Edible exosome-like nanoparticles from Portulaca oleracea L prevent DSS-induced colitis via the promotion of double-positive CD4 + CD8 + T cells

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Abstract

Plant-derived exosome-like nanoparticles (PDENs) have been paid great attention in the treatment of ulcerative colitis (UC). As a proof of concept, we show that Portulaca oleracea L-derived exosome-like nanoparticles (PELNs) are isolated and identified from edible Portulaca oleracea L with desirable nano-size (~ 160 nm) and a negative zeta potential value (-31.4mV). Moreover, we find that oral administration of PELNs can suppress the expressions of pro-inflammation cytokines (TNF-α, IL-6, IL-12, and IL-1β), and increase the levels of anti-inflammatory cytokine (IL-10) in dextran sulphate sodium (DSS)-induced mice and PHLPP2−/− mice spontaneous colitis contributing to alleviate colitis. Furthermore, orally administered PELNs can display great stability in gastrointestinal tract environment, and specifically targeted to desired inflammatory site in mice colitis. In addition, oral administration of PELNs can effectively maintain the diversity and balance of gut microbiota, and specifically induce the differentiation of double-positive CD4+CD8+ T cells (DP CD4+CD8+ T cells). In summary, our findings suggest that orally administered PELNs brings a novel and natural colon-targeted agent, and open a new therapeutic avenue in the management of UC.

Introduction

Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease with persistent damage of colonic epithelial mucosa, which characterizes by a relapsing and remitting course\(^1\). Its clinical manifestations are abdominal pain, diarrhea, blood in the stool, and weight loss\(^2\). Moreover, UC has been recognized as a global disease with a steady upward trend worldwide, leading to a huge economic burden on healthcare\(^3\). In addition, patients with long-standing UC tend to develop colorectal cancer (CRC)\(^4\). Unfortunately, the pathogenesis and exact etiology of UC remain obscure so far. However, current research suggests that the pathogenesis of UC may be involved in genetic, immunological, and environmental factors\(^5\)–\(^8\). The IBD armamentarium mainly depends on non-targeted therapies, such as 5-aminosalicylates, glucocorticoid, and immunosuppressive agents, as well as targeted biologic therapies, such as anti-TNF antibodies (Infliximab (IFX), Adalimumab (ADL), Golimumab (GOLI), and Certolizumab pegol (CZP)), against the p40 subunit of IL-12 and IL-23 (Ustekinumab), and JAK signaling pathway inhibitors (Tofacitinib)\(^7\).

Unfortunately, intolerance or non-response to drugs, adverse events, and toxicity prevent UC patients from getting remission\(^9\). Therefore, it is urgent to develop new medications with preferable therapeutic efficacy and limited side effects.

Recently, edible plant-derived exosome-like nanoparticles (PDENs) are nanosized vesicles derived from edible plants, such as ginger, grape, carrot, and broccoli, and have been identified\(^10\)–\(^13\). Accumulated studies have demonstrated that PDENs can not only contribute in plant cells crosstalk, but also connect inter-kingdom communication due to containing bioactive molecules, such as lipids, protein, and nucleic acid\(^14\),\(^15\). Moreover, PDENs have several advantages of non-toxicity, low immunogenicity, and excellent biocompatibility\(^16\),\(^17\). Therefore, PDENs provide great promise for clinical application in the treatment of several diseases, including UC. For instance, grape-derived exosome-like nanoparticles (GDENs) promoted...
dramatic proliferation of intestinal stem cells, and contributed to an intense acceleration of mucosal epithelium regeneration, consequently prohibiting progression of DSS-induced mouse colitis\(^{11}\). Moreover, oral administration of GDENs can target to intestinal epithelial cells (IECs) and macrophages, which resulted in the survival and proliferation of IECs, and decreased the expressions of the pro-inflammatory cytokines, such as TNF-\(\alpha\), IL-6 and IL-1\(\beta\), and increased the levels of the anti-inflammatory cytokines, including IL-10 and IL-22 in DSS-induce experimental colitis\(^{10}\). In addition, a randomized controlled trial has currently been designed to investigate the effectiveness and safety of GDENs for the patients with UC\(^{18}\). Notably, PDENs will be a promising alternative in the treatment of UC.

Portulaca oleracea L. (purslane, POL) is one of the most commonly used medicinal herb, which has a cosmopolitan distribution and widely used in many countries\(^{19}\). Several studies have revealed that POL exerts a wide range of pharmacological effects, such as anti-inflammatory, antioxidant, immune-regulating, and antitumor activities\(^{20}\). Furthermore, POL can effectively attenuate DSS-induced colitis by prohibiting the oxidative stress response of nitric oxide and superoxide dismutase, and decreasing the levels of pro-inflammatory cytokines, including TNF-\(\alpha\), IL-1\(\beta\), and IL-6\(^{21-23}\). In this study, we, for the first time, isolated and purified the natural exosome-like nanoparticles from POL using ultracentrifugation and sucrose gradient centrifugation. Subsequently, we demonstrated that oral administration of POL-derived exosome-like nanoparticles (PELNs) can specifically target to inflammatory sites in the DSS-induced UC mouse model. Moreover, PELNs can effectively alleviate colitis through suppressing the expression of pro-inflammatory cytokines, and promoting the secretion of anti-inflammatory cytokines. Mechanistically, PELNs can effectively maintain the diversity and balance of gut microbiota, and induce the differentiation of DP CD4\(^+\)CD8\(^+\)T cells.

