# Oral administration of *Parabacteroides distasonis* antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition.

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## SUPPLEMENTARY MATERIALS AND METHODS
### Preparation of bacterial lysates and bacterial components

Cultures were grown on Wilkins-Chalgren Anaerobe Agar (Oxoid, Basingstoke, UK) and enriched with defibrinated blood. After 24 hours (h) of incubation in an anaerobic atmosphere (Oxoid), a small portion of agar was cut out and placed into Brain Heart Infusion Broth (Oxoid) for 48 h at 37°C in an anaerobic atmosphere. The cells were harvested by centrifugation (4000 x g, 30 min) and washed twice with sterile PBS to minimise contamination of the sample with the culture media.

After disruption of the bacteria with the French press, part of the lysate was separated by centrifugation (8500 x g, 30 min) into two fractions, membranous (insoluble; mPd) and cytoplasmic (soluble; cBd). The lysate and its fractions were lyophilised and diluted to a working concentration of 15 g/l. Lipopolysaccharide of the *P. distasonis* was isolated by phenol-water extraction according to the procedure described by Westphal *et al* [1]. DNA from *P. distasonis* was isolated using a shortened version of the cetyltrimethylammonium bromide (CTAB) DNA isolation method described previously [2]. Sterility of all components was verified by both aerobic and anaerobic cultivation before administration.

### Evaluation of colitis

Colitis was evaluated on the last day of the experiment using a clinical activity score, histological score and colon length measurement. The clinical activity score represents the sum of separate scores ranging from 0 to 4 and was calculated using the following parameters: body weight decrease (none 0 points, weight loss of 1 to 5% as 1 point, 5 to 10% as 2 points, 10 to 20% as 3 points, and 20% as 4 points), stool consistency (solid 0 points, loose stool that did not stick to the anus 2 points, and 4 points for liquid stools that did stick to the anus), and bleeding (none 0, positive guaiacum reaction 2 points, and 4 points for gross bleeding). These scores were added and divided by 3, forming a total clinical score that ranged from 0.0 (healthy) to 4.0 (maximal activity of colitis), as described previously by Cooper *et al* [3]. Postmortem, the entire colon was removed (from caecum to anus) and placed without tension on a ruler and colon length measured. Colon descendens were fixed in 4% buffered formalin and embedded in paraffin for histological evaluation. Sections were stained with hematoxylin/eosin. Four transversal sections, separated with 100 μm gaps, were evaluated from each sample. Histological scoring was performed for each section in a blinded fashion by 2 expert pathologists (K. K. and P. R.) and a score combining the degree of leukocyte infiltration in lamina propria and submucosa and the extent of mucosal defect (Table S1 and Fig. S1) was obtained. The final score represents the mean of four sections ranging from 0 (no signs of colitis) to 3 (severe colitis). Evaluation of acute DSS-induced colitis in parenterally (s.c. subcutaneous, i.p. intraperitoneal) treated BALB/c mice is summarised in table S6.

