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Supplementary Information for

Neutralization of IL-1α ameliorates Crohn's disease-like ileitis by functional alterations of the gut microbiome

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Supplementary Materials and Methods

Intersubject Preexperimental Fecal homogeneization (IsPreFeH)

We reported a novel strategy, 'IsPreFeH', to control for the confounding effects associated with the large variability that exists in the microbiome composition across mouse cages in experimental facilities[1]. This preferred method in which all animals are gavaged with feces, has immense benefits over cohousing[2]. The intervention experiment described below commenced 7 days after the completion of the IsPreFeH protocol.

Anti-IL-1α treatment

Study 1 was conducted on SPF SAMP mice (26-wk-old) (n=6/group). These mice were injected intraperitoneally with FLO1 (XBiotech USA Inc., Austin, TX), an anti-IL-1α monoclonal antibody (100μg; 2x/wk for 4-wks). These mice were compared to age/gender-matched SAMP mice treated with dexamethasone (Dex, 1mg/kg/day for 1-wk) and vehicle-treated controls. This experiment was conducted twice. The same treatment was conducted 3 times also in 4-wks-old SAMP mice (n=6/group) (Study 2). GF SAMP mice (n=6/group) were treated with FLO1 (100μg; 2x/wk for 4wks) and compared to vehicle-treated controls. This experiment was also conducted 3 times. For all experiments, microbiota was normalized before the beginning of the study as previously described[2]. To validate the neutralizing activity of FLO1 mAb, we tested the ability of the antibody (100μg/mL) to block IL-1β production induced by recombinant mIL-1α (r-m-IL1α 10ng/mL, R&D System, Minneapolis, MN) in mesenteric lymph node (MLN) cells isolated from 20-wks old SAMP mice. Cells, plated at the concentration of 10^6 cells, were incubated with a combination of 10ng/mL of r-m-IL-1α and 1.0μg/mL of LPS (InvivoGen, San Diego, CA) and in the presence or absence of FLO1 for 48hrs. Supernatants were collected for IL-1β measurement (Figure S1).

DSS-induced colitis model

Induction of acute colitis was achieved in SAMP mice with 7 days of 3% dextran sodium sulfate (DSS) (TdB Consultancy AB; Batch no. DB001-37) in drinking water. Mice were allowed to drink ad libitum with new DSS-containing water every 3 days. Mice returned to normal drinking water after 7 days of DSS and were allowed to recover for 2 weeks. Survival and body weight loss, calculated as the percentage change relative to baseline, were monitored during the treatment period. The experiment was performed 3 times.

Histology

Ilea and colons were removed from mice, flushed of fecal contents with cold PBS, opened longitudinally, and fixed in Bouin's solution (Ricca Chemical, Arlington, TX) for 24hrs. Fixed tissues were processed, paraffin embedded, sectioned at 3–4μm, and stained with hematoxylin and eosin (H&E). All processed sections were subsequently evaluated by a GI pathologist in a blinded fashion. Histological evaluation of inflammation severity was determined by using a semiquantitative scoring system as previously described[3].

Tissue culture

We discovered that the ileitis in SAMP mice is characterized by the formation of cobblestones that resemble the typical lesions of CD[1]. Therefore, abnormal ileal tissue characterized by cobblestones formation (involved areas) and normal mucosa (uninvolved areas) were isolated by examining the 3D structural pattern of ileal tissue via stereomicroscopy (SM)29. Both involved/uninvolved areas were collected and cultured in complete RPMI 1640 medium (HyClone Laboratories) with 10% FBS and 1x penicillin/streptomycin. Tissue samples were incubated in a humidified 5% CO_2 at 37°C for 24hrs. Supernatants were subsequently collected and stored at 80°C for further assays.

IL-1α Staining

Paraffin sections of mouse ilea were rehydrated and incubated in a 5% normal donkey serum containing blocking solution for 30min at RT. Subsequently, the slides were incubated for 1hr at RT with 15μg/mL goat polyclonal Ab against murine IL-1α (R&D Systems) in the blocking solution. The slides were then washed 3x in PBS and incubated with an Alexa 488–conjugated donkey anti-goat IgG (1:1000; Molecular Probes, Eugene, OR) secondary Ab and counter-stained with Vectashield Mounting Medium for fluorescence with 4′,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA). All images were acquired using a TCS-SP laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

MPO Activity Assay

Colon specimens and both involved/uninvolved areas from ilea were dissected via SM and assayed for MPO activity as previously described[1]. Specimens were collected, weighed, diluted 20-fold in 0.5% hexadecyl-trimethil-ammonium-bromide buffer, homogenized with a Polytron PT 10–35 tissue homogenizer. Ten µl of each sample were added in triplicate into a 96-well plate and exposed to substrate solution (200µL; 5mM potassium phosphate buffer containing 0.0005% H2O2 and 0.167mg/mL O-dianisidine). The average rate of absorbance changes at 450nm over 5 sequential 30-s intervals was used to calculate MPO activity.

qRT-PCR

Colon samples and SM-micro-dissected samples from ilea were placed in RNAlater (Applied Biosystems, Forest City, CA), left at 4°C overnight, then stored at -80°C. Total RNA was isolated using the RNAeasy Mini kit (Qiagen