**Methods**

**Isolation and purification of PELNs from POL**

POL were washed with running water, placed into phosphate-buffered saline (PBS). The POL was cut into small pieces, and homogenized in a blender. Initially, low-speed centrifugation (500-3,000 g for 10–15 min) is used to remove plant fibers and large particles. Then, medium-speed centrifugation (3,000–10,000 g for 20–40 min) is applied for removal of large debris and intact organelles. Finally, high-speed centrifugation (10,000-150,000 g for 1.5-2 h) is performed to yield PELNs pellet. The pellet was resuspended in sterile PBS, and purified on a sucrose gradient (8, 30, 45, and 60% sucrose in 20 mM Tris-Cl PH 7.2) followed by centrifugation at 150,000 g for 2h at 4°C by SW41 rotor (Beckman Coulter, Fullerton, CA, USA). Fractions of PELNs -specific density were then pooled together, and subsequently concentrated via ultracentrifugation (100,000 g for 4 h, 4°C). The band in the 45/60% layer was collected, and washed 3 times. The resulting EV pellet was resuspended in PBS and stored at -80°C until use (Figure S1a).
Physicochemical Characterization

PELNs samples were analyzed by a NanoSight NS300 technology (Malvern Instruments, Malvern, United Kingdom) to calculate diameter size and concentration distribution. Dynamic light scattering (DLS) (Malvern Instruments, Malvern, UK) was employed to determine the zeta potential of PELNs. Cryo-electron micrographs of PELNs samples were collected using a transmission electron microscope Glacios (Thermo Fisher Scientific, USA), equipped with a Ceta camera at accelerating voltage of 200 kV. Grid mapping and image acquisition were performed using EPU (Thermo Fisher Scientific, USA) at a nominal magnification of x8,500. High magnification images were recorded at x73,000 nominal magnification (0.2 nm pixel size) with a -3.0 µm defocus value. Electron dose was controlled below 30e-/Å-2 to minimize radiation damage during image acquisition.

Lipidomic Analysis

Lipid was extracted by Bligh and Dyer methods. PELNs (100 ug) were added into a 10 ml glass tube, then mixture of methanol: dichloromethane (2 ml:0.9 ml v / w) were added and rotated for 30s. After incubated for 30 min at room temperature, then the higher performance liquid chromatography grade water and 0.9 ml dichloromethane were added. The sample was gently inverted for 10 times, and then centrifuged at the speed of 1200 rpm for 10 minutes. The organic lower phase (dichloromethane) was collected, condensed in nitrogen to dry, and then reassembled into methanol: dichloromethane (1:1 v / w) containing 10 mM of ammonium acetate. The extracted lipid was detected by Waters Acquity UPLC tandem Thermo Fisher Q Exactive quadrupole Orbitrap mass spectrometry with ACQUITY UPLC BEH C18 (2.1*100 mm 1.7 um) column. Lipids were separated by ultrahigh performance liquid chromatography with gradient time of 16 minutes, and detected in positive and negative ion mode respectively. The data acquisition modes of mass spectrometry were used as follows: first-stage full scan (Full Scan, m _ max z 200,1200) and data-dependent secondary mass spectrometry scanning (dd-MS2, TopN = 10). Then the data were extracted by Thermo Scientific LipidSearch software, qualitatively through the lipid search lipid database, and integral to get the peak area, representing the relative content of lipids.

In vitro stability of PELNs

A stomach-like solution was composed of 18.5% (w/v) HCl (pH 2.0) and pepsin solution (80 mg/ml in 0.1 N of HCl, pH 2.0, Sigma) and 4 mg/ml of pancreatin (Sigma), which added to 1 ml (1 mg/ml) of PELNs in PBS with 37°C for 4 h. The pH value of the stomach-like solution was adjusted to pH ~ 6.5 with 1N NaHCO₃, and referred to as a small-intestine-like solution. PELNs were incubated for additional 60 min in the intestinal solution. The stability of PELNs was evaluated by measuring particle size and surface charge using the method described above.

Animals
C57BL/6 male mice (9 weeks of age, 25-26g) were provided by SPF (Biotechnology Co., Ltd. Beijing, China). We have previously demonstrated that PHLPP2 deficient (PHLPP2^{-/-}) mice developed spontaneous colitis\textsuperscript{25}. And PHLPP2^{-/-} mice were obtained from Beijing View solid Biotech Co., Ltd. (Beijing, China). All the mice were housed in a pathogen-free facility on a 12 h light/dark cycle. All the animal experiments were approved by the Animal Care Committee of the Shenzhen People's Hospital, Shenzhen, China.

