcoholic fatty liver disease [23–27]. Deficiency of MR in T cells mitigates angiotensin II-induced hypertension and pressure overload-induced cardiac hypertrophy [28, 29]. However, the function of MR in Tregs has not been explored. Considering the significant role of Treg in IBD, it is of great interest to explore whether Treg MR participates in regulating host-microbiota homeostasis during the development of IBD.

In this study, we investigated whether and how Treg MR deficiency affects the progression of IBD. First of all, we studied the effects of Treg MR knockout (KO) on the severity of dextran sulphate sodium (DSS)-induced colitis, cytokine production and inflammatory cell infiltration in colons. Then, we analysed impacts of KO on the composition of gut microbiota after induction of colitis. Subsequently, we performed faecal microbiota transplantation experiment preceded the treatment of DSS to evaluate the involvement of gut microbiota. Finally, we conducted a microbiota depletion experiment to test whether MR in Tregs regulated the severity of colitis in a gut microbiota-dependent manner.

MATERIALS AND METHODS

Animals

Treg MR knockout mice were generated by crossing $MR^{flox/flox}$ mice [30] with Foxp3-Cre mice. $Foxp3^{YFP\text{-}Cre}$ mutant mice express a knocked-in yellow fluorescent protein/iCre-recombinase fusion protein from the *Foxp3* locus without disrupting expression of the endogenous *Foxp3* gene [31]. $Foxp3^{YFP\text{-}Cre}$ (WT) and $MR^{flox/flox}Foxp3^{YFP\text{-}Cre}$ (KO) mice of C57BL/6 strain were used. Wild-type C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and maintained in a SPF facility with unlimited access to water and food. 6–8 weeks old age- and sex-matched mice were used for experiments.

All animal experiments were approved by the Institutional Review and Ethics Board of Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (HKDL 2016-303).

DSS-induced colitis model

For colitis induction, WT and KO mice were treated with 3% DSS (36–50 kDa, MP Biomedical) in drinking water for 6 days followed with 1 day regular drinking water. The disease activity index (DAI) was scored daily according to the average of three parameters: weight loss (0–4), stool consistency (0, 2, 4), and stool blood (0, 2, 4) [32]. Mice were sacrificed on day 7, spleen weight and colon length were recorded.

Flow cytometry

Single-cell suspensions were centrifuged, stained with Live/Dead Fixable Viability Stain 510, pre-incubated with anti-mouse CD16/32 and then stained with antibodies for the detection of cell surface antigens. For intracellular staining, cells were first stimulated with stimulation cocktail (BioLegend) for 5 h, and then fixed and permeabilized using intracellular cytokine staining kit (eBioscience) before the detection of IFNγ and IL17A, or fixed and permeabilized using a transcription factor

staining buffer set (eBioscience) before Foxp3 staining. Flow cytometry data were acquired using LSR Fortessa (BD Biosciences) and analysed by FlowJo software (Tree star). The antibodies were listed in Table S2.

Faecal microbiota analysis

Fresh faecal samples were collected and stored at −80°C. Genomic DNA was extracted using OMEGA Soil DNA Kits (Omega Bio-Tek) according to the manufacturer's instructions. After DNA extraction, the full length of 16S rRNA genes was amplified to construct PCR-based libraries and samples were sequenced using an Illumina platform at Personal Biotechnology Co., Ltd (Shanghai, China). Sequencing analysis was performed on QIIME2 platform according to previously described protocols [33].

Statistical analysis

All data were shown as mean ± SEM and analysed using Prism (GraphPad Software). Two-way ANOVA analysis followed by Bonferroni correction was used for all experiments that had four groups and two factors. Student's *t*-test analysis was used to compare means that were detected in independent samples between two groups. All data are presented as mean ± SEM. Results were considered significantly different if $p < 0.05$.

RESULTS

Treg MR deficiency protects mice from DSS-induced colitis

To study the role of Treg MR during the development of acute colitis, wild type control (WT, $Foxp3^{YFP\text{-}Cre}$) mice and Treg MR knockout (KO, $MR^{flox/flox}Foxp3^{YFP\text{-}Cre}$) mice were exposed to 3% DSS in drinking water for 6 days followed by 1-day regular drinking water or treated with regular drinking water for 7 days (Figure 1a). QRT-PCR analysis revealed efficient depletion of MR was restricted to Treg cells isolated from the spleen of KO mice (Figure S1). WT and KO mice had similar body weights and body lengths during the first 8 weeks of life (Figure S2). Under DSS exposure, KO mice exhibited significantly less body weight change (Figure 1b), lower disease activity index (DAI) scores (Figure 1c), and longer colon length (Figure 1d) compared to WT mice while there was no significant difference in the body weight change, DAI scores and colon length of WT and KO mice treated with H_2O. DSS-induced splenomegaly was relieved in KO mice (Figure 1e). Histological analysis revealed that epithelial tissue damage and inflammation were significantly attenuated in DSS-treated colons of KO mice compared to WT mice (Figure 1f,g). WT and KO mice showed comparable body weight change, colon length, spleen weight and histological change when treated with H_2O (Figure 1b–g). These data demonstrated a clear protection of Treg MR deficiency on DSS-induced colitis.