**Fig. S1** Histological examples of different grades of mucosal damage in DSS treated mice (H&E stained colon descendens; magnification, ×40). See table S1 for detailed description.
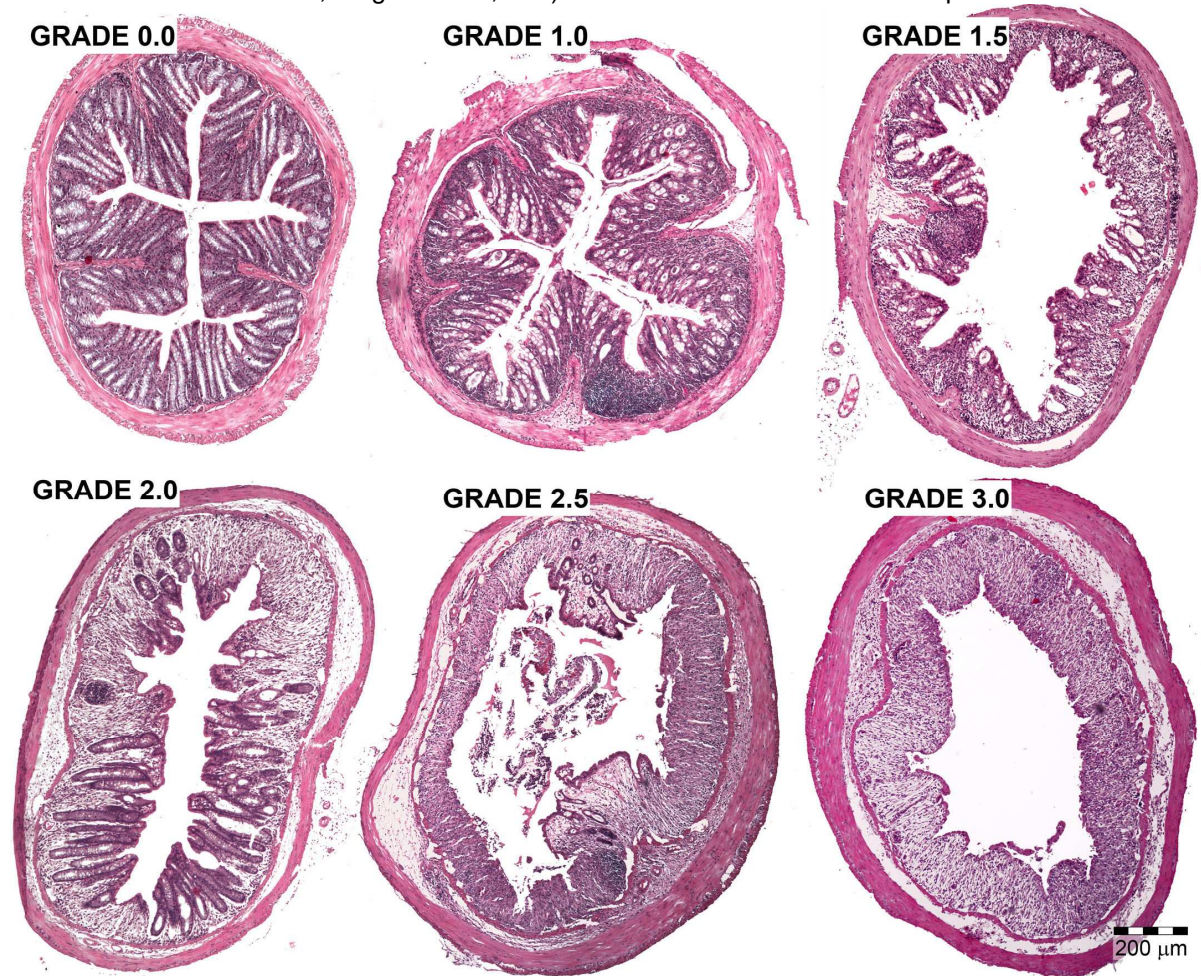


**Table S1.** Detailed description of individual histological grades.

| Grade | | Description |
|---|---|---|
| **0** | **normal mucosa** | Thin colon wall without oedema or infiltration, crypt without defects with well-preserved mucus production. |
| **0.5** | **borderline** | Discrete focal infiltration by the crypt basis without any defect in mucosa. Also encountered in some control animals. |
| **1.0** | **mild** | Extension of cellular infiltrate to the superficial layer of lamina propria and to submucosa. Mild oedema of lamina propria and flattening of crypts without defects of epithelium. |
| **1.5** | **medium** | Confluence of inflammatory cells and oedema in lamina propria and patchy infiltrate in submucosa. The mucosa is markedly flat with discrete errosion(s) or ulcers covering less than 10% of colon diameter. |
| **2.0** | **medium to severe** | Same as above, but the ulcers extend to 10%-50% of diameter, mostly with the purulent exudate in the lumen. Crypts are regressed and the mucus production is suppressed. |
| **2.5** | **very severe** | Same as above, but the ulcers cover over 50% of diameter. Massive inflammatory infiltration and oedema of both lamina propria and submucosa with pseudoabscesses and intravascular leukostasis. |
| **3.0** | **extreme** | Same as above, but with subtotal/total denudation of the mucosa. |

**Determination of serum and faecal antibodies**

Faecal pellets were collected and processed as previously described [4]. We then used indirect ELISA, optimised in our laboratory, to compare serum or fecal antibody (IgG, IgM and IgA) titres against *P. distasonis* lysate between PBS and mPd-treated groups.