In vivo therapeutic outcomes of PELNs against mice colitis

C57BL/6 male mice were randomly divided into 4 groups: the healthy control group, the DSS group, the DSS + 50mg/g PELNs-L, the DSS + 100mg/g PELNs-H. The mice were given 3\% DSS in their drink for continuous 7 days. Mice in the PELNs treatment group were also orally gavaged per mouse for 5 days at day 2. Body weight, feces, and physical activity were monitored daily. A disease severity score was calculated as described previously. On the day 7, mice were euthanized with CO\textsubscript{2} inhalation. Blood sample were collected from the orbits of mice for ELISA. Colon tissues, feces, blood serum and major organs (heart, liver, spleen, lung, and kidney) were harvested for further examinations.

PHLPP2^{-/-} mice were randomly divided into 3 groups: the healthy control group, the DSS group, the oral administration PELNs-H group following the previous results. The therapeutic effect of PELNs for DSS-induced PHLPP2^{-/-} mice colitis was tested using the method described above.

Pelns Labeling

IRDye 800CW near-infrared fluorescent dyes (IRDye® 800CW NHS Ester) were used for PELNs labeling. Briefly, 0.21mM fluorescent IRDye 800CW solution added into 1 mg PELNs (1 ml in PBS), and incubated with 0.2M sodium biocarbonate buffer (pH8.3) buffer for 2 h at room temperature. Then, the labeled PELNs were subject to 100-kDa ultracentrifuge filter to remove the free dye.

Biodistribution Of Orally Administerated Pelns

To investigate the in vivo biodistribution of PELNs, UC mice model were orally administrated with IRDye 800CW-labeled PELNs (100mg/g) to track the distribution of PELNs in gastrointestinal tract (GIT). At different time points after gavage (3, 6, 12, and 24h), mice were sacrificed by CO\textsubscript{2} inhalation and colon tissues were obtained for fluorescence imaging by an IVIS spectrum imaging system (Hopkinton, USA).

16s Rdna Sequencing

Extraction of fecal DNA and analysis of gut microbiota
After sacrifice, the intestinal contents of mice were removed from the colon, frozen with liquid nitrogen immediately and transferred to the −80°C refrigerator for subsequent DNA extraction and microbial analysis. The HiPure Stool DNA Kit was used to extract total fecal DNA (Magen, Guangzhou, China). Primers used to amplify were: 341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT. Amplified products were purified by Phusion High-Fidelity PCR Master Mix (New England Biolabs, Beverly, USA). Sequencing libraries were generated by the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, USA). The quality of the constructed library was evaluated using the Agilent Bioanalyzer 2100 system and Qubit® 2.0 Fluorometer (Thermo Scientific, Carlsbad, USA), the Illumina HiSeq 2500 platform used for sequencing was supported by Tianjin Novogene Bioinformatics Technology Co., Ltd.

### 16s Rdna Bioinformatics Analyses

Operational taxonomic units (OTUs) analysis

Effective tags of OTUs ≥ 97% were clustered together with using UPARSE software (version 9.2.64). Each cluster was represented by the most abundant tag sequence of each cluster. The number of OTUs, exclusive OTUs, and shared OTUs of each group are displayed in the form of update plot which was performed by R project (version 2.2.1).

Diversity analysis

α-diversity of ACE index, Chao1 index, Simpson index, and Shannon index were calculated in QIIME (version 1.9.1). PD-whole tree index was calculated in picante (version 1.8.2). The Tukey's HSD test was used to evaluate α-indices among three groups and Welch's t test was used between two groups. β-diversity analysis was performed by the VennDiagram package of the R project which used to analyze element distribution, while the Vegan package in the R project was utilized for principal component analysis (PCA), and PCoA (principal coordinates analysis) and NMDS (non-metric multi-dimensional scaling) of Bray-Curtis distances were calculated for multivariate statistical techniques.

Community composition analysis

A naive Bayesian model using RDP classifier (version 2.2) based on SILVA databases was used to classify sequences based on organisms. The community composition was visualized as a stacked bar plot using ggplot2 package (version 2.2.1) in the R project.

Welch's t test

Welch's t test was used to evaluate significant variances in terms of mean abundance between two groups which meet the conditions that total repeat samples in each group ≥ 3 and the number of species tags/total tags of at least one sample ≥ 0.1%.
**Rna Extraction And Qrt-pcr**

The RNA extraction and qRT-PCR were carried out as previously described\(^{25,35}\). RNA was extracted from colon samples using the TRIzol Reagent (Thermo, USA). The quantity of RNA was measured using NanoDrop 2000 (Thermo Scientific, Massachusetts, USA). The reverse transcription program used the PrimeScript RT Master Mix (Takara Biomedical Technology (Beijing, China). The primers were available in Table S1.

**Enzymelinked Immunosorbent Assay (Elisa)**

The levels of IL-6, IL-12, IL-1\(\beta\), TNF-\(\alpha\) and IL-10 in the serum and colonic tissue were quantified using commercial ELISA kits according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd).