MR deficiency decreases production of pro-inflammatory cytokines and infiltration of immune cells in colons of mice with colitis

Consistent with the alleviation of colitis in KO mice, DSS-induced expression of pro-inflammatory cytokines, including IL1β, IFNγ, IL6 and TNFα was decreased at gene level in colons of KO mice compared to WT mice after DSS treatment (Figure 2a). Moreover, DSS-induced expression of monocyte-attracting chemokine CCL2, neutrophil-attracting chemokines such as CXCL1 and CXCL2, and T cell-attracting cytokines such as CXCL9 and CXCL10 was decreased in colons of KO mice (Figure 2b). WT and KO mice under H_2O treatment showed comparable expression of inflammatory cytokines and chemokines (Figure 2a,b). Flow cytometry analysis revealed significantly decreased frequency and number of $CD45^+$ leukocytes in colonic lamina propria (cLP) of KO mice compared to WT mice under DSS exposure (Figure 2c,d). The percentage and total numbers of $CD11b^+Ly6C^{hi}$ monocytes and $CD11b^+Ly6G^+$ neutrophils were also notably decreased in cLP of KO mice compared to WT mice at day 7 post DSS exposure (Figure 2e–h and Figure S3a). Similarly, the percentages and numbers of $CD4^+IFN\gamma^+$ T cells and $CD8^+IFN\gamma^+$ T cells were significantly lower in cLP of KO mice (Figure 2i–l and Figure S3b), although the percentages of $CD4^+$ and $CD8^+$ T cells in $CD3e^+$ T cells were both similar between WT and KO mice after DSS exposure (Figure S4a). In mice treated with H_2O, the percentages and total numbers of $CD45^+$ leukocytes, monocytes, neutrophils, and $IFN\gamma^+$ T cells in cLP were similar between WT and KO mice (Figure 2c–l). Moreover, the percentage of Treg cells and expression of Foxp3 in Treg cells, as well as the percentage and number of $CD4^+IL17A^+$ T cells in cLP were comparable between WT and KO mice (Figure S4b–g). Interestingly, flow cytometry analysis revealed that the percentages of $CD11b^+Ly6G^+$ neutrophils and $CD11b^+Ly6C^{hi}$ monocytes were notably decreased in intraepithelial compartments of KO mice compared to WT mice at day 7 post DSS exposure (Figure S5a–d). These data suggested that Treg MR

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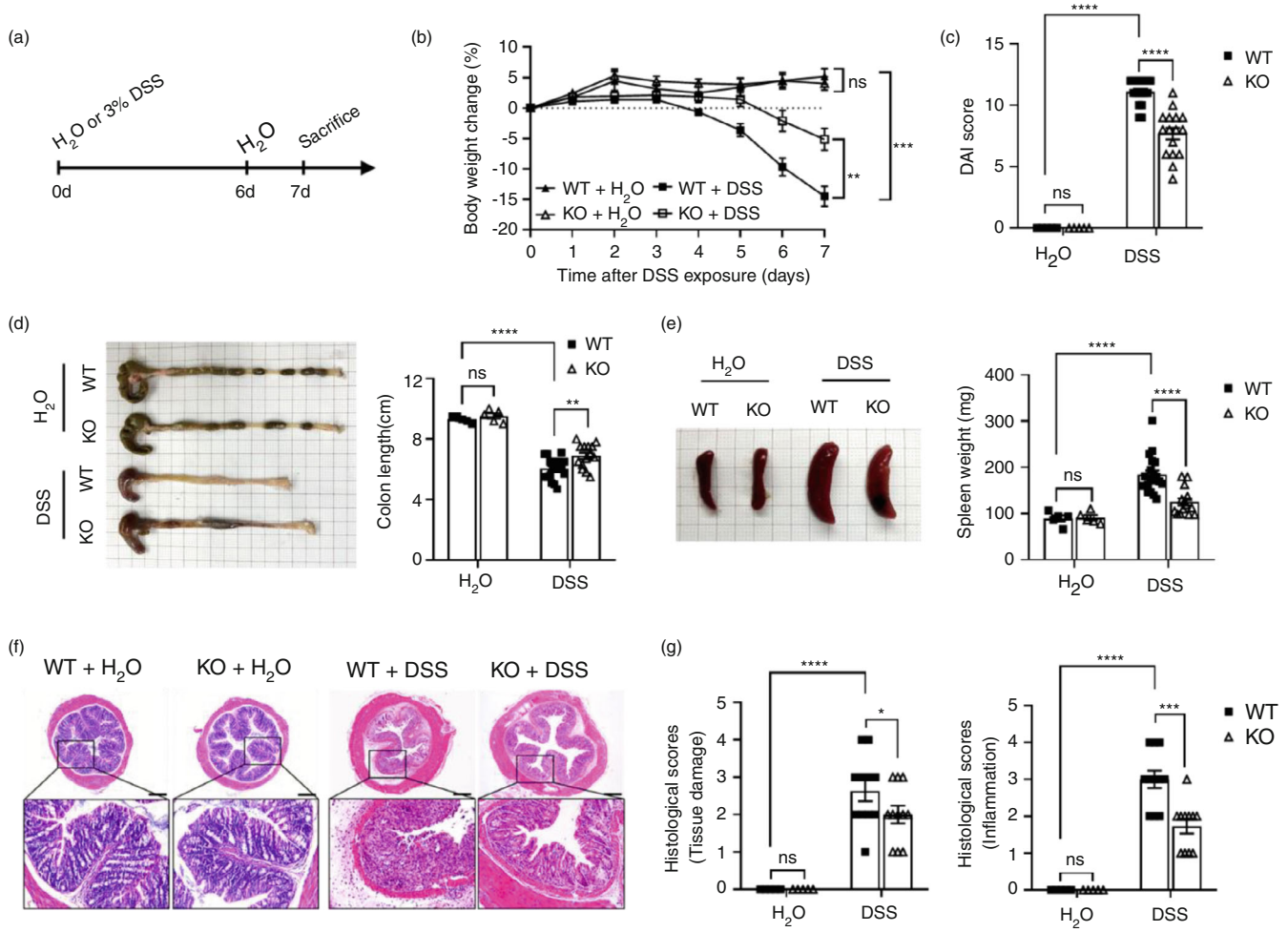