Briefly, 96-well ELISA plates (Nunc, Roskilde, Denmark) coated overnight with mPd (100 µl/well at 10 mg/l in PBS) and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in PBS were incubated for 2 h with serum samples diluted 1:50 (1% BSA was used as a blank, normal mouse serum as a negative control and a pool of the hyper-immune mouse sera as a positive control). After washing (three times with PBS containing 0.05% Tween 20 (Sigma-Aldrich)), secondary antibodies (50 µl/well) were added and incubated for 1 h at room temperature. We used horseradish peroxidase (HRP)-labelled anti-mouse IgG (The Binding Site Ltd, Birmingham, UK) diluted 1:2000 in 1% BSA, HRP-labelled anti-mouse IgM (The Binding Site Ltd) diluted 1:500 in 1% BSA, or biotinylated anti-mouse IgA (Sigma-Aldrich) diluted to a concentration of 1:2000 in 1% BSA and 5% fetal bovine serum (BioClot GmbH, Aidenbach, Germany). After a washing step, we added 50 µl/well of streptavidin-HRP (R&D Systems Inc., Minneapolis, MN) diluted 1:200 in 1% BSA into the IgA plate. The plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich) and the optical density (OD) was measured at 450 nm. The OD of the background (1% BSA) was subtracted and resulting adjusted ODs of the treated groups were compared with those of PBS-treated groups. We used 10 mice per group for the analysis of serum and 5 mice per group for analysis of coproantibodies.

**Determination of serum haptoglobin levels**

The level of the acute-phase protein haptoglobin was determined in mouse serum using a modified Human Haptoglobin ELISA Quantitation Kit (GenWay Biotech, Inc., San Diego, CA). Antibodies used in this kit have high cross-reactivity with mouse haptoglobin; the recovery for the mouse reference serum was 94%. The kit was used according to the manufacturer's recommendation, with minor modifications. Briefly, a 96-well ELISA plate (Nunc) was coated with Chicken anti-Human Haptoglobin antibody (100 µl/well at 5 mg/l) diluted in 0.05 M Carbonate-Bicarbonate buffer (pH 9.6) and incubated for 1 h at room temperature. After washing (three times with PBS containing 0.05% Tween 20), the plate was blocked with 1% nonfat dry milk in PBS. The samples were diluted 1:1000 in 1% nonfat dry milk and incubated for 1 h at room temperature. Serial dilutions of mouse reference haptoglobin serum (ICL, Inc., Newberg, OR, USA) were used as calibrator instead of the pure human haptoglobin provided in the kit. Then the plates were washed five times and incubated with HRP conjugated detection antibody (100 µl/well at 61.3 µg/l) for 1 h. The plates were developed with TMB (Sigma-Aldrich) and the OD was measured at 450 nm. The quantitative determination was performed between 39-2500 µg/l.

**Gut tissue fragment culture**

Five sections of mouse intestine were obtained (Peyer's patches, jejunum, ileum, caecum and colon), cut open longitudinally, washed in PBS containing penicillin and streptomycin and weighed.

The tissue fragments were then cultivated for 48 h in a humidified incubator at 37°C and 5% $CO_2$ in RPMI-1640 (Sigma-Aldrich) containing 10% fetal bovine serum (BioClot GmbH, Aidenbach, Germany) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich). The supernatants were collected and stored at -20°C until analysis. During our preliminary experiments, we found that there is still significant production of IL-10 and TGF-β after 48h cultivation (Fig. S2).