**Flow Cytometry (Fcm)**

FCM was used to analysis the population DP CD4\(^+\)CD8\(^+\)T cells in the colon samples. The procedure carried out as previously described\(^{35}\).

**Immunofluorescence (If)**

IF was performed to detect the population DP CD4\(^+\)CD8\(^+\)T cells in the colon samples as previously described\(^{25,35}\).

**Statistical Analysis**

Comparison of multiple experimental groups was carried out by one-way or two-way ANOVA. A t test was calculated to compare the means of two groups. The p values < 0.05*, P < 0.01**, P < 0.001***, P < 0.0001**** were considered to be statistically significant.

**Results**

**Characterization of PELNs**

The PELNs were isolated from POL, and which abundantly accumulated at the 8/30% interface (band 1), and 30/45% interface (bang 2) of the sucrose gradient (Figure S1a). The size distribution of PELNs was characterized as exosome-like nanoparticles, and confirmed by electron microscopic examination (Figure S1b). Moreover, the hydrodynamic particle sizes of PELNs ranged from 30 to 400 nm, with an average size of 180 nm (Figure S1c). In addition, Zeta potential measurements demonstrated that PELNs had a negative zeta potential of -31.4 mV (Figure S1d). The lipidomic analysis showed that PELNs mainly
contained digalactosyldiacylglycerol (DGDG, 24.47%), triglyceride (TG, 17.09%), and phosphatidylcholine (PC 9.28%) (Figure S1e).

To examine the stability of PELNs in the GIT, PELNs were incubated in different aqueous solutions that mimicked a stomach-like solution and a small-intestine-like solution, and then analyzed changes in their zeta potential and size. Their zeta potential had a reduction in negative charge in stomach-like solution, whereas which was weakly positively changed in small-intestine-like solution (Figure S1f). The results showed that the heterogeneity in their size was increased in both a stomach-like and small-intestine-like solution comparing with that incubated in the water for 2 h (Figure S1g). Strikingly, these results indicated that PELNs can maintain integrity, and resist to digestion during their passage through the GIT.

**Oral Administration Of Pelns Protects Mice Against Dss-induced Colitis**

To investigate their anti-inflammatory effects of PELNs, mice were randomly divided into four groups: healthy control group, DSS control group, PELNs-H group, and PELNs-L group abovementioned. Protocol was available in Fig. 1a investigating the potential of PELNs to alleviate UC. The body weight was gradually increased in healthy control group, whereas which was dramatically decreased in DSS control group during the entire experiment (Fig. 1b). Nevertheless, PELNs had the therapeutic potential against weight loss (Fig. 1b). Treatment with PELNs can significantly prevent the body weight loss compared to DSS control group (Fig. 1b). Moreover, PELNs-H seemed to be more effective in the prevention of the body weight loss than PELNs-L (Fig. 1b). Consistently, PELNs exerted an important role in the reduction of disease activity index (DAI) (Fig. 1c). The PELNs groups have significantly lower DAI than that in the DSS control group (Fig. 1c). Furthermore, the DAI in the PELNs group is comparable with that in the healthy control group (Fig. 1c). As expected, PELNs remarkably reduced a reduction of colon shortening (Fig. 1d and 1e). PELNs had significantly longer colon comparing to the DSS control group (Fig. 1d and 1e). Furthermore, the colon length of the PELNs-H group was slightly longer than that in the PELNs-L group, the fecal condition and mucosa of the colon in PELNs-H group was better than that in PELNs-L group, whereas which was appreciably shorter than that in the healthy control group (Fig. 1d and 1e). In addition, hematoxylin-eosin staining (H&E) and histological scores also confirmed that PELNs decreased accumulation of immune cells and damage of colonic epithelial barrier (Fig. 1g and 1f). Interestingly, the histological scores were significant lower in the PELNs-H group than that in the PELNs-L group (Fig. 1g). Therefore, PELNs-H have greater anti-inflammatory therapeutic effect compared with PELNs-L in the treatment of DSS-induced mice colitis.

As known, the levels of pro-inflammatory cytokines play a crucial role in intestinal inflammation. Interestingly, pro-inflammatory cytokines (IL-6, IL-12, IL-1β, and TNF-α) were dramatically elevated in DSS control group compared with the healthy control group in colon samples from C57 mice colitis using qRT-PCR (Fig. 2a, 2b, 2c, 2d). However, treatment of PELNs remarkably inhibited the expression of these pro-inflammatory cytokines (Fig. 2a, 2b, 2c, 2d). On the contrary, IL-10, as an anti-inflammatory, was
considerably decreased in DSS control group compared with the healthy control group, whereas which was sharply increased in the PLENs administered group (Fig. 2e). As expected, an ELISA assay demonstrated that the secretion amount of these pro-inflammatory cytokines exhibited a remarkable increase in the DSS control group compared with the healthy control group in blood samples from C57 mice colitis (Fig. 2f, 2g, 2h, 2i). However, the levels of these pro-inflammatory cytokine (IL-6, IL-12, IL-1β, and TNF-α) secretion were significantly reduced in the PELNs group (Fig. 2f, 2g, 2h, 2i). Accordingly, the secretion level of IL-10 was detected, and revealed that PLENs promoted the secretion profiles of IL-10 (Fig. 2j). Moreover, PELNs-H have more excellent performance in the decrease of pro-inflammatory cytokines and increase of anti-inflammatory cytokines than PELNs-L.

