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Early-life fingolimod treatment improves intestinal homeostasis and pancreatic immune tolerance in non-obese diabetic mice

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Fingolimod has beneficial effects on multiple diseases, including type 1 diabetes (T1D) and numerous preclinical models of colitis. Intestinal dysbiosis and intestinal immune dysfunction contribute to disease pathogenesis of T1D. Thus, the beneficial effect of fingolimod on T1D may occur via the maintenance of intestinal homeostasis to some extent. Herein, we investigated the role of fingolimod in intestinal dysfunction in non-obese diabetic (NOD) mice and possible mechanisms. NOD mice were treated with fingolimod (1 mg·kg⁻¹ per day, i.g.) from weaning (3-week-old) to 31 weeks of age. We found that fingolimod administration significantly enhanced the gut barrier (evidenced by enhanced expression of tight junction proteins and reduced intestinal permeability), attenuated intestinal microbial dysbiosis (evidenced by the reduction of enteric pathogenic Proteobacteria clusters), as well as intestinal immune dysfunction (evidenced by inhibition of CD4⁺ cells activation, reduction of T helper type 1 cells and macrophages, and the expansion of regulatory T cells). We further revealed that fingolimod administration suppressed the activation of CD4⁺ cells and the differentiation of T helper type 1 cells, promoted the expansion of regulatory T cells in the pancreas, which might contribute to the maintenance of pancreatic immune tolerance and the reduction of T1D incidence. The protection might be due to fingolimod inhibiting the toll-like receptor 2/4/nuclear factor-κB/NOD-like receptor protein 3 inflammasome pathway in the colon. Collectively, early-life fingolimod treatment attenuates intestinal microbial dysbiosis and intestinal immune dysfunction in the T1D setting, which might contribute to its anti-diabetic effect.

Keywords: fingolimod; type 1 diabetes; gut barrier; enteric pathogens; TLR2-NF-κB-NLRP3

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INTRODUCTION

Fingolimod is commonly used to treat multiple sclerosis [1]. Notably, fingolimod also exerts therapeutic benefits against type 1 diabetes (T1D) and has been a subject of recent investigations related to the treatment of T1D and its associated complications. However, its clinical efficacy has not been determined, suggesting that the antidiabetes mechanism of fingolimod is complex and limited and requires further study [2–4]. Compared to healthy controls, T1D subjects exhibit increased intestinal inflammation and impaired intestinal barrier function [5–7], which promotes the translocation of gut luminal bacteria and initiates systemic inflammation, including pancreatic inflammation [6, 8], thus contributing to the onset and progression of T1D [9–11]. The shift of intestinal homeostasis toward a proinflammatory state predates increased susceptibility to T1D [7, 12]. T1D is caused by expansion of CD4⁺ interferon-gamma γ (IFN-γ) + cells (Th1 cells) or a decrease in the number or function of regulatory T cells (Tregs) [13–15]. Interestingly, fingolimod ameliorates colitis in numerous preclinical models [1, 16] due to its roles in inducing Tregs and inhibiting lymphocyte proliferation and IFN-γ secretion [1, 17, 18]. Furthermore, fingolimod prevents the loss of tight junction proteins (TJPs) in a variety of barrier-related diseases

[19, 20]. These studies suggest that fingolimod may enhance the gut barrier and maintain intestinal homeostasis in the setting of T1D, which may contribute to preventing T1D. However, the effects of fingolimod on intestinal homeostasis in the setting of T1D are poorly understood. The present study aimed to elucidate the microbial and immune mechanisms of fingolimod in the gastrointestinal system in T1D and to investigate the influence of fingolimod on the pancreatic immune system in the setting of T1D.

MATERIALS AND METHODS

Animals and T1D mouse model

The NOD mouse model is a classical model that recapitulates the hallmarks of T1D in patients [21]. Three-week-old female NOD/LtJ mice (Su Pu Si Biotechnology, Co., Ltd., Suzhou, China) were housed in the animal facility of Jiangnan University (Wuxi, China). All mice were housed in individual ventilated caging systems (TECNIPLAST, Italy) under specific pathogen-free conditions at a controlled temperature (24 ± 1 °C) on a 12 h light-dark cycle. The animals were anesthetized with pentobarbitone sodium (90 mg/kg, Sigma-Aldrich, MO, USA) and euthanized. All studies were

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approved by the Institutional Animal Ethics Committee of Jiangnan University (JN No. 20131205) and carried out in compliance with national and international guidelines for the Care and Use of Laboratory Animals.

Fingolimod treatment

To evaluate the effect of fingolimod on the onset of T1D, mice were treated daily with 1 mg/kg fingolimod (MCE, New Jersey, USA) in 100 μ L sterile water or an equal volume of sterile water by gavage from weaning (3-weeks-old) to the age of 31 weeks ($n = 12$ /group), and their glucose levels were monitored. In the present study, "early-life fingolimod treatment" means treatment with fingolimod from weaning to the age of 31 weeks. The mice were euthanized when a diagnosis of diabetes was made or at the end of the study.

In a parallel study, NOD control and fingolimod-treated mice were euthanized at 12 weeks of age ($n = 12$ /group) after 9 weeks of fingolimod treatment. The pancreatic lymph nodes (PaLNs), mesenteric lymph nodes (MLNs), pancreas, blood, and spleen were collected for immune cell detection by flow cytometry. The pancreas was collected for histological evaluation by hematoxylin and eosin staining.

To track the migration of immune cells adoptively transferred into NOD mice, we transferred fluorescently labeled splenocytes to the recipient mice by intraperitoneal injection ($n = 5$ /group).

For the assessment of gut barrier function, 10-week-old female NOD mice were given either 1 mg/kg fingolimod in 100 μ L sterile water or an equal volume of sterile water by gavage daily for 3 weeks ($n = 5$ /group).

Blood glucose measurement

A glucometer (Roche Diagnostics, Almere, Netherlands) was used to measure glucose levels from tail vein samples. NOD mice with hyperglycemia (>11.1 mmol/L) on two consecutive daily readings were considered diabetic [22–27]. NOD mice with hyperglycemia (>11.1 mmol/L) likely require urgent institution of insulin therapy [28, 29]. Thus, in the present study, this diagnostic criterion was adopted.

Intestinal permeability

Intestinal permeability was assessed by measuring fluorescein isothiocyanate (FITC)-dextran (FD20-250 mg, average molecular weight: 20,000, Sigma-Aldrich, MO, USA) accumulation in serum. At the end of the experiment, the mice received 50 mg/100 g of FITC-dextran in phosphate-buffered saline (PBS) by gavage after 4 h of fasting. Four hours after gavage, serum was collected, diluted at 1:10 in PBS, and analyzed with the Thermo Scientific™ Varioskan™ LUX reader (Thermo Fisher Scientific, MA, USA).