## Macrophage cell line culture

Mouse macrophage RAW 264.7 cells, originally obtained from the American Type Culture Colection (ATCC TIB-71), were cultured in Dulbecco´s modified Eagle´s medium (Institute of Molecular Genetics AS CR, Prague, Czech Republic) supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany), penicillin (100 U/ml), streptomycin (100 mg/l, Sigma-Aldrich), 4.5 g/l glucose, 1.5 g/l sodium bicarbonate and 4 mM glutamine (Institute of Molecular Genetics AS CR). The cells were cultured in a humidified incubator at 37°C and 5% $CO_2$. Cell viability was evaluated by flow cytometry. The cell density was then adjusted to $10^6$ cells/ml and the cells were seeded in the wells of flat-bottom, 96-well plates (200 µl/well). The plates were incubated with LPS (*Salmonella typhimurium*, 1 mg/l, Sigma-Aldrich), LPS together with either bacterial lysate from *P. distasonis*, or mPd or cPd or DNA for 24 h (37°C, 5% $CO_2$). To address the question of dose dependence, serial decreasing dilutions of lysate and mPd were used, ranging from 1 µg/l to 100 fg/l. Supernatants were collected and stored at - 20°C until analysis. The supernatants were screened semiquantitatively with the RayBio$^{TM}$ Mouse Cytokine Array 3 (Raybiotech, Inc., Norcross, GA), capable of detecting 62 cytokines, or quantitatively for TNF-$\alpha$ with ELISA (Invitrogen Corp., Carlsbad, CA), similarly as described below.

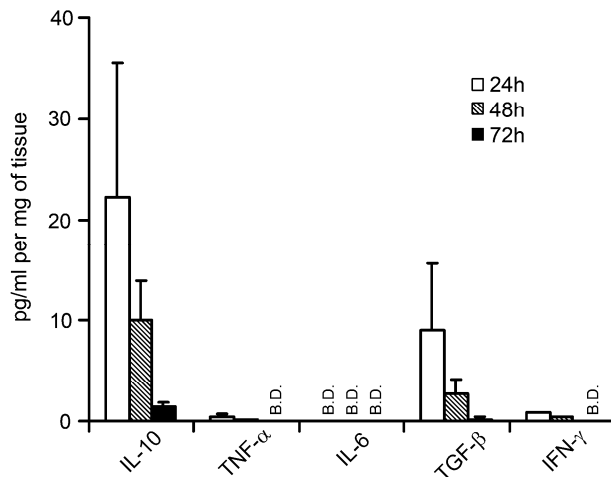## Determination of cytokine production

To determine the changes in cytokine spectra induced by DSS treatment and mPd therapy in the colon of mice, we used the RayBio$^{TM}$ Mouse Cytokine Array II (Raybiotech, Inc.) (see Table S2 for array layout). For this purpose, we used three samples of media after 48 h of colon cultivation (see above) from healthy, DSS/PBS (sham) and DSS/mPd-treated groups of mice. Chemiluminescence was detected by a luminescence detector LAS-1000 (Fujifilm, Tokyo, Japan), and quantitation of spots was performed by AIDA (3.28, Raytest, Straubenhardt, Germany) software as described previously [5]. Values from different arrays were first normalized using the intensity of positive controls, which are made of biotinylated antibody directly spotted on the array. Furthermore, the levels of selected cytokines were determined using commercially available ELISA sets purchased from Invitrogen (TNF-$\alpha$, IFN-$\gamma$, TGF-$\beta$, IL-10; Invitrogen Corp.) or R&D Systems (IL-6; R&D Systems Inc., Minneapolis, MN). All tests were performed according to the manufacturers' recommendations.

**Table S2.** Layout of the RayBio$^{TM}$ Mouse Cytokine Array II

| | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 2 | Positive control | Positive control | Negative control | Negative control | 6-Ckine | CTACK | Eotaxin | G-CSF | GM-CSF | IL-2 | IL-3 | IL-4 |
| 3 4 | IL-5 | IL-6 | IL-9 | IL-10 | IL-12p40p70 | IL-12p70 | IL-13 | IL-17 | IFN-$\gamma$ | KC | Leptin | MCP-1 |
| 5 6 | MCP-5 | MIP-1$\alpha$ | MIP-2 | MIP-3$\beta$ | RANTES | SCF | sTNFRI | TARC | TIMP-1 | TNF-$\alpha$ | TPO | VEGF |
| 7 8 | Blank | Blank | Blank | Blank | Blank | Blank | Blank | Blank | Blank | Blank | Blank | Positive control |