**Oral Administration Of Pelns Protects Phlpp2 Mice Spontaneous Colitis**

To further evaluate the anti-inflammatory effects of PELNs in a chronic colitis model, PHLPP2−/− mice were chosen due to we previously demonstrated that PHLPP2−/− mice spontaneously developed colitis with a time-dependent within 4 weeks post weaning. Subsequently, PHLPP2−/− mice were randomly divided into 3 groups: healthy control group, DSS control group and PELNs-H group. Surprisingly, PELNs-H treated mice failed to develop spontaneous colitis, owing to which can effectively prevent the body weight loss, colon shortening, immune cells infiltration, and histological scores compared with PHLPP2−/− mice not treated PELNs-H (Fig. 3).

Strikingly, qRT-PCR and ELISA results showed that oral administration of PELNs-H effectively suppressed the expressions of pro-inflammatory cytokines (IL-6, IL-12, IL-1β, and TNF-α), in colon tissues and blood samples of PHLPP2−/− mice spontaneous colitis. However, PLENs prominently increased the expression of IL-10 (Figure S2).

**In vivo distribution of PELNs**

The accumulation of oral nanotherapeutics in the desired colon is critical to exert therapeutic effectiveness against UC. To determine the PELNs biodistribution after oral administration, mice with UC were gavaged with IRDye 800CW-labeled PELNs (100 mg/g) for 3, 6, 12 and 24h. Near-infrared imaging revealed that IRDye 800CW-labeled PELNs was visible in the colon at the 3 h, and gradually increased at the 6 h, whereas fluorescent signals detected was steadily decreased in the colon at the 24 h (Fig. 4). However, no conspicuous fluorescence signals were detected in the colon when PELNs administered the healthy mice (Fig. 4). Subsequently, the accumulation of fluorescent signals in the heart, liver, spleen, lung, and kidney was examined, and found that there were very few fluorescent signals detected in these vital organs when PELNs treated mice colitis at 3, 6, 12 and 24h (Fig. 4). Collectively, PELNs can specially target to the inflamed colon in the treatment of mice colitis.

**Pelns Distinctly Altered The Diversity Of The Intestinal Microflora**
The intestinal microbiota profiles were analyzed by 16S rDNA sequencing. The abundance of gut microbiota could be reflected by calculating the number of OTUs and further be observed the richness and evenness through different α-diversity indexes. As shown in Fig. 5a, the OTUs of colitis mice was only 590 and was up-regulated significantly by PELNs. Further analyzed the effect of PELNs on α-diversity through five indicators, including ACE (Fig. 5b), Chao 1 (Fig. 5c), PD-tree (Fig. 5d), Simpson (Fig. 5e) and Shannon (Fig. 5f), and found that DSS induced significant decrease of α-diversity (ACE, P = 0.00002; Chao 1, P = 0.000016; PD-tree, P = 0.000257; and Shannon, P = 0.000015), while PELNs could significantly up-regulate (ACE, P = 0.000775; Chao 1, P = 0.000707; PD-tree, P = 0.001010; Simpson, P = 0.107145; and Shannon, P = 0.003565). β-diversity usually used to judge the difference between groups of gut microbiota, the β-diversities of the intestinal microbiota across both cohorts were assessed using PCA (Fig. 5g), PCoA (Fig. 5h), and NMDS (Fig. 5i), consistent results were obtained with NMDS, PCA and PCoA (PCA1 + PCA2 = 79.44 > 50%, PCoA1 + PCoA2 = 52.40 > 50%, and stress = 0.078 < 0.1,) which showed that PELNs significantly change the microbial structure of colitis mice and approach to the healthy group.