Western blot analysis

Colon tissues were added to 2-mL centrifuge tubes, lysed with RIPA buffer containing protease inhibitors (Beyotime, Shanghai, China), and ground with a high-throughput tissue homogenizer (SCIENTZ-48, Ningbo, China). The colon homogenates were centrifuged at $8000 \times g$ for 15 min at 4 °C. The supernatant was used for Western blotting. The total protein concentration was quantified by a BCA protein quantitation assay kit (Beyotime, Shanghai, China). Equal amounts of total proteins were loaded on a polyacrylamide SDS-PAGE gel. The proteins were transferred to polyvinylidene fluoride membranes and then blocked with blocking buffer for 1 h at room temperature. Primary antibodies, including ZO-1, ZO-2, Occludin, Toll-like receptor 2 and 4 (TLR2 and 4), pyrin domain-containing protein 3 (NLRP3), p-NF- κ B p65 (p-P65), cleaved-IL-1 β (IL-1 β), caspase-1-p20 (p20), and β -actin antibodies (the antibody information is listed in Table S1), were added at 4 °C overnight. After being washed, the PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) at room temperature

for 2 h. The protein bands were visualized with Western Lightning Plus-ECL (Pierce, Rockford, IL, USA). β -actin was used as an internal standard to control for unwanted sources of variation, and relative protein expression values were expressed as the fold relative to the mean of the controls by comparison to the corresponding control value, and the control value was normalized to 1.0. Protein expression levels were quantitated with ImageJ.

Preparation of single-cell suspensions

When the mice were euthanized, the PaLNs, MLNs, and pancreas were collected and immediately placed in cold PBS.

For macrophage detection, fresh pancreatic tissues were cut into small pieces, digested with 0.75 mg/mL collagenase P (Roche Basel, Switzerland) at 37 °C for 15 min, homogenized with gentle MACS™ Dissociators (MiltenyiBiotec, BergischGladbach, Germany) and filtered with a 70- μ m filter [22, 30]. For T-cell detection, fresh pancreatic tissues were harvested, filtered with 70- μ m polypropylene mesh, ground using a 2.5-mL syringe plunger and washed with PBS (containing 10% fetal bovine serum) [22, 30].

MLNs were harvested and filtered with 70- μ m polypropylene mesh, ground using a 2.5 mL syringe plunger and washed with PBS [22, 30].

After the mice were euthanized, their spleens were harvested and dissociated into single-cell suspensions with gentle MACS™ Dissociators (MiltenyiBiotec, BergischGladbach, Germany). After centrifugation at $300 \times g$ for 5 min, red blood cell lysis buffer (Fcmacs, Nanjing, China) was added to lyse the red blood cells. After the cells were rested for 15 min at room temperature and away from light, they were centrifuged and resuspended in PBS.

After the mice were euthanized, blood was collected in an anticoagulant tube. After centrifugation at $300 \times g$ for 5 min, red blood cell lysis buffer was added to lyse the red blood cells. After the cells were rested for 15 min at room temperature and away from light, they were centrifuged and resuspended in PBS.

Flow cytometry

Mice were sacrificed, and single-cell suspensions were prepared from pancreatic, PaLNs, blood, spleen, and MLN tissues as described in previous studies [22, 31] and stained for 30 min at 4 °C after FcyRII/III was blocked with an anti-CD16/CD32 monoclonal antibody. The antibody information is listed in Table S2. According to the manufacturer's protocol, for the detection of intracellular (cytoplasmic) IFN- γ , cell suspensions were incubated at 37 °C for 6 h with Cell Stimulation Cocktail (eBioscience, San Diego, CA, USA), stained and fixed. For Treg staining, cells were first surface stained, fixed and stained for intracellular (nuclear) forkhead box P3 (Foxp3). Stained cells were analyzed on an Attune NxT flow cytometer (Thermo Fisher Scientific, Massachusetts, USA). The gating strategy for flow cytometry is listed in Fig. S1–15.

Assessment of donor cell distribution

Donor cell distribution was assessed as previously described [32]. Spleens were harvested from adult NOD donors and immediately dissociated into single-cell suspensions with gentle MACS™ Dissociators (MiltenyiBiotec, BergischGladbach, Germany). After centrifugation at $300 \times g$ for 5 min, red blood cell lysis buffer was added to lyse the red blood cells. After the cells were rested for 15 min at room temperature and away from light, they were centrifuged and resuspended in PBS. The splenocytes were labeled with the cell proliferation dye eFluor 450 (CPD450) (eBioscience, San Diego, CA, USA). CPD450-labeled cells were then injected into the peritoneal cavity. The recipients were killed 20 h after donor cell injection, and the pancreas, PaLNs, MLNs, blood, and spleen were harvested and processed into single-cell suspensions. After counting, the single-cell suspensions were subjected to cytofluorimetric analysis with an Attune NxT flow cytometer (Thermo Fisher Scientific, Massachusetts, USA).

Histopathological examination of the pancreas

Freshly harvested pancreatic tissues were collected, fixed in NEG-50 (Thermo Scientific, Massachusetts, USA), and stored at -80°C until cut into frozen sections. Eight-micrometer sections were stained with hematoxylin and eosin following a standard procedure [33]. Then, they were scanned with a digital slide scanner (3DHISTCH, Hungary). Morphological changes in islets were evaluated based on the percentage of cell infiltration. Islets that exhibited no insulinitis, few infiltrated mononuclear cells, peri-insulinitis, 50% islet infiltration, and $>50\%$ islet infiltration were scored as 1, 2, 3, 4, and 5, respectively.

Collection of feces, DNA extraction, PCR amplification, and Illumina MiSeq sequencing

Fresh feces were collected from mice at 12 weeks of age and immediately stored at -80°C until used for the extraction of fecal microbial genomic DNA with the Fast DNA Spin Kit for Soil (MP Biomedicals, cat. # 6560-200, California, USA) following the manufacturer's instructions. In detail, 50 mg of frozen stool was added to a Lysing Matrix A tube, and then 1.0 mL CLS-TC was added to the sample tube. The mixture was homogenized in the FastPrep Instrument for 40 s at a speed setting of 6.0 and then centrifuged at $14,000 \times g$ for 5–10 min to pellet debris. Next, the supernatant was transferred to a 2.0-mL microcentrifuge tube, an equal volume of Binding Matrix was added, and the samples were mixed and incubated with gentle agitation for 5 min at room temperature on a rotator. Then, the suspension was transferred to a SPIN™ filter, filtered and centrifuged ($14,000 \times g$, 1 min) twice. Subsequently, the pellet was resuspended gently with 500 μL prepared SEWS-M and centrifuged ($14,000 \times g$, 1 min). The contents of the Catch Tube were discarded, and the Catch Tube was replaced and centrifuged ($14,000 \times g$, 1 min) without any addition of liquid. The collection tubes were replaced with new, clean tubes, the binding matrix above the SPIN™ filter was resuspended in 100 μL DES to elute DNA, and the samples were centrifuged at $14,000 \times g$ for 1 min to collect the eluted DNA into