Abbreviations used in this table stand for the following cytokines: 6-Ckine, 6-Cysteine chemokine; CTACK, Cuteaneous T-cell attracting chemokine; G-CSF, Granulocyte-colony stimulating factor; GM-CSF, Granulocyte-macrophage colony stimulating factor; IFN (Interferon) -$\gamma$, IL (Interleukin) -2, -3, -4, -5, -6, -9, -10 , -12p40p70 (detects both p70 and p40), -12p70 (detects only whole cytokine IL-12), -13, -17, KC, Growth-regulated alpha protein precursor; MCP (Monocyte chemoattractant protein)-1, -5; MIP (Macrophage inflammatory protein)-1$\alpha$, -2, -3$\beta$, RANTES, Regulated upon activation, normal T cell expressed, and presumably secreted; SCF, Stem call factor; sTNFRI, Soluble tumor necrosis factor-$\alpha$ receptor 1; TARC, Thymus and activation-Regulated chemokine; TIMP (Tissue inhibitor of metalloproteinases)-1; TNF (Tumor necrosis factor)-$\alpha$; TPO, Thrombopoietin; VEGF, Vascular endothelial growth factor

**Fig. S2** Cytokine production by colon tissue of healthy mice during the first, second and third days of *ex vivo* cultivation. Three colon samples were cultivated for 72 h in complete RPMI medium. Every 24 h, the tissue was gently washed in fresh media and transferred to the new cultivation well for next 24 h. The supernatant after the first, second and third 24 h of cultivation was stored for cytokine analysis.

**Fig. S3** Showing gating strategy in 3 mice (rows) from DSS/PBS and 3 mice from DSS/mPd treated group.
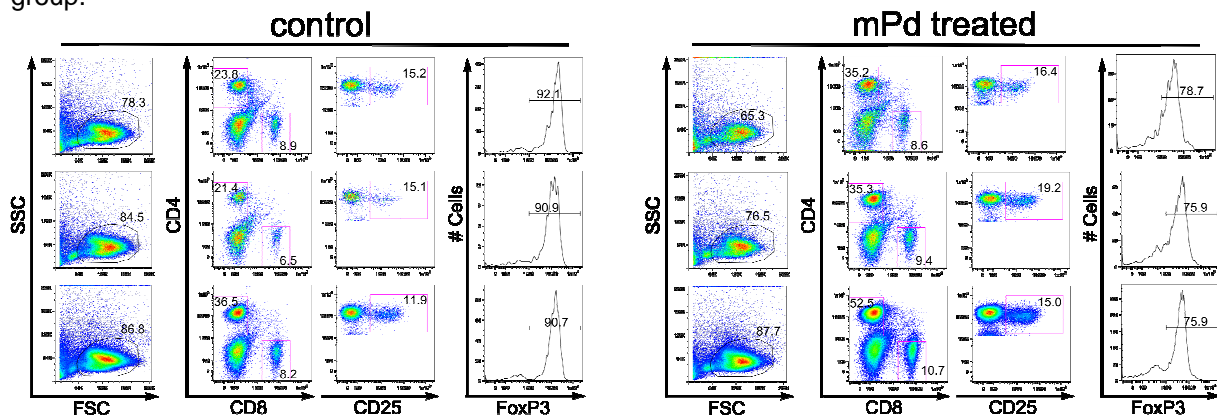


Total number of $T_{regs}$ is increased in mesenteric lymph nodes (MLN) of mPd treated mice is increased in cells (mean±SD; 3.40±0.50 vs. 4.81±0.30; P=0.014 for $CD4^+CD25^+FoxP3^+$ or 4.29±0.26 vs. 5.36±0.10; P=0.019 for $CD4^+FoxP3^+$). First column shows gating on cells, second gating on CD4 and CD8 expression on these cells, third gating on $CD4^+CD25^+$ cells and fourth is a histogram of FoxP3 expression on these $CD4^+CD25^+$ cells.

## Evaluation of microbiota changes with PCR-DGGE

Total bacterial DNA was isolated from mice faecal samples by using the ZR Fecal DNA Kit[TM] (Zymo Research Corp., Orange, CA) according to the manufacturer's description. Fragments of 16S rRNA genes were amplified from total bacterial DNA with primers 338GC and RP534 [6].