**Pelns Improve Dss Induced Changes In Dominant Microbiota And Mediated Significant Changes In Microbial Structure**

As shown in Fig. 6a, the relative abundance of *Firmicutes* (51.26% vs. 19.36%, P = 0.000141), *Bacteroidetes* (20.70% vs. 16.91%, P = 0.374828), *Patescibacteria* (3.92% vs. 0.41%, P = 0.006606), *Tenericutes* (0.92% vs. 0.10%, P = 0.089697), *Actinobacteria* (0.46% vs. 0.13%, P = 0.071755), *Lachnospiraceae* (24.75% vs. 5.53%, P = 0.000056), *Ruminococcaceae* (10.81% vs. 3.18%, P = 0.001079), *Muribaculaceae* (12.08% vs. 5.09%, P = 0.008093), *Lactobacillaceae* (8.23% vs. 1.81%, P = 0.002318), *Moraxellaceae* (14.34% vs. 8.82%, P = 0.502718), *Erysipelotrichaceae* (3.64% vs. 1.32%, P = 0.004203), *Lachnospiraceae_NK4A136_group* (15.23% vs. 2.53%, P = 0.000263), *Lactobacillus* (8.22% vs. 1.81%, P = 0.002318), *Acinetobacter* (14.34% vs. 8.82%, P = 0.502718), *Ruminococcaceae_UCG-014* (4.30% vs. 0.51%, P = 0.001301), *Candidatus_Saccharimonas* (3.92% vs. 0.41%, P = 0.006644), *Alistipes* (2.61% vs. 2.02%, P = 0.514264), *Acinetobacter_calcoaceticus* (14.11% vs. 8.43%, P = 0.484667), *Lactobacillus_gasseri* (3.95% vs. 0.40%, P = 0.027309), *Lactobacillus_murinus* (2.80% vs. 1.30%, P = 0.177553), *Lactobacillus_reuteri* (1.12% vs. 0.03%, P = 0.000201), *Bacteroides_caecimuris* (1.82% vs. 1.20%, P = 0.531045), *Helicobacter_sp_MIT_03-1616* (0.20% vs. 0.18%, P = 0.759178), were notably elevated in mice treated with PELNs in comparison to colitis group, while that of *Proteobacteria* (18.79% vs. 49.81%, P = 0.004250), *Deferribacteres* (2.28% vs. 9.67%, P = 0.010716), *Epsilonbacteraeota* (0.76% vs. 2.15%, P = 0.188453), *Verrucomicrobia* (0.10% vs. 0.42%, P = 0.221802), *Cyanobacteria* (0.05% vs. 0.09%, P = 0.253980), *Enterobacteriaceae* (1.37% vs. 39.56%, P < 0.0001), *Deferribacteraceae* (2.28% vs. 9.67%, P = 0.010716), *Bacteroidaceae* (2.31% vs. 7.14%, P = 0.070016), *Rikenellaceae* (3.19% vs. 3.83%, P = 0.631456), *Escherichia-Shigella* (1.27% vs. 39.13%, P < 0.0001), *Mucispirillum* (2.28% vs. 9.67%, P = 0.010716), *Bacteroides* (2.31% vs. 7.14%, P = 0.070016), *Staphylococcus* (0.17% vs. 3.67%, P = 0.164005), *Bacteroides_thetaiotaomicron* (0.31% vs. 5.87%, P = 0.029962), *Staphylococcus_sciuri* (0.12%
vs. 3.61%, P = 0.162046), *Mucispirillum_sp_69* (0.75% vs. 3.15%, P = 0.011562), *Enterococcus_faecalis* (0.13% vs. 2.17%, P = 0.037956) were decreased.

Welch's t test was used to analyze the microbial changes induced by PELNs. There was a higher abundance of the phyla (Fig. 6b) *Firmicutes* and *Patescibacteria*, while decreased abundance of *Proteobacteria* and *Deferribacteres*. The relative proportion of the family (Fig. 6c) *Lachnospiraceae*, *Ruminococcaceae*, *Muribaculaceae*, *Lactobacillaceae*, *Saccharimonadaceae*, *Erysipelotrichaceae*, *Erygerthellaceae*, *Enterococcaceae*, and *Bacillaceae* increased remarkably, and that of *Enterobacteriaceae*, *Deferribacteraceae*, *Enterococccaceae*, and *Tannerellaceae* decreased compared to the colitis group. Furthermore, the abundant genera (Fig. 6d) relative to the PELN group were *Lachnospiraceae_NK4A136_group*, *Lactobacillus*, *Ruminococcaceae_UCG-014*, *Candidatus_Saccharimonas*, *Ruminiclostridium_6*, *Dubosiella*, *Parasutterella*, *Alloprevotella*, *GCA-900066575*, *Ileibacterium*, *Photobacterium*, *Turicibacter*, *Lachnospiraceae_UCG-006*, *Ruminococcus_1*, *Bilophila*, *Enterorhabdus*, *Butyricoccus*, *ASF356*, *UBA1819*, *Harryflintia*, *GCA-900066225*, *Muribaculum*, and *Acetatifactor*, whereas *Escherichia-Shigella*, *Mucispirillum*, *Enterococcus*, *Rikenellaceae_RC9_gut_group*, and *Parabacteroides* had low abundance. At the species level (Fig. 6e), PELNs drastically elevated the relative proportion of *Lactobacillus_gasseri*, *Lactobacillus_reuteri*, *Alistipes_inops*, *Ileibacterium_valens*, *Photobacterium_damselae_subsp_damselae*, and *Ruminococcus_flavefacien*, and lowered that of *Bacteroides_theaiotaomicron*, *Mucispirillum_sp_69*, *Enterococcus_faecalii*, and *Parabacteroides_goldsteinii*. (The Welch's t test results of Control group vs. DSS group are provided as supplementary materials Figure S3).