FIGURE 1 Treg MR deficiency alleviates DSS-induced colitis in mice. (a) Schematic presentation of the experimental design. (b) Body weight change and (c) disease activity index (DAI) scores of *Foxp3*$^{YFP\text{-}Cre}$ (WT) and *MR*$^{flox/flox}$*Foxp3*$^{YFP\text{-}Cre}$ (KO) mice treated with H_2O or DSS. (d) Representative pictures of colons and quantification the length on day 7 post H_2O or DSS exposure. (e) Representative pictures of spleens and quantifications of spleen weight. (f) Representative photomicrographs of haematoxylin and eosin (H&E)-stained colon sections from WT and KO mice on day 7 post H_2O or DSS exposure. Scale bar, 200 μm. (g) Quantification of histological scores exemplified in (f). Two-way ANOVA (b, c, d, e and g) was used for statistical analysis. ns, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

deficiency inhibited infiltration of inflammatory cells and production of pro-inflammatory cytokines to alleviate DSS-induced colitis in mice.

MR deficiency in Treg cells alters the composition of gut microbiota in DSS-treated mice

Mutiple studies have reported that gut microbiota plays an important role in the pathogenesis of IBD. Treg cells have been demonstrated to regulate gut microbiota and IBD.[12,19] We therefore investigated whether gut microbiota mediated the effects of Treg MR deficiency in colitis. We first analyzed the impacts of Treg MR deficiency on the composition of gut microbiota of colitis mice using 16S rRNA gene sequencing. Analysis of beta diversity by principal coordinate analysis (PCoA) based on Bray-Curtis distance showed that KO and WT samples were clearly segregated into two clusters (Figure 3a). MR deficiency in Treg cells led to substantial alterations in the composition of the gut microbiota at family level, including increases in the abundance of Akkermansiaceae and Bacteroidaceae and decreases in the abundance of Clostridiaceae and Prevotellaceae (Figure 3b). LEfSe analysis ranked Akkermansia muciniphila, Bacteroides sartorii, Bacteroides caecimuris and Bacteroides acidifaciens the most elevated species in gut microbiota of KO mice compared to WT mice (Figure 3c). Consistently, relative abundances of *B. sartorii, A. muciniphila, B. acidifaciens*