the clean Catch Tube after the tubes were incubated at 55°C for 5 min. The DNA was used for downstream applications and stored at -80°C until use. The V3 and V4 regions of 16S rRNA were PCR-amplified using specific primers. The reaction conditions were as follows: 95°C for 5 min; 95°C for 30 s, 64°C for 30 s, 72°C for 30 s for 40 cycles, and a final incubation at 72°C for 10 min. The PCR products were excised from a 1.5% agarose gel, purified by Gene Clean Turbo (MP Biomedicals, cat. # 111102400) and quantified by the Quant-iTPicoGreen dsDNA Assay Kit (Life Technologies, cat. # P7589) following the manufacturer's instructions. Libraries were prepared using the TruSeq DNA LT Sample Preparation Kit (Illumina, cat. # FC-121-2001, San Diego, USA) and sequenced for 500 + 7 cycles on the Illumina MiSeq platform using the MiSeq Reagent Kit (500 cycles-PE, cat. # MS-102-2003) [30].

Statistics

The data are presented as the mean \pm standard error of the mean (SEM) and were analyzed using GraphPad Prism 7 software (San Diego, CA, USA). Two-tailed Student's *t*-test was used for two independent groups. A *P*-value ≤ 0.05 was considered statistically significant. Principal component analysis (PCoA) figure was generated and analyzed by the Vegan 2.0 package (2.5.4) and ade4 package (1.7.13). Significantly different clusters of bacterial communities (PCoA) were explained by ANOSIM. Cumulative diabetes incidence was calculated using Kaplan–Meier estimation. Statistical significance was evaluated by the log-rank test.

RESULTS

Early-life fingolimod treatment attenuates intestinal microbial dysbiosis in diabetes-prone NOD mice

The NOD mouse model is a classical model that recapitulates the hallmarks of T1D in patients [21]. Gut microbial dysbiosis contributes to the disease pathogenesis of T1D [9, 11, 34]. 16S rRNA gene sequencing was used to determine how fingolimod modulates the gut microbiome in the setting of T1D. As shown in Fig. 1a, PCoA

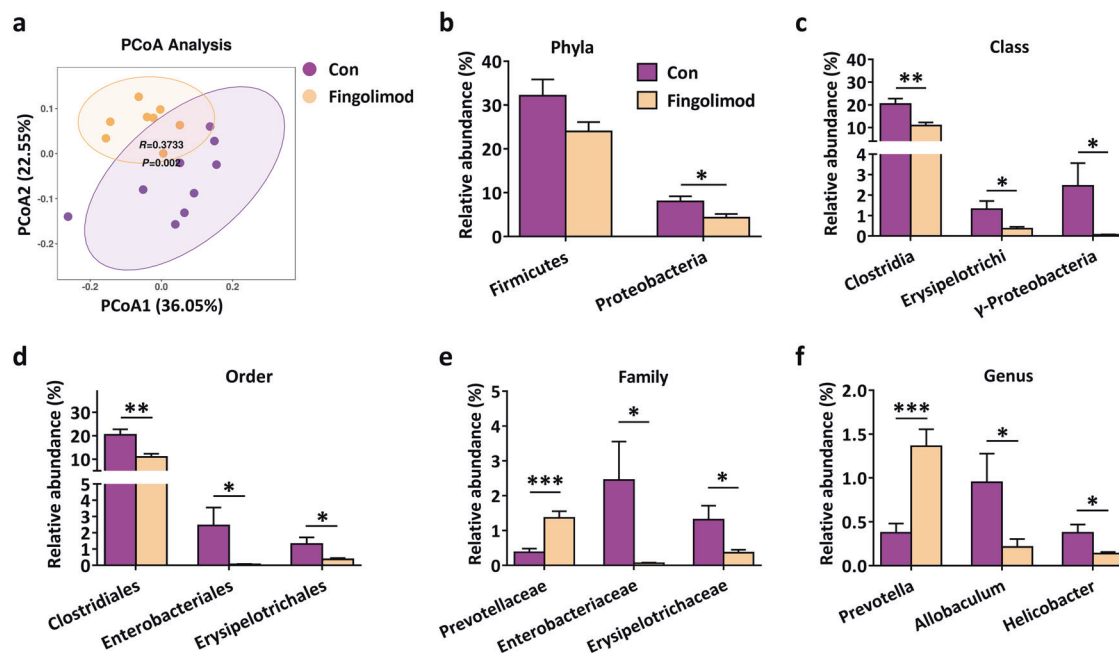


Fig. 1 Early-life fingolimod treatment attenuates intestinal microbial dysbiosis in diabetes-prone NOD mice. Mice were treated daily with 1 mg/kg fingolimod in 100 μL sterile water or an equal volume of sterile water by gavage from weaning. Fresh feces were collected when the mice were 12 weeks of age. (a) Principal coordinate analysis (PCoA) plot of weighted UniFrac distances, with each dot representing a colonic community; the percentage of variation explained by each principal coordinate is shown in parentheses. The relative abundances of the main altered phyla (b), classes (c), orders (d), families (e), and genera (f) in control and fingolimod-treated mice. The data are the mean \pm SEM ($n = 8-9$ mice/group). **P* < 0.05 versus Con, ***P* < 0.01 versus Con, ****P* < 0.001 versus Con.

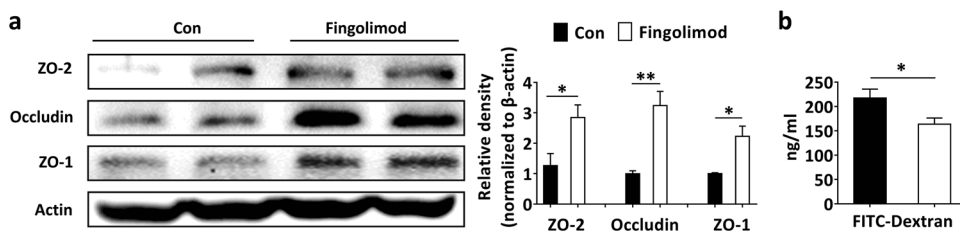


Fig. 2 Early-life fingolimod treatment enhances the intestinal barrier in diabetes-prone NOD mice. (a) The expression of colonic TJPs (ZO-2, Occludin, and ZO-1) was measured by Western blotting, and gray value analysis was performed with ImageJ. Colonic tissue was collected from 12-week-old mice. (b) Assessment of intestinal barrier integrity by oral administration of FITC-dextran. The data shown are the mean \pm SEM ($n = 4$ experiments with two pooled mice in each experiment/group in a; $n = 5$ experiments/group in b). * $P < 0.05$ versus Con, ** $P < 0.01$ versus Con.