**PELNs exert therapeutic effect of mice colitis by inducing the differentiation of DP CD4^+^CD8^+^ T cells**

As reported, DP CD4^+^CD8^+^ T cells can maintain a steady-state of the gut mucosa, and inhibit pro-inflammatory cytokine release during pathogenic infection. Moreover, we previously demonstrated that the population of DP CD4^+^CD8^+^ T cells was significantly decreased in UC mice colon samples. Consequently, we investigated whether the oral administration of PELNs have an effect in the differentiation of DP CD4^+^CD8^+^ T cells in mice colitis. Strikingly, the population of DP CD4^+^CD8^+^T cells was significantly increased in the PELNs group compared to the DSS control group using FCM (1.1% vs. 0.75%, P < 0.01, Fig. 7a). Nevertheless, there was significant difference of the population of DP CD4^+^CD8^+^T cells was detected between the PELNs group and the healthy group (1.1% vs. 2.45%, P < 0.05, Fig. 7a). Similarly, IF revealed that the population of DP CD4^+^CD8^+^T cells was dramatically increased in the PELNs group compared to the DSS control group (Fig. 7b). Furthermore, the population of DP CD4^+^CD8^+^T cells was comparable among the PELNs group and the healthy group (Fig. 7b).

**Biosafety Of Orally Administered Pelns**
To evaluated the biosafety of orally administered PELNs, the blood serum samples and vital organs (heart, liver, spleen, lung, and kidney) were obtained, when mice were daily given 50mg/g and 100mg/g PELNs for 5 days. H&E staining for histological analysis of the vital organs found no obvious abnormalities of organs damage in PELNs-administered group (Fig. 8a). H&E staining of the vital organs in the healthy control group, the DSS group, PELNs-L group and PELNs-H group was shown in Figure S4. Moreover, blood cell count, cardiac enzymes, glutamic pyruvic transaminase (ALT), aspartate aminotransferase (AST), Serum Creatinine (CREA), and Serum Urea (UREA) did not significantly change between the normal control group, DSS group and PELNs-administered group (Fig. 8b, 8c, 8d, 8e).

Discussion

In the last few years, extensive studies have shown that PDENs attract considerable attention for UC treatment due to their unique physicochemical properties and desired-site targeting capabilities. In this study, PELNs from edible POL were isolated and identified with characteristics of nano-sized and negative zeta potential. Moreover, an analysis of potential components revealed that PELNs contained several of bioactive substances, such as nucleic acid, lipids and proteins. Furthermore, PELNs can not only suppress the expression of pro-inflammatory cytokines (IL-6, IL-12, IL-1β, and TNF-α), but also increase the levels the anti-inflammatory cytokines (IL-10), contributing to mitigate the DSS-induced mice colitis. In addition, PELNs had great stability in mimicked GIT environment, and specifically targeted to inflammatory site in mice colitis. Therefore, our findings suggested that the oral administration of PELNs could be a promising alternative for the colitis therapeutics.

It is well-accepted that the dysregulation in the composition and diversity of gut microbiota are involved in the pathogenesis of UC, which characterizes by reduced α-diversity, depletion in the abundance of Firmicutes, Clostridium cluster, and an enrichment abundance of Actinobacteria and Proteobacteria. Moreover, a systematic review found that a significantly decreased abundance of Eubacterium rectale and Akkermansia was reported, whereas an increased levels of E. coli was detected in UC patients. Normally, the metabolites of gut microbiota, such as butyrate and short-chain fatty acids (SCFAs), play a crucial role in intestinal cell proliferation and anti-inflammatory effect, whereas dysbacteriosis display decreased amount of these metabolites resulting in colitis. Consequently, fecal microbiota transplantation (FMT) is emerging as a promising alternative in the treatment of the patients with UC by increasing the abundance of SCFAs producing bacteria in the intestine, thus indirectly increasing the content of SCFAs, which improve intestinal epithelial repair effectively. Recently, accumulated studies have shown that PDENs modulate the composition and function of the gut microbiota, and represent a novel therapeutic approach for the patients with UC. For instance, Zu MH et al. found that edible “green” tea-derived nanoparticle can restore the microbial homeostasis, and mitigate the colonic inflammation in mice colitis. In addition, edible ginger-derived exosome-like nanoparticles can be taken up by gut microbiota, maintain the balance of microbiota, and attenuate the colitis in experimental mice colitis. In this study, PELNs significantly increased the α-diversity and altered the structure of the intestinal microflora. The relative abundance of Firmicutes was notably elevated in mice treated with PELNs, while
that of Proteobacteria were decreased. The SCFAs producing bacteria including Lachnospiraceae_NK4A136_group, Lactobacillus, Ruminococcaceae_UCG-014, Ruminiclostridium_6, Lachnospiraceae_UCG-006, Ruminococcus_1, and Butyricicoccus were upregulated whereas conditional pathogen including Escherichia-Shigella, Enterococcus, and Parabacteroides were down regulated. In total, PELNs can restore the diversity of gut microbiota, decrease the level of harmful microbiota, and increase the abundance of Probiotic.