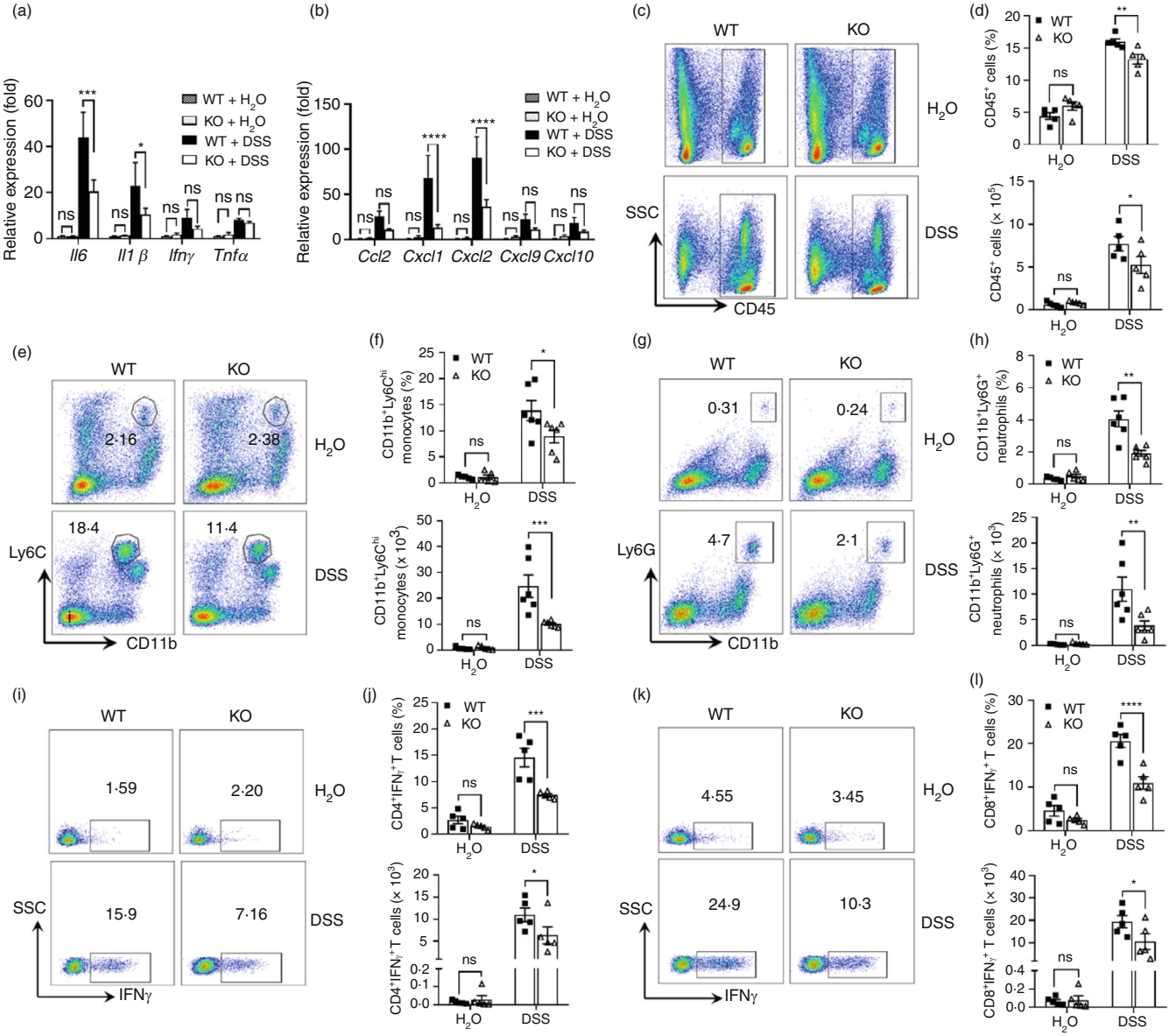

FIGURE 2 Treg MR deficiency decreases production of pro-inflammatory cytokines and infiltration of immune cells in colons of mice with colitis. (a) QRT-PCR analysis of inflammatory genes in colons. (b) QRT-PCR analysis of monocyte-attracting *Ccl2*, neutrophil-attracting *Cxcl1* and *Cxcl2*, as well as T cell-attracting *Cxcl9* and *Cxcl10* in colons. (c) Representative flow cytometry analysis of $CD45^+$ leukocytes in colonic lamina propria (cLP) of H_2O- or DSS-treated mice. (d) Quantifications of the percentage (in live cells) and total number of $CD45^+$ leukocytes in cLP. (e) Representative flow cytometry analysis of $CD11b^+Ly6C^{hi}$ monocytes in cLP. (f) Quantifications of the percentage (in $CD45^+$ cells) and total number of monocytes in cLP. (g) Representative flow cytometry analysis of $CD11b^+Ly6G^+$ neutrophils in cLP. (h) Quantifications of the percentage (in $CD45^+$ cells) and total number of neutrophils in cLP. (i) Representative flow cytometry analysis of $CD4^+IFN\gamma^+$ T cells in cLP. (j) Quantifications of percentage (in $CD4^+$ cells) and total number of $CD4^+IFN\gamma^+$ T cells in cLP. (k) Representative flow cytometry analysis of $CD8^+IFN\gamma^+$ T cells in cLP. (l) Quantifications of percentage (in $CD8^+$ cells) and total numbers of $CD8^+IFN\gamma^+$ T cells in cLP. Two-way ANOVA were used for statistical analysis. $n = 5$–8. ns, not significant; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

and Bacteroides intestinalis were significantly elevated in microbiota of KO mice (Figure 3d). Random forest analysis ranked *B. sartorii* and *A. muciniphila* the top 2 species that differentiated KO from WT (Figure 3e). We have also compared gut microbiota between WT and KO mice treated with H_2O and found there were slight differences in the composition between the two groups (Figure S6). LEfSe analysis ranked *A. muciniphila* and *B. acidifaciens* the most elevated species in gut microbiota of KO mice compared to WT mice treated with H_2O (Figure S6c) and the differences were amplified under DSS exposure (Figure 3c).

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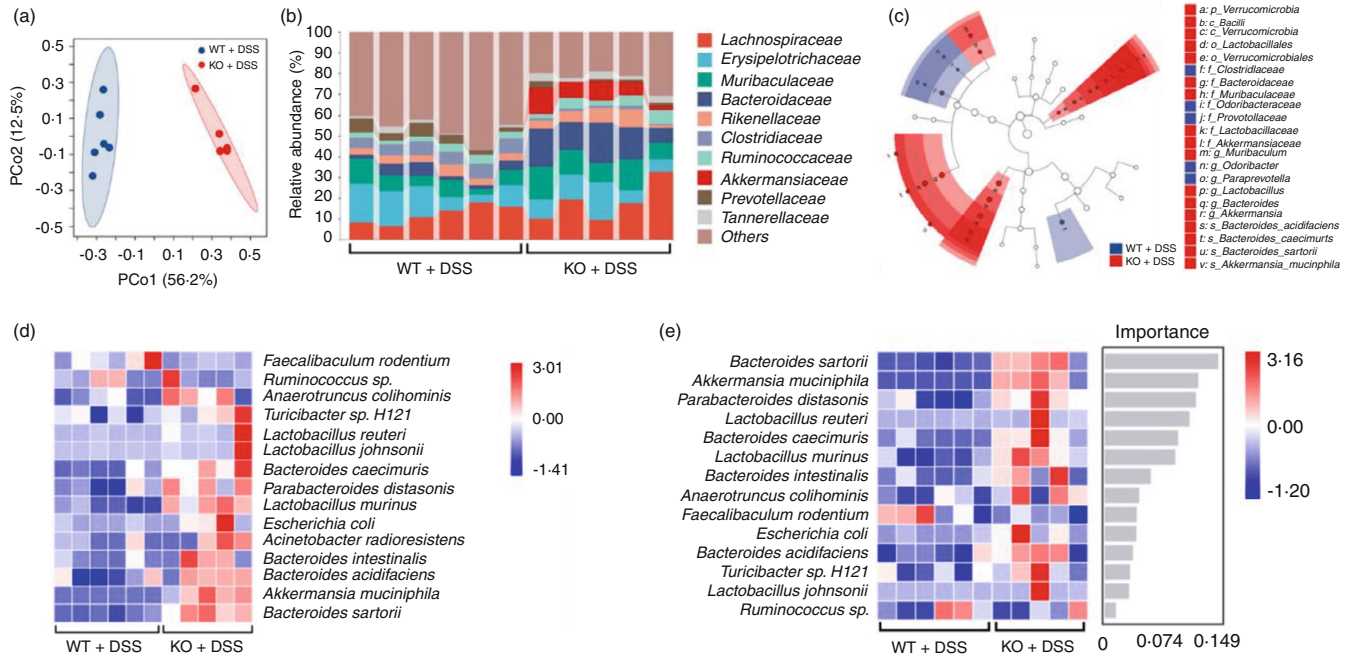