PCR products were separated and analysed on the DCode[TM] Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). The denaturing gradient was 35 – 60% and the electrophoresis was carried out for 18 h at 55 V. The gel was stained in SYBR[®] green I dye for 30 minutes and observed in a Vilber Lourmat system under UV light.

Banding patterns were converted to a binary matrix, taking into account the presence or absence of the individual bands. This binary matrix was used to calculate the distance matrix between individual samples [7] and a dendrogram comparing all 30 samples was obtained with UPGMA (unweighted pair-group method with arithmetic averages) using FreeTree software [8].

The similarity between the DGGE profiles obtained from a single mouse at different time points was determined by calculating Dice's similarity coefficient ($D_{SC}$ =[2j/(a+b)] x 100), where j is the number of DGGE bands found in both profiles, a is the number of bands at first time point, and b is the number of bands at the second time point. A $D_{SC}$ value of 100% indicates that the samples are identical.

To identify the bacteria, bands of interest were cut from the gel, eluted with $dH_2O$ and amplified with PCR. The PCR product was purified with the QIAquick PCR purification kit and analysed on the 3100 Avant Genetic Analyser (Applied Biosystems Inc., Foster City, CA).

**Quantitative PCR**

Faecal samples were weighed and total bacterial DNA was extracted by using a ZR Fecal kit (Zymo Research, USA) according to the manufacturer's protocol. Real-time PCR analyses were performed on the Mx3005P system (Stratagene, USA) with the qPCR 2x SYBR Master Mix (Top-Bio, Czech Republic). The qPCR reactions were performed in a 20 µL volume, and the primer concentrations were 0.5 µM each. The following bacterial groups were monitored: all *Eubacteria* (with primers Uni331F+Uni797R)[9], *Bacteroides-Prevotella* group (primers Bac303F+Bac708R)[9] and *P. distasonis* (Bd180F+Uni797R)[10]. We used these amplification conditions: initial denaturation at 95°C (3 min), 35 cycles of denaturation at 95°C (30 seconds) and annealing/elongation (30 seconds) (Table S3), and one final cycle at 95°C (30 seconds) followed by a dissociation curve from 55°C to 95°C (1°C per cycle of 10 s). DNA isolated from a known number of cells from pure cultures of *Bacteroides vulgates* and *P. distasonis* were used as qPCR standards. Because the weight and the consistency of the stool differed among the samples, the results were normalised to the total number of *Eubacteria* and expressed as a percentage of total *Eubacteria*.

**Table S3.** PCR primer sets used in the study.

| Target organism | Primer set | Sequence (5'-3') | Product size (bp) | Annealing temp (°C) | Reference |
|---|---|---|---|---|---|
| All eubacteria (PCR-DGGE) | 338GC | CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCGCCGCCGC ACTCCTACGG GAGGCAGCAG | 196 | 58 | [6] |
| | RP534 | ATTACCGCGG CTGCTGG | | | [6] |
| All eubacteria (qPCR) | Uni331F | TCCTACGGGAGGCAGCAGT | 466 | 58 | |
| | Uni797R | GGACTACCAGGGTATCTATCCTGTT | | | [9] |
| *Bacteroides-Prevotella* group | Bac303F | GAAGGTCCCCCACATTG | 418 | 56 | |
| | Bac708R | CAATCGGAGTTCTTCGTG | | | |
| *Parabacteroides distasonis* | Bd180F | AAT ACC GCA TGA AGC AGG | 617 | 62 | [10] |
| | Uni797R | GGACTACCAGGGTATCTATCCTGTT | | | [9] |

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**SUPPLEMENTARY TABLES**

**Table S4**. Evaluation of acute DSS colitis in orally treated BALB/c mice.

| Experimental group | Colon length (cm) | Disease activity index | Histological grade |
|---|---|---|---|
| PBS | 9.31±0.88 | 3.33±0.49 | 1.44±0.71 |
| *P. distasonis* lysate | 10.85±1.26** | 0.90±0.77** | 0.61±0.50** |
| *B. ovatus* lysate | 9.48±0.80 | 2.63±1.15 | 1.26±0.35 |
| *V. alcalescens* lysate | 9.21±0.66 | 1.58±0.66 | 1.58±0.66 |

Values are expressed as means ± standard deviation (10 mice per group). One-way ANOVA with Dunnett's multiple comparison test was used to evaluate differences between experimental groups and PBS-treated controls (*P<0.05, **P<0.01).