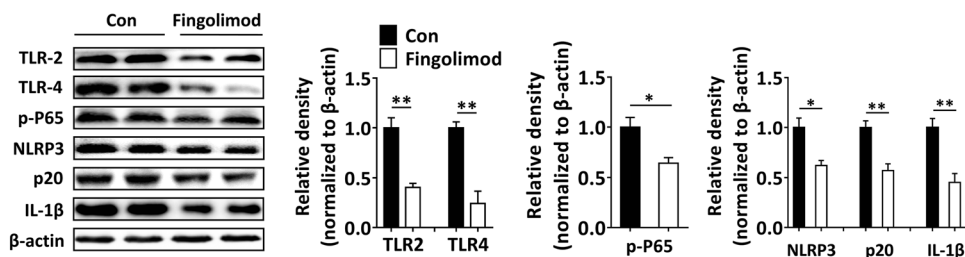


Fig. 3 Early-life fingolimod treatment inhibits the activation of colonic TLR2/4-NF- κ B-NLRP3 in diabetes-prone NOD mice. Mice were treated daily with 1 mg/kg fingolimod in 100 μ L sterile water or an equal volume of sterile water by gavage from weaning. Colonic tissue was collected from 12-week-old mice. Western blot analysis of colonic TLR2, TLR4, p-P65, NLRP3, p20, and IL-1 β expression and gray value analysis by ImageJ. The data are the mean \pm SEM ($n = 4$ experiments with two pooled mice in each experiment/group). * $P < 0.05$ versus Con, ** $P < 0.01$ versus Con.

demonstrated that overall, the gut microbiota was significantly modified by fingolimod (ANOSIM, $r = 0.3733$, $P = 0.002$). Proteobacteria, particularly those belonging to the class γ -Proteobacteria and the family Enterobacteriaceae within this class, are associated with intestinal or systemic inflammation [35, 36]. In the healthy intestine, the abundance of the phylum Proteobacteria is low [35, 37]. In the present study, the abundances of taxonomic groups from the same phylogenetic lineage (Proteobacteria, γ -Proteobacteria, Enterobacteriales, and Enterobacteriaceae) decreased consistently in response to fingolimod treatment (Fig. 1b, c, d, e). Infection with the pathogen *Helicobacter pylori* contributes to T1D development [38, 39]. Our data suggested that fingolimod treatment effectively reduced the abundance of *Helicobacter* (also a pathogen of Proteobacteria) (Fig. 1f). *Allobaculum* is an important functional phylotype in metabolic dysbiosis [36]. In the present study, the abundance of the genus *Allobaculum* (Firmicutes phylum; Erysipelotrichi class; Erysipelotrichales order; Erysipelotrichaceae family) in fingolimod-treated mice was significantly lower than that in NOD controls (Fig. 1b–f). Furthermore, fingolimod treatment significantly increased the abundance of *Prevotella* (a short-chain fatty acid producer that is beneficial in T1D) (Fig. 1f) [37, 40]. Collectively, the data suggest that in diabetes-prone NOD mice, early-life fingolimod treatment attenuates intestinal microbial dysbiosis, specifically reducing the abundances of enteric pathogenic Proteobacteria clusters.

Early-life fingolimod treatment enhances the gut barrier in diabetes-prone NOD mice

Impaired intestinal barrier function occurs before T1D onset, and colonic barrier dysfunction further aggravates T1D [6, 7, 41]. TJPs, including ZO-2, Occludin, and ZO-1, participate in the regulation of intestinal barrier function. In the present study, fingolimod treatment significantly enhanced the colonic barrier in the setting of T1D, evidenced by increased expression of TJPs (Fig. 2a) and decreased passage of FITC-dextran from the gut lumen to the blood (Fig. 2b).

Early-life fingolimod treatment inhibits the activation of the colonic TLR2/4-NF- κ B-NLRP3 pathway in diabetes-prone NOD mice

Activation of the TLR2/4, NF- κ B, and NLRP3 inflammasome pathways are critical for the maintenance of homeostasis against pathogenic infections [42, 43]. TLRs are critically involved in the initiation of innate cellular immune responses and subsequent adaptive immune responses to microbial pathogens that may contribute to T1D development [44]. Commensal bacteria modulate intestinal inflammation, presumably through the TLR2/4 pathway [45, 46]. In the present study, colonic TLR2 and TLR4 expression was significantly inhibited in the fingolimod-treated mice compared with the NOD control mice (Fig. 3). In addition, the NLRP3 (the downstream signaling protein of TLRs) inflammasome is required for the secretion of IL-1 β and IFN- γ and the differentiation of IFN- γ ⁺ T cells [47, 48]. IL-1 β induces beta-cell apoptosis under the control of the transcription factor NF- κ B, subsequently contributing to insulinitis lesions in T1D [48]. Furthermore, the inhibition of NLRP3 not only suppresses the activation of T cells and the differentiation of IFN- γ ⁺ Th1 cells but also impairs the migration of diabetogenic T cells to the pancreas [49]. In the present study, early-life fingolimod treatment inhibited the activation of NF- κ B (downstream of TLR2/4) and the downstream NLRP3/p20/IL-1 β pathway in diabetes-prone NOD mice (Fig. 3).

Early-life fingolimod treatment attenuates intestinal immune dysfunction in diabetes-prone NOD mice

In diabetes-prone NOD mice, macrophages play a major role in protecting against translocation of microbes from the gut, and the number of macrophages increases rapidly 1–2 weeks after weaning [5]. In the present study, early-life fingolimod treatment significantly inhibited the recruitment of macrophages (Fig. 4a). Furthermore, fingolimod effectively reduced CD69 expression on CD4⁺ cells in the MLNs (Fig. 4b). We then detected T1D-associated T-cells in the MLNs and found that fingolimod significantly