FIGURE 3 Treg MR deficiency alters the composition of gut microbiota in mice with colitis. Faeces were taken from mice treated with DSS for 7 days and gut microbiota was analysed using 16S rRNA gene sequencing. (a) Assessment of beta diversity by principal coordinate analysis (PCoA) based on Bray-Curtis distance. (b) Relative abundances of gut microbiota at family level. (c) Taxonomic cladogram of gut microbiota based on linear discriminant analysis (LDA) effect size (LEfSe). The threshold of LDA score was 4. (d) Heatmap showing changes in the relative abundances of bacterial taxa at the species level between WT and KO mice (top 15). (e) Random forest analysis of gut microbiota at species level. $n = 6{:}5$

MR in Treg cells regulates the severity of DSS-induced colitis in a gut microbiota-dependent manner

To determine whether Treg MR deficiency-induced alterations of gut microbiota were necessary for the improvement of colitis, WT and KO mice were treated with antibiotics (ABX) to deplete gut microbiota before the initiation of DSS. In those without microbiota depletion (treated with H_2O), DSS-induced colitis phenotypes and immune cell infiltration were significantly alleviated in KO compared to WT mice (Figure 4a–o), consistent with our earlier observations. Importantly, ABX treatment abolished the differences between WT and KO mice, resulting in comparable body weight change, colon length, spleen weight, and DAI scores between WT and KO mice after DSS exposure for 7 days (Figure 4a–d). Mice in WT + DSS + ABX and KO + DSS + ABX groups displayed indistinguishable colonic expression of inflammatory genes as well as epithelial tissue damage and inflammation revealed by histological analysis (Figure 4e–g). Furthermore, there was no significant difference in the percentage or number of monocytes, neutrophils, $CD4^+IFN\gamma^+$ T cells, or $CD8^+IFN\gamma^+$ T cells in cLP between WT + DSS + ABX and KO + DSS + ABX groups (Figure 4h–n). Meanwhile, antibiotics treatment abolished the compositional difference in microbiota between WT and KO mice under DSS exposure (Figure S7). These results showed that gut microbiota was required for Treg MR deficiency to protect mice from DSS-induced colitis.

Gut microbiota of KO mice protects mice from DSS-induced colitis

Finally, we studied whether the gut microbiota of KO mice was sufficient to exert a protective role on colitis. We transferred faeces from WT and KO mice to ABX-treated mice via daily oral gavage for 1 week. After DSS exposure, recipient mice that had received faecal microbiota from KO mice (KO Mb) were notably protected from DSS-induced colitis, as evidenced by reduced weight loss (Figure 5a), increased colon length (Figure 5b), decreased spleen weight (Figure 5c) and improved DAI scores (Figure 5d) compared to recipient mice received faecal microbiota from WT mice (WT Mb). Expression of inflammatory genes, including IL1β, IFNγ, IL6 and TNFα were also decreased in colons of mice in the KO Mb group (Figure 5e). Histological analysis revealed that mice in the KO Mb group had significantly less epithelial tissue damage and