**Table S5**. Evaluation of acute DSS colitis in orally treated BALB/c mice.

| Experimental group | Colon length (cm) | Disease activity index | Histological grade |
|---|---|---|---|
| PBS | 7.83±0.55 | 3.78±0.27 | 1.81±0.28 |
| *B. vulgatus* lysate | 8.13±1.06 | 2.87±1.04 | 1.44±0.89 |
| *B. stercoris* lysate | 8.60±0.60 | 3.40±0.80 | 1.33±0.41 |
| *B. stercoris* confidence level | | | |
| *Capnocytophaga spp.* lysate | 9.00±0.70 | 3.13±0.65 | 0.63±0.25** |
| *B. thetaiotamicron* lysate | 8.90±0.81 | 2.13±0.96 | 1.25±0.47 |

Values are expressed as means ± standard deviation (5 mice per group). One-way ANOVA with Dunnett's multiple comparison test was used to evaluate differences between experimental groups and PBS-treated controls (*P<0.05, **P<0.01).

**Table S6**. Evaluation of acute DSS colitis in parenterally treated BALB/c mice.

| Experimental group | Colon length (cm) | Disease activity index | Histological grade |
|---|---|---|---|
| PBS/IFA s.c. | 6.02±0.46 | 3.40±0.37 | 1.61±0.84 |
| mPd/IFA s.c. | 6.20±0.51 | 2.87±0.80 | 1.43±0.53 |
| PBS/IFA i.p. | 6.72±0.54 | 3.13±0.69 | 1.69±0.81 |
| mPd/IFA i.p. | 6.72±0.83 | 2.73±0.89 | 1.29±0.62 |

Values are expressed as means ± standard deviation (5 mice per group). One-way ANOVA with Dunnett's multiple comparison test was used to evaluate differences between experimental groups and PBS-treated controls (*P<0.05, **P<0.01).