DP CD4+CD8+ T cells originate from the conventional CD4+ T cells regulating by the transcriptional reprogramming process36. DP CD4+CD8+ T cells play an important role in immune tolerance and immune responses against gut antigens48. Moreover, DP CD4+CD8+ T cells have functions of intestinal inflammation repression, IL-10 production, and bacterial invasion prevention49. We have previously discovered that the amount of DP CD4+CD8+ T cells in colon tissues is substantially decreased compared the DSS-induced colitis with the health control35. Consequently, DP CD4+CD8+ T cells could be an effective target for the treatment of colitis. In this study, we demonstrated that the oral administration of PELNs can prominently promote the differentiation of DP CD4+CD8+ T cells in colonic tissues of the DSS-induced mice colitis. Therefore, our work provided prospective insight on developing PELNs for the treatment of UC.

Conventional therapeutic agents for UC are limited in clinical widespread application due to their potential systematic complications7. Although targeted biologic therapies take several benefits for the patients with UC, which have many disadvantages of huge burden, non-response, and adverse events7. However, edible PDENs have numerous advantages of non-toxicity, non-immunogenicity, and abundant availability in the treatment of UC50,51. In this study, oral administration of PELNs has excellent safety performance for the experimental mice.

In summary, our findings revealed a novel, natural, and biosafety PELNs originated from POL. Moreover, oral administration of PELNs can decrease the expressions of pro-inflammatory cytokines, and increase the levels of anti-inflammatory cytokines contributing to alleviate mice colitis. In addition, orally administered PELNs can maintain the homeostasis of gut microbiota, and induce the differentiation of DP CD4+CD8+ T cells in mice colitis. Therefore, PELNs may be an effective alternative in the treatment of UC.

Declarations

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Author Contributions

D.F.L and Y.J.L conceived and designed the experiments. M.Z.Z and H.M.X performed most of the experiments. Y.J.L prepared and characterized exosome-like nanoparticles. J.X, M.Z.Z and N.N.Y assisted in flow cytometry. Y.Z, C.M.T assisted with in vivo assays. D.F.L, M.Z.Z and Y.J.L wrote the manuscript. L.S.W, J.Y and Y.Q.N supported this study and approved the final version of the manuscript.

Ethics approval and consent to participate

All the animal experiments were approved by the Animal Care Committee of the Shenzhen People's Hospital, Shenzhen, China.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript and its additional files.

Competing interests Funding

The authors declare no competing interests.

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References


Figures
Figure 1

Oral administration of PELNs protects mice against DSS-induced colitis. a, Protocol for DSS-induced colitis and PELNs administration; b, Changes of body weight over time, normalized to the percentage of the day-zero body weight; c, Disease activity index (DAI); d, e, Colon length; f, Histological scores; g, H&E-stained colon sections. *P<0.05, **P<0.01, ***P<0.001 ****P<0.0001.
Pro-inflammatory cytokines and anti-inflammatory cytokine expression profiles. a, b, c, d, e, qRT-PCR detecting the levels of IL-6, IL-12, IL-1β, TNF-α and IL-10 in colonic samples; f, g, h, i, j, ELISA testing the expression profiles of IL-6, IL-12, IL-1β, TNF-α and IL-10 in blood serum. *P<0.05, **P<0.01, ***P<0.001 ****P<0.0001.
Figure 3

Oral administration of PELNs protects PHLP2<sup>−/−</sup> mice spontaneous colitis. a, Changes of body weight; b, Disease activity index (DAI); c, Colon length; d, Histological scores; e, H&E-stained colon sections.
**Figure 4**

**In vivo distribution of PELNs.** Fluorescence images of the gastrointestinal tract revealing *in vivo* biodistribution of orally administered IRDye 800CW-labeled PELNs at different time points (3, 6, 12, and 24 h).
Figure 5

PELNs alters diversity of gut microbiota in mice models of colitis. a, Upset Polt of OTUs; b, ACE index; c, Chao 1 index; d, PD-tree index; e, Simpson index; f, Shannon index; g, PCA (Principal Component Analysis); h, PCoA (principal co-ordinate analysis). Sum of PCoA1 and PCoA2 more than 50%, indicating significant difference; i, NMDS (Non-metric Multi-Dimensional Scaling). Stress value less than 0.1, indicating significant difference. **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. 
Figure 6

**PELNs alters gut microbiota structures across different levels in mice with colitis.** a, Stacked bar plot depicts the structure of gut microbiota in each group of mice. Left to right: phylum, family, genus, and species; b-e, Welch’s t test analysis of PELNs mediated differential microbial changes at the phylum, family, genus, and species level.
**Figure 7**

**PELNs inducing the differentiation of DP CD4^+CD8^+ T cells.** a, Flow cytometry detecting the population of DP CD4^+CD8^+ T cells in colonic samples; b, Immunofluorescence showing the population of DP CD4^+CD8^+ T cells in colonic samples. Data are representative FCM images or expressed as the mean ± SEM of each group. *P<0.05, **P<0.01.
Figure 8

**Biosafety of orally administered PELNs.** a, H&E-stained of vital organs (heart, liver, spleen, lung, and kidney) when administering orally PELNs; b, c, Liver function; d, e, Kidney function.

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