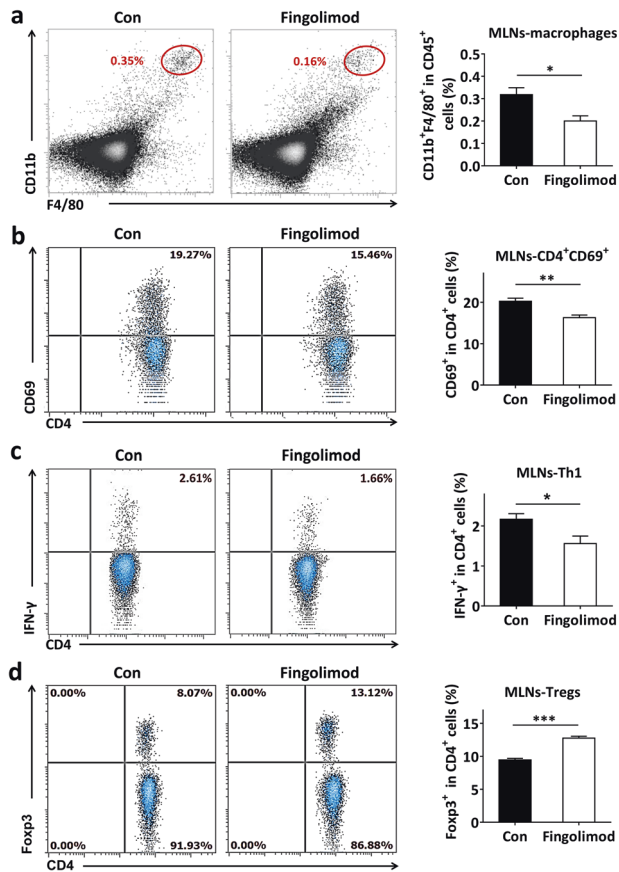


Fig. 4 Early-life fingolimod treatment attenuates intestinal immune dysfunction in diabetes-prone NOD mice. Mice were treated daily with 1 mg/kg fingolimod in 100 μ L sterile water or an equal volume of sterile water by gavage from weaning. The MLNs were collected from 12-week-old mice. The frequency of stained cells was detected by flow cytometry. (a) The frequency of $CD45^+ F4/80^+ CD11b^+$ cells among $CD45^+$ cells (macrophages) in the MLNs. (b) The frequency of $CD69^+ CD4^+$ T-cells among $CD4^+$ cells in the MLNs. (c) The frequency of $CD4^+ IFN-\gamma^+$ cells among $CD4^+$ cells (Th1 cells) in the MLNs. (d) The frequency of $CD4^+ Foxp3^+$ among $CD4^+$ cells (Tregs) in the MLNs. The data are the mean \pm SEM ($n = 5-6$ experiments with two pooled mice in each experiment/group). * $P < 0.05$ versus Con, ** $P < 0.01$ versus Con, *** $P < 0.001$ versus Con.

reduced the frequency of pathogenic Th1 cells (Fig. 4c) and enhanced the frequency of protective Tregs (Fig. 4d). Collectively, these results suggest that early-life fingolimod treatment attenuates intestinal immune dysfunction in the setting of T1D.

Early-life fingolimod treatment inhibits the migration of peritoneal $CD45^+$ leukocytes and $CD4^+$ cells to the pancreas in diabetes-prone NOD mice

The recruitment of autoreactive T cells to islets is a critical step in beta-cell destruction during the pathophysiological process of T1D [50]. To track the migration of immune cells adoptively transferred into NOD mice, we transferred fluorescently labeled $CPD450^+$ splenocytes to recipient mice by intraperitoneal injection. Twenty hours later, the pancreas, PaLNs, blood, MLNs, and spleen were harvested from the recipient mice and examined by flow cytometry. The donor cells did not proliferate within this short period of time after transfer. We observed fewer $CPD450^+$ cells (donor cells) in the pancreas (Fig. 5a), PaLNs (Fig. 5b), and blood (Fig. 5c) of fingolimod-treated mice than in those of NOD control mice. However, fingolimod treatment did not affect the number of $CPD450^+$ cells (donor cells) in the MLNs or spleen (Fig. 5d, e), suggesting that the impacts of fingolimod on the pancreas and its

draining lymph nodes may be specific to some extent. More importantly, significantly lower densities of $CPD450^+ CD45^+$ leukocytes (Fig. 5f) and $CPD450^+ CD4^+$ cells (Fig. 5g) were found in the PaLNs of fingolimod-treated mice than in those of NOD control mice. These data demonstrate that fingolimod may exert its immunosuppressive activity at least in part by inhibiting the migration of intraperitoneal $CD45^+$ leukocytes, as well as $CD4^+$ cells to the pancreas and its draining lymph nodes.

Early-life fingolimod treatment contributes to the maintenance of pancreatic immune tolerance in diabetes-prone NOD mice
Impairment of the gut barrier leads to bacterial leakage into the PaLNs, which enhances the activation of autoreactive T-cells and promotes the differentiation of Th1 cells in the PaLNs [5, 6]. In the pancreas, fingolimod treatment significantly suppressed the recruitment of macrophages (Fig. 6a) and inhibited the activation of $CD4^+$ cells (evidenced by reduced expression of the activation marker CD69) (Fig. 6b). T1D-associated T cells were detected in the pancreas to verify the effects of fingolimod-induced attenuation of intestinal dysfunction on pancreas-directed autoimmunity. The results showed that fingolimod treatment effectively inhibited the recruitment of pathogenic Th1 cells (Fig. 6c) and enhanced the frequency of protective Tregs (Fig. 6d). These results demonstrate that early-life fingolimod treatment contributes to the maintenance of pancreatic immune tolerance in the setting of T1D.

Early-life fingolimod treatment prevents T1D in diabetes-prone NOD mice

The effect of fingolimod on T1D development was studied by evaluating the time of onset and the incidence of diabetes in NOD mice. As shown in Fig. 7a, early-life fingolimod treatment delayed the onset of T1D and reduced the incidence of T1D. Compared with no treatment, early-life fingolimod treatment resulted in a delay in T1D onset (Fig. 7a). At 31 weeks of age, 41.67% (5/12) of NOD control mice were diabetes-free, whereas 75% (9/12) of fingolimod-treated mice were diabetes-free (Fig. 7a). In addition, the average insulinitis score of fingolimod-treated mice was significantly lower than that of NOD controls (Fig. 7b-c and Fig. S16).

DISCUSSION

In the present study, early-life fingolimod treatment attenuated intestinal microbial dysbiosis and intestinal immune dysfunction, subsequently contributing to pancreatic immune tolerance in NOD mice, a model of T1D. This protective effect may be due to enhancement of the gut barrier and inhibition of the activation of the colonic TLR2/4-NF- κ B-NLRP3 pathway by early-life fingolimod treatment, which may be attributed to reductions in the abundances of pathogenic Proteobacteria clusters. This study demonstrates that early-life fingolimod treatment may affect diabetes progression via modulation of gut microbiota composition and provides us with alternative treatment options for T1D patients.