## SUPPLEMENTARY FIGURES

**Fig. S4.** Cytokine profiling of the supernatants after the cultivation of untreated RAW264.7 cells or cells after treatment with LPS, Pd+LPS or mPd+LPS, as measured by RayBio Mouse Cytokine Antibody Array 3. Only cytokines positive at least in one sample are shown.
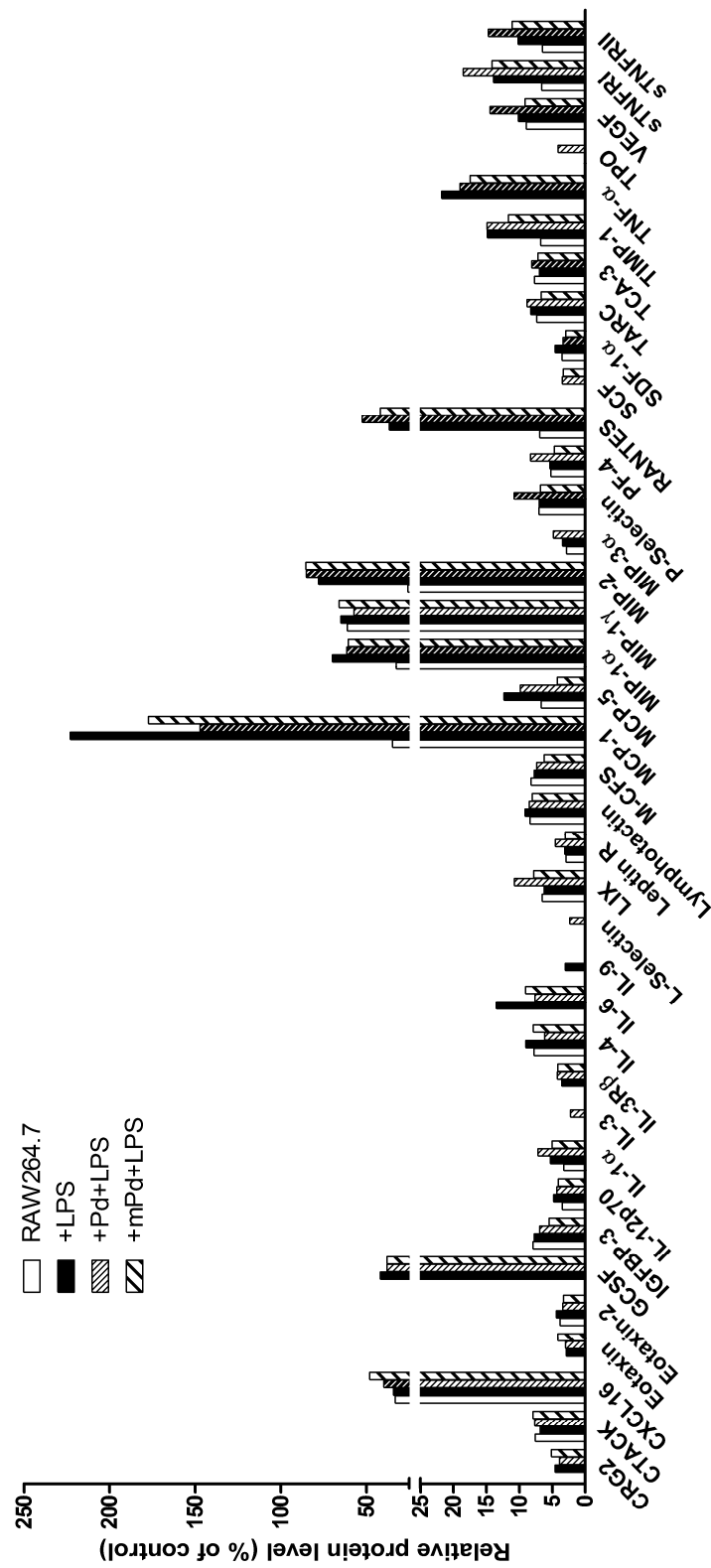


As compared with LPS-activated cells, the Pd and mPd decrease TNF-α, IL-6, MCP-1 and MCP-5, and increase in CXCL16.

**Fig. S5.** The effect of Pd and mPd on TNF-$\alpha$ production by LPS-activated macrophage cell line RAW 264.7 was measured by ELISA. TNF-$\alpha$ production with sterile PBS with 1 mg/l of LPS is set at 100%. Data are means of five independent experiments. Error bars are SEM. P < 0.05: [a] (for LPS+Pd versus controls); [b] (for LPS+mPd versus controls) using ANOVA with a Dunnett's post-hoc test.
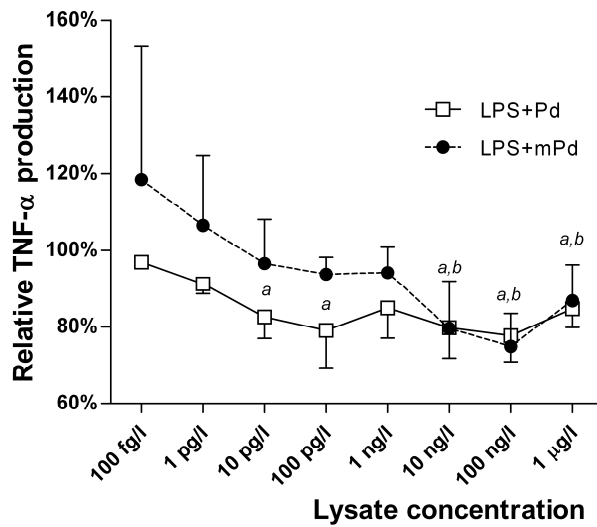


**Fig. S6**. Pretreatment with mPd decreases cytokine production (pg/mg of tissue) in different parts of the gut in orally treated SCID mice as measured by ELISA. **P<0.01: DSS/mPd versus DSS/PBS-treated mice; n = 5 mice per group.