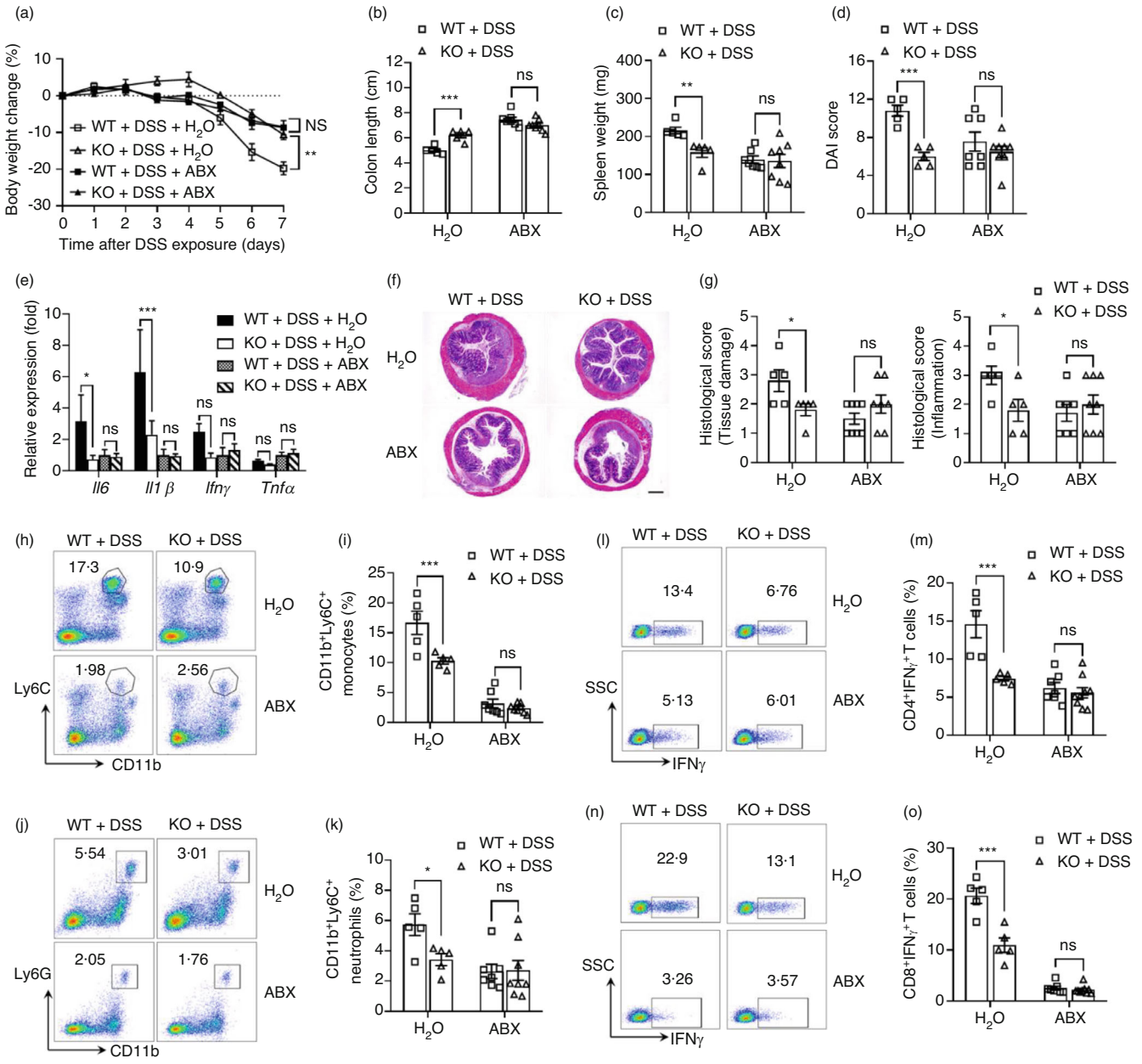

FIGURE 4 Treg MR regulates DSS-induced colitis in a gut microbiota-dependent manner. (a) Body weight change, (b) colon length, (c) spleen weight and (d) DAI scores of H_2O- or ABX-treated WT and KO mice treated with DSS for 7 days. (e) QRT-PCR analysis of *Il1β*, *Ifnγ*, *Il6* and *Tnfα* in colons. (f) Representative micrographs of HE-stained colon tissues and quantification of histological scores. Scale bar, 200 μm. (g) Quantification of histological scores exemplified in (f). (h) Representative flow cytometry analysis of $CD11b^+Ly6C^{hi}$ monocytes in cLP of H_2O- or ABX-treated WT and KO mice under DSS exposure. (i) Quantification of the percentage (in $CD45^+$ cells) of monocytes in cLP. (j) Representative flow cytometry analysis of $CD11b^+Ly6G^+$ neutrophils in cLP of H_2O- or ABX-treated WT and KO mice under DSS exposure. (k) Quantification of the percentage (in $CD45^+$ cells) of neutrophils in cLP. (l) Representative flow cytometry analysis of $CD4^+IFN\gamma^+$ T cells in cLP. (m) Quantifications of the percentage (in $CD4^+$ cells) of $CD4^+IFN\gamma^+$ T cells. (n) Representative flow cytometry analysis of $CD8^+IFN\gamma^+$ T cells in cLP. (o) Quantifications of the percentage (in $CD8^+$ cells) of $CD8^+IFN\gamma^+$ T cells. Two-way ANOVA was used for statistical analysis. $n = 5{:}8$. ns, not significant; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

inflammation (Figure 5f,g). Flow cytometry detected significant decrease in percentage and number of infiltrating monocytes in cLP of mice in the KO Mb group (Figure 5h,i). However, the percentage and number of neutrophils in cLP were similar between WT Mb and KO Mb groups (Figure 5j,k). Analysis of gut microbiota after FMT using 16S rRNA gene sequencing revealed different microbial composition between WT Mb and