T1D is an autoimmune disease, and the gut microbiota and gut immune system appear to contribute to its pathogenesis. Expansion of $CD4^+ IFN-\gamma^+$ Th1 cells or a decrease in the number or function of Tregs leads to beta-cell destruction and T1D [13-15]. Intriguingly, increased expression of IFN- γ , reduced expression of Foxp3 (a transcription factor of Tregs), and reduced or impaired Tregs are found in the intestine and its draining lymph nodes in addition to the pancreas in T1D patients [51-53]. Previous studies have shown that relieving intestinal inflammation or improving the intestinal microbiota may contribute to the treatment of T1D [7, 54]. Fingolimod has beneficial effects on both T1D and colitis. In addition, in patients with multiple sclerosis, oral administration of fingolimod is effective and more commonly used than intravenous injection of fingolimod, suggesting that restoring

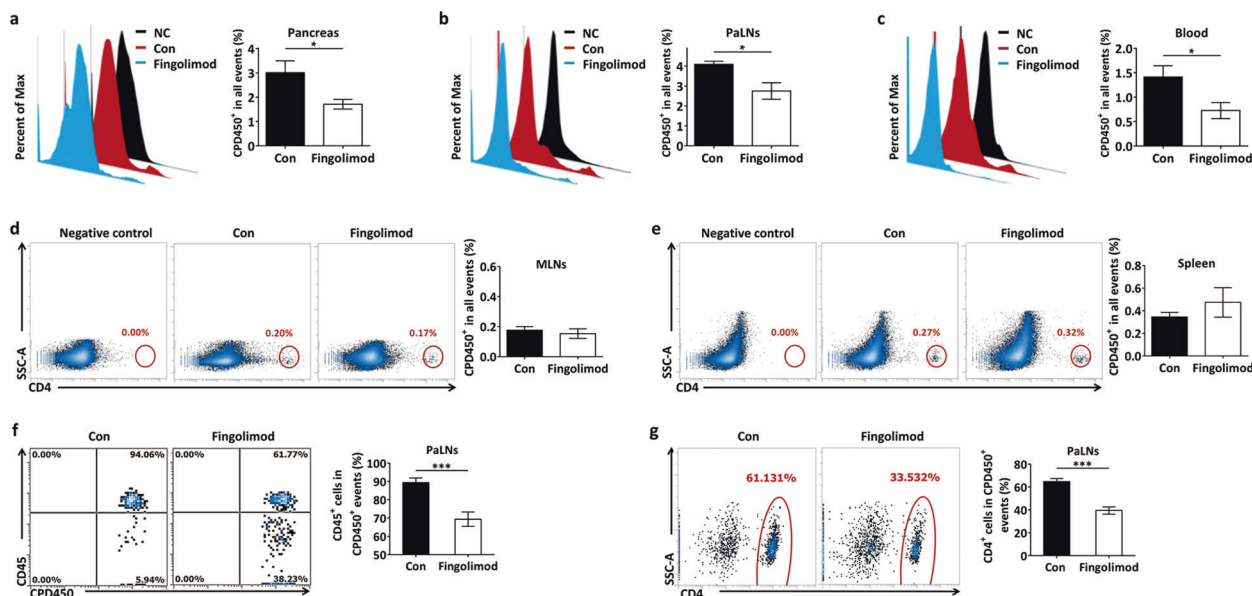


Fig. 5 Early-life fingolimod treatment inhibits the migration of peritoneal CD45⁺ leukocytes and CD4⁺ cells to the pancreas in diabetes-prone NOD mice. Recipient mice were treated daily with 1 mg/kg fingolimod in 100 μ L sterile water or an equal volume of sterile water by gavage from weaning to 12 weeks of age. CPD450-labeled cells were then injected into the peritoneal cavity of the recipient mice. The recipients were killed 20 h after donor cell injection. The pancreas, PaLNs, blood, MLNs, and spleen were harvested and subjected to flow cytometry analysis. (a) The frequency of CPD450⁺ cells among total pancreatic cells. (b) The frequency of CPD450⁺ cells among total PaLNs cells. (c) The frequency of CPD450⁺ cells among total blood cells. (d) The frequency of CPD450⁺ cells among total MLNs cells. (e) The frequency of CPD450⁺ cells among total spleen cells. (f) The frequency of CD45⁺ cells among total CPD450⁺ cells in the PaLNs. (g) The number of CD4⁺ cells among total CPD450⁺ cells in the PaLNs. The data are the mean \pm SEM ($n = 5$ mice per group/group). * $P < 0.05$ versus Con, *** $P < 0.001$ versus Con.

intestinal homeostasis may be partly responsible for fingolimod's therapeutic effects. Furthermore, fingolimod ameliorates colitis in numerous preclinical models, including oxazolone [55] /2,4,6-trinitrobenzene sulfonic acid/dextran sulfate sodium [56]/colitogenic T-cell transfer-induced colitis model, models of memory T-cell-mediated autoimmune inflammatory bowel diseases [57], Th1-cell-mediated colitis model [17] and interleukin-10 gene-deficiency-mediated colitis model [18]. Moreover, fingolimod treatment bolsters the integrity of the blood–brain barrier and enhances the expression of TJPs in diabetic retinopathy and a variety of barrier-related diseases [19, 20, 58]. Based on the present study in conjunction with these previous studies, the inhibitory effect of fingolimod on anti-islet cell autoimmunity may also depend on its modulation of intestinal homeostasis during early life to some extent.

Normally, the intestinal epithelium provides a protective barrier that prevents both pathogenic and commensal bacteria from escaping from the intestinal lumen and activating the systemic immune system. Infection with enteric bacterial pathogens results in a loss of gut barrier integrity [59]. Macrophages play a significant role in guarding parenteral tissue against microbial threats from the gut [5]. Compared to healthy control mice, T1D mice display an altered gut microbiota, increased intestinal inflammation, and increased intestinal permeability [5–7], as well as increased translocation of intestinal bacteria to the PaLNs [5–7]. Subsequently, increased gut bacterial leakage leads to an increase in the number of macrophages in the peritoneal cavity [5–7] and promotes the activation of CD4⁺ cells (evidenced by increased expression of CD69) and excessive differentiation of pathogenic Th1 cells in both the intestine and pancreas, which may contribute to the pathogenesis of T1D [5, 6, 10, 59]. These studies reveal that changes in the intestinal environment may initiate an innate immune response extraintestinally in the setting of T1D. Fingolimod, as a sphingosine-1-phosphate receptor agonist, prevents the loss of TJPs [1, 19, 20] and inhibits the proliferation

of activated lymphocytes and the expression of CD69 on T-cells [60–62]. Furthermore, fingolimod increases the anti-inflammatory properties of macrophages, thus enhancing the immune suppression function of Tregs and maintaining the gut tolerogenic milieu [63]. Based on the present study in conjunction with these previous studies, fingolimod may enhance the gut barrier, thus reducing the recruitment of macrophages and inhibiting the activation of CD4⁺ cells and the proliferation of pathogenic Th1 cells.

Continuous TLR2 and TLR4 signaling occurs in the microenvironment of the large intestine, where commensal bacteria are extremely abundant [46]. TLR2 and TLR4 are located on gut epithelial cells, as well as on cells of the immune system (especially macrophages and dendritic cells in the lamina propria) [64–66]. Compared to those in the healthy colon, the expression levels of TLR2 and TLR4 are increased in intestinal macrophages in inflamed mucosa [67, 68]. In both diabetes-prone NOD mice and T1D patients, the TLR (particularly TLR2/4)-NF- κ B-NLRP3/IL-1 β pathway is critically involved in the initiation of host cellular immune responses against enteric pathogens (especially Enterobacteriaceae) and the development of T1D [42, 44, 47, 69]. Interestingly, several studies have demonstrated that fingolimod effectively inhibits the TLR2/4-NF- κ B signaling pathway [70] and blocks the activation of NLRP3 and the production of IL-1 β [71, 72]. In infection with Enterobacteriaceae (a cluster of enteric pathogens, such as *Escherichia coli*, *Citrobacter rodentium*, *Salmonella enterica ssp.*, *Enterica serovar Typhimurium*, and *Salmonella typhimurium*), the activation of mammalian TLR2/4 on the cell surface and within endosomes triggers NF- κ B activation and is critical for the activation of their downstream noncanonical NLRP3 inflammasome pathway in macrophages [42, 69]. Furthermore, the TLR2-NLRP3 pathway is critical to specific immune regulation in infection with the bacterial pathogen *Helicobacter pylori* [73, 74]. In the setting of T1D, NLRP3 deficiency not only reduces the number of macrophages and suppresses T-cell activation and

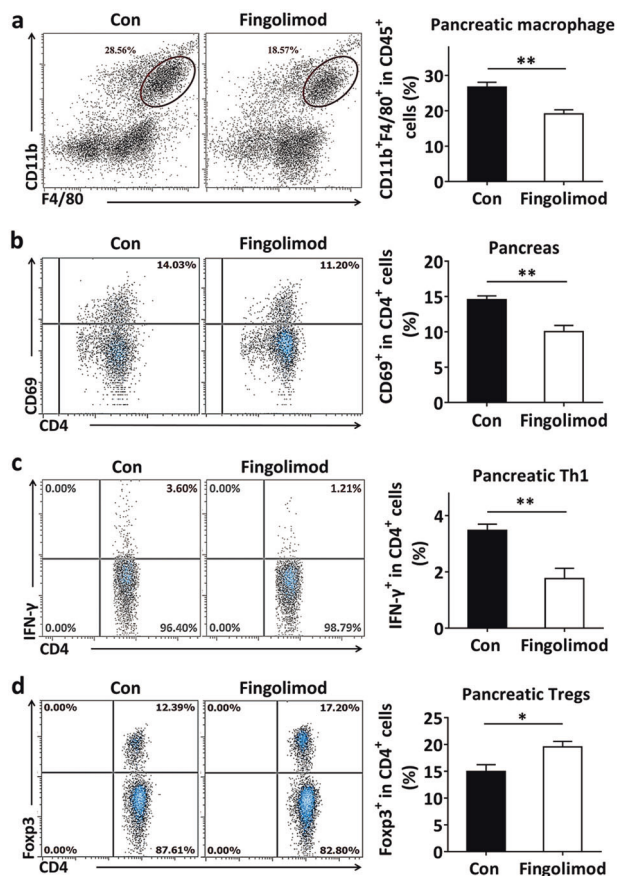


Fig. 6 Early-life fingolimid treatment contributes to pancreatic immune tolerance in diabetes-prone NOD mice. Mice were treated daily with 1 mg/kg fingolimid in 100 μ L sterile water or an equal volume of sterile water by gavage from weaning. The pancreas was collected from 12-week-old mice. The number of stained cells was detected by flow cytometry. (a) The frequency of CD45⁺F4/80⁺CD11b⁺ cells among CD45⁺ cells (macrophages) in the pancreas. (b) The frequency of CD69⁺CD4⁺ T-cells among CD4⁺ cells in the pancreas. (c) The frequency of CD4⁺IFN- γ ⁺ cells among pancreatic CD4⁺ cells (Th1 cells). (d) The frequency of CD4⁺Foxp3⁺ cells among pancreatic CD4⁺ cells (Tregs). The data are the mean \pm SEM ($n = 5-6$ experiments with two pooled mice in each experiment/group). * $P < 0.05$ versus Con, ** $P < 0.01$ versus Con.

Th1-cell differentiation but also impairs the migration of pathogenic T-cells to islets [49], which are thought to be beneficial in reducing the risk of T1D. Based on the present study in conjunction with these previous reports, fingolimid may reduce the abundances of enteric pathogenic Proteobacteria clusters (especially Enterobacteriaceae), subsequently inhibiting the activation of the colonic TLR2/4-NF- κ B-NLRP3 pathway and the development of T1D.

Multiple immunosuppressive drugs cause islet dysfunction, limiting their use for T1D therapy [75–77]. The conservation of the insulin secretory function of islets after high-dose fingolimid treatment indicates that this drug is not overtly toxic to islets [75–77], demonstrating that fingolimid can serve as a potential therapeutic for T1D. Nevertheless, there are still many problems associated with the use of fingolimid, such as the high-dose required, its apparent side effects on the entire body, and the low concentration of the drug in target organs [1, 78], suggesting that the development of new therapeutic strategies based on fingolimid should be pursued further. In recent years, many efforts have been made to address these issues. The graphene-fingolimid complex has a drug-releasing effect [79]. Interestingly, compared with oral fingolimid application, local application of

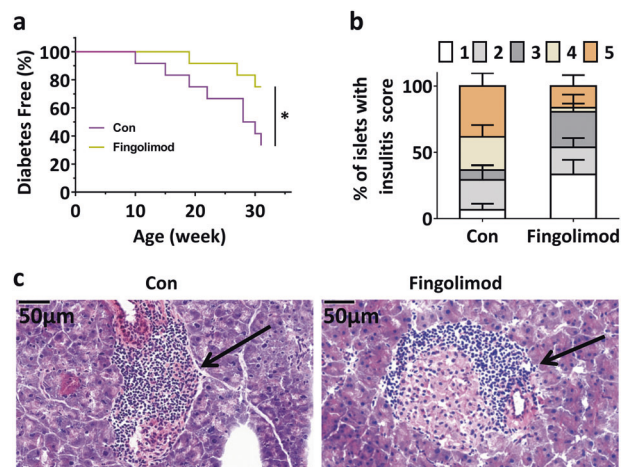


Fig. 7 Early-life fingolimid treatment prevents T1D in diabetes-prone NOD mice. To evaluate the effect of fingolimid on the onset of T1D, NOD mice were randomly assigned to two groups and received fingolimid or an equal volume of sterile water by gavage from weaning to 31 weeks of age, and glucose levels were monitored. (a) Delayed onset and reduced incidence of diabetes in fingolimid-treated mice. Mice were diagnosed with diabetes when blood glucose levels exceeded 11.1 mmol/L on 2 consecutive days. (b) Insulinitis scores for NOD controls and fingolimid-treated mice. Percentage of islets with a given score in 12-week-old NOD controls and fingolimid-treated mice (1: no infiltration; 2: few mononuclear cells infiltrated; 3: peri-insulinitis; 4: <50% islet infiltration; 5: >50% islet infiltration). (c) Histological examination of pancreatic islet infiltration by immune cells in female NOD mice at 12 weeks of age. The data are the mean \pm SEM ($n = 12$ mice/group in a; $n = 8$ mice/group in b, c). * $P < 0.05$ versus Con.