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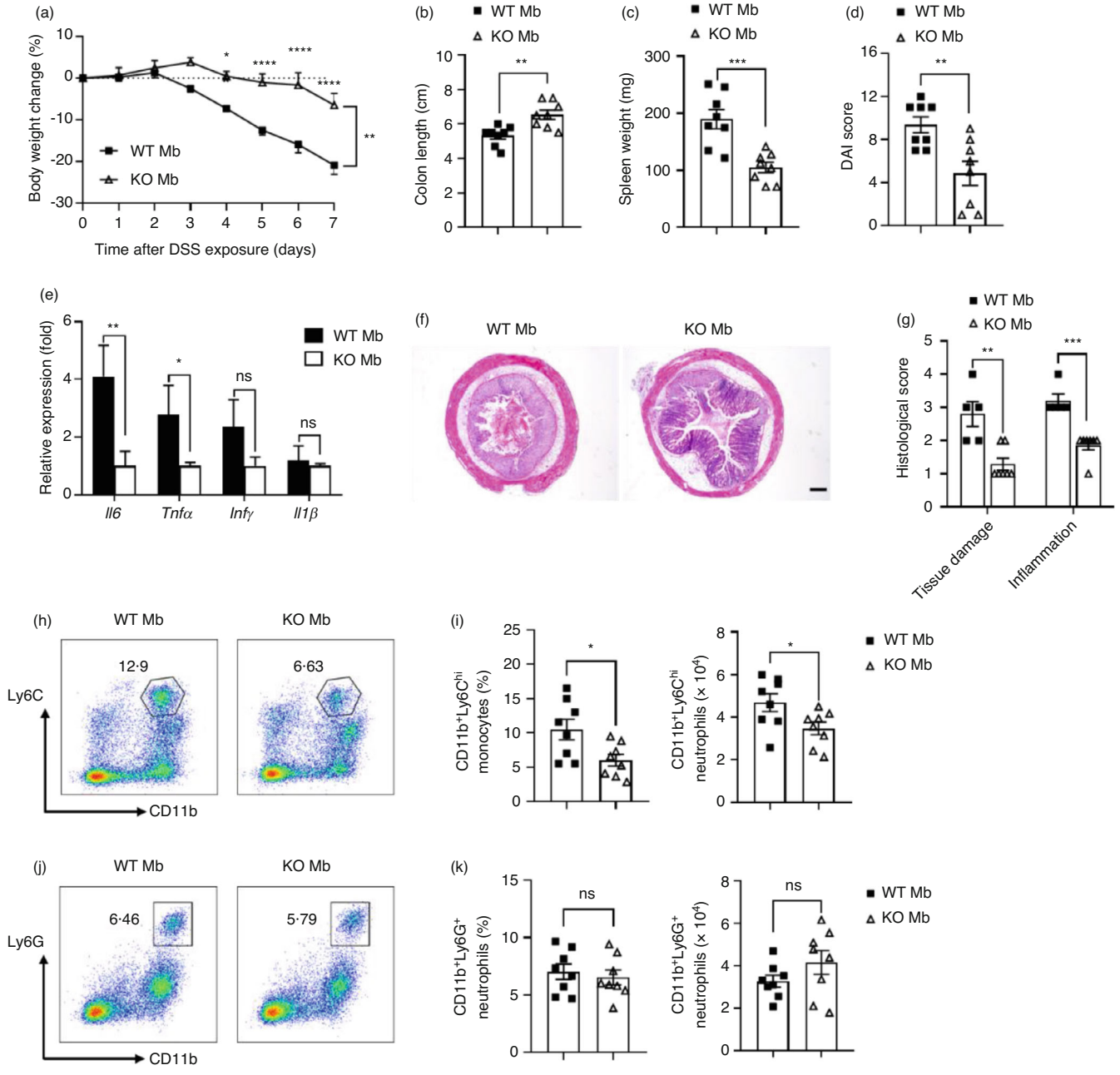

FIGURE 5 Gut microbiota of KO mice protects mice from DSS-induced colitis. (a) Body weight change, (b) colon length, (c) spleen weight, and (d) DAI scores of C57BL/6J mice pre-treated with ABX, received FMT from WT mice (WT Mb) or KO mice (KO Mb), and treated with DSS for 7 days. (e) QRT-PCR analysis of *Il1β*, *Ifnγ*, *Il6* and *Tnfα* in colons. (f) Representative photomicrographs of H&E-stained colon sections. Scale bar, 200 μm. (g) Quantification of histological scores exemplified in (f). (h) Representative flow cytometry analysis of $CD11b^{+}Ly6C^{hi}$ monocytes in cLP of DSS-treated mice. (i) Quantifications of the percentage (in $CD45^{+}$ cells) and total number of monocytes in cLP. (j) Representative flow cytometry analysis of $CD11b^{+}Ly6G^{+}$ neutrophils in cLP. (k) Quantifications of the percentage (in $CD45^{+}$ cells) and total number of neutrophils in cLP. Two-way ANOVA (a) or Student's *t*-test (b–e, g, i and k) was used for statistical analysis. $n = 8{:}8$. ns, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

KO Mb groups, and the differences were similar to those between WT and KO mice (Figure 3 and Figure S8), suggesting successful transplantation of gut microbiota. Taken together, these data demonstrated that Treg MR deficiency-induced alterations of gut microbiota were sufficient to alleviate colitis.

DISCUSSION

Although functions of MR in many immune cells have been reported in cardiovascular diseases and metabolic syndromes [24, 27, 28], the role of MR in Tregs and its functions in IBD have not been elucidated. Through the

current study, we demonstrated that Treg MR deficiency ameliorated DSS-induced colitis with attenuated colonic inflammation and immune cell infiltration in a gut microbiota-dependent manner.