the graphene-fingolimid releasing system can alleviate the harmful effects of fingolimid on the systemic immune system and achieve better blood glucose control [79]. Furthermore, either delayed or abrogated efficacy is observed when >0.5% w/w fingolimid is packaged in a fingolimid-three-dimensional polydimethylsiloxane scaffold (which modulates local delivery of fingolimid in a sustained-release manner), despite the fact that drug release rates are estimated to be ~80-fold less than published systemic delivery reports where no detrimental effects were noted [80]. Moreover, a preconditioning implant scheme with fingolimid nanofibers effectively prevents exposure of islets to harmful concentrations of fingolimid while simultaneously preserving the therapeutic effects of fingolimid on diabetes [81]. Previous studies and our data suggest that the selection of an appropriate administration method and dose can mitigate the adverse effects of fingolimid and allow better blood glucose control, suggesting the need for further basic medical studies and prospective randomized clinical trials to determine the proper dose and administration route of fingolimid for T1D therapy and bringing a new understanding to the field of the potential for fingolimid as a therapeutic for T1D.

Furthermore, multiple immunoregulatory drugs, including fingolimid, are most effective when applied very early in the clinical course of multiple sclerosis, an autoimmune disease [82]. In the present study, our data indicated that oral administration of fingolimid from early life has beneficial effects in the NOD mouse model of T1D, suggesting that it may represent an effective new therapeutic strategy for T1D. This is consistent with previous reports that fingolimid can prevent T1D if administered before or during stimulation and expansion of autoreactive T cells or in the early stages of insulinitis [3, 4]. The effectiveness of fingolimid is diminished in each successive stage of diabetes [3, 4]. Thus, it is possible that an early window of therapeutic opportunity may exist in which the biology of the disease can be modified for a

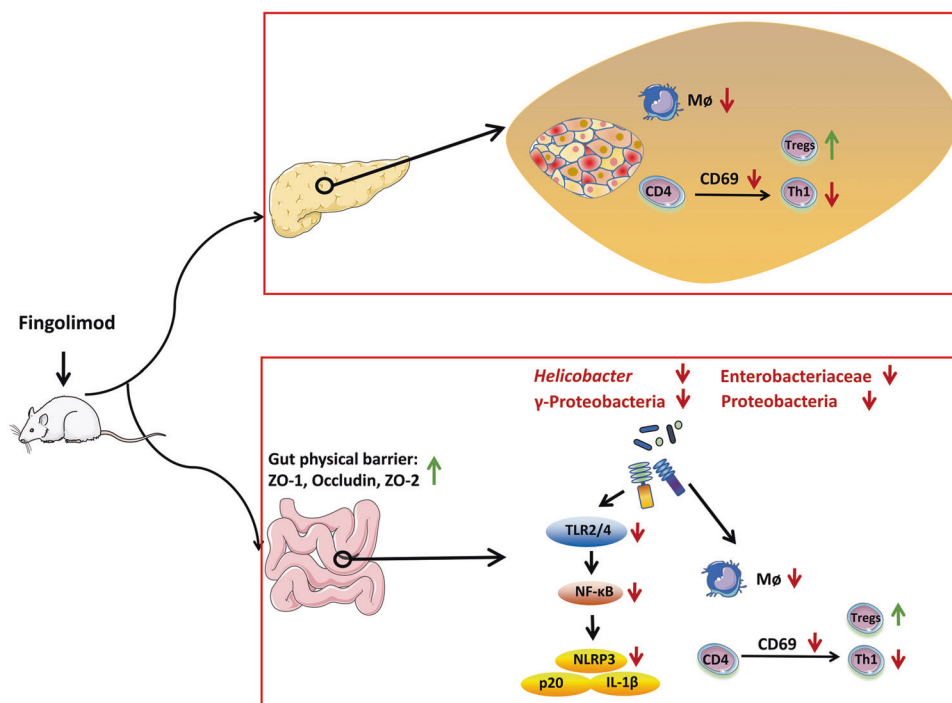


Fig. 8 Graphical abstract. In diabetes-prone NOD mice, early-life fingolimod treatment enhances the gut barrier and attenuates intestinal microbial dysbiosis and intestinal immune dysfunction, thus suppressing CD4⁺ T-cell activation and Th1-cell differentiation in the pancreas and promoting the expansion of Tregs. This protective effect of fingolimod may be due to its inhibition of the activation of the colonic TLR2/4-NF-κB-NLRP3 pathway to some extent, which may be mediated by decreases in pathogenic Proteobacteria clusters.

long-term benefit. Furthermore, the number of peritoneal macrophages increases rapidly 1–2 weeks after weaning in NOD mice [5], suggesting that the gut barrier changes around weaning (3-weeks-old) in the setting of T1D. During early life, the intestinal environment undergoes significant developmental changes, and until the entire ecosystem is established, minor intestinal modifications in this critical stage can have significant consequences for intestinal and systemic immune homeostasis [12, 83, 84]. Therefore, effective fingolimod intervention may have to begin in early life. It is also possible that fingolimod intervention in early life is sufficient to protect against T1D.

Collectively, early-life fingolimod treatment maintains intestinal homeostasis (attenuates intestinal microbial dysbiosis and intestinal immune dysfunction) and enhances the gut barrier, thus suppressing the activation of CD4⁺ cells and the differentiation of Th1 cells by inhibiting the activation of the colonic TLR2/4-NF-κB-NLRP3 signaling pathway (Fig. 8). These results indicate that intestinal microbiota and intestinal immunity may play an essential role in the anti-inflammatory effect of fingolimod in the setting of T1D, providing novel insights into the antidiabetes mechanisms of fingolimod and complementing the conclusions of previous studies, which deserve clinical investigation in the future.

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AUTHOR CONTRIBUTIONS

LLP, JS, and LLJ designed the study. LLJ, MZ, and HL conducted the studies. LLJ, MZ, and HL analyzed the data. LLJ wrote the paper. All authors read and approved the final paper.

ADDITIONAL INFORMATION

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