Our data demonstrated that abrogation of Treg MR in mice resulted in a broad range of beneficial effects in DSS-induced colitis. Treg MR deficiency improved a variety of colitis parameters, including loss of body weight, increase of DAI, shortening of the colon, increase of spleen weight, and histological pathology of the colon. Further analyses suggested that Treg MR deficiency relieved the severity of IBD with down-regulated expression of pro-inflammatory cytokines and less colonic recruitment of inflammatory leukocytes. Recent studies have revealed important roles of MR in leukocytes such as macrophages and T lymphocytes in cardiovascular diseases [24, 26, 28, 29]. Spironolactone, an MR antagonist, has been reported to alleviate inflammation by suppressing the expression of cytokines in a rat model of 2,4,6-trinitrobenzenesulfonic acid-induced colitis [34]. On the other hand, it has been reported that spironolactone treatment may increase mortality in patients with both Crohn's disease and *Clostridium difficile* infection, and a mouse model of colitis induced by *Salmonella typhimurium* infection [35]. The conflicting results may be attributable to different models (colitis only vs. combination of colitis with superimposed infection). Our study used a genetic mouse model in combination with a simple colitis model to clearly illustrate the effective protection against colitis by blockade of MR in Treg cells. The conclusion regarding functions of Treg MR in IBD may be further strengthened in future studies using interleukin-10-knockout mice, which display the most similar characteristics to human Crohn's disease [36].

We also identified that MR deficiency in Tregs had a profound impact on the composition of gut microbiota, which might have ultimately mediated the protective effects of Treg MR deficiency on IBD. Treg MR deficiency dramatically altered the composition of the gut microbiota in IBD mice, resulting in two completely separated clusters for the KO and WT mice. Among the species that differentiated KO from WT mice, *A. muciniphila* has been widely reported to play protective roles in metabolic disorders and tumour immunotherapy [37, 38]. It has also been demonstrated that administration of *A. muciniphila* ameliorates DSS-induced colitis by promoting the production of short-chain fatty acids through their anti-inflammatory functions [39, 40]. Commensal *Bacteroides* is one of the most numerically prominent genera in the mammalian gastrointestinal tract [41]. The results of human studies have shown that the relative abundance of *Bacteroides* in IBD patients is markedly lower than that in healthy individuals [42]. In our study, the relative abundances of *Bacteroides* species, including *B. sartorii*, *B. caecimuris*, *B. intestinalis*, and *B. acidifaciens* elevated in the gut microbiota of the KO mice. Germ-free mice mono-associated with single strain *B. sartorii* has been reported to suppress inflammation in a spontaneous colitis mouse model [43]. *B. caecimuris*, which is positively correlated with the frequency of effector IL10^{+} Tregs in hepatocellular carcinoma related to non-alcoholic fatty liver disease, has been shown to produce short-chain fatty acid and exert immunomodulatory function [44]. *B. intestinalis* has been proposed to exert anti-inflammatory activity in vitro experiment [45]. The protective effect of *B. acidifaciens* in ameliorating insulin resistance has been observed in obese mice [46]. On the other hand, *Bacteroides* species, generally beneficial microbes, may become opportunistic pathogens under ectopic colonization [41]. Collectively, our observations demonstrated that Treg MR deficiency regulated dysbiosis of the gut microbiota in IBD, particularly increased the abundances of beneficial commensal microbes.

Importantly, depletion of gut microbiota by ABX treatment abolished the protection of Treg MR deficiency on IBD. Our results also showed that ABX treatment alleviated DSS-induced colitis in both WT and KO mice, particularly in WT mice. ABX has been shown to either exacerbate or alleviate DSS-induced colitis. For example, one report has shown that 4 weeks of ABX treatment increases mortality and morbidity of DSS-induced colitis in mice [47]. However, another report has shown that 12 days of ABX significantly alleviates DSS-induced colitis in mice [48]. The discrepancy is likely due to the length of ABX treatment, the dose of DSS, the genetic background of mice, and other differences. The length of ABX may be a major determinant. Our study used 2 weeks of ABX, similar to the second report mentioned above, which may explain why our results were similar to the results in this report [48]. Furthermore, FMT from KO mice alleviated IBD compared to FMT from WT mice, illustrating that modulation of the gut microbiota was both sufficient and necessary for Treg MR deficiency to exert protective effects on IBD.

In summary, we have identified that Treg MR plays a pivotal role in DSS-induced colitis through the regulation of immune cell infiltration in a gut microbiota-dependent manner. These findings support that specific perturbation of Treg MR may be a promising approach to treat IBD.

AUTHOR CONTRIBUTIONS

Ting Liu and Sheng-Zhong Duan designed the study, conducted data analysis and wrote the manuscript. Ting Liu, Yu-Lin Li, Lu-Jun Zhou, Xue-Nan Sun, Yong-Li Wang, Lin-Juan Du, Yuan Liu, Hong Zhu, Bo-Yan Chen, Hui-Lin Ye and Shi-Jia Huang performed experiments. Xiaoxia Wang and Bin Li contributed to mouse colonies,

reviewed and edited the manuscript. Yu-Lin Li, Lu-Jun Zhou, Yong-Li Wang, Shuo Xu and Yan Liu conducted data analysis, reviewed and edited the manuscript. Sheng-Zhong Duan is the guarantor of this work and, as such, has full access to all the data of the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

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CONFLICT OF INTEREST

The authors disclose no conflict of interest.

DATA AVAILABILITY STATEMENT

These sequence data have been submitted to the Sequence Read Archive of NIH with accession number PRJNA794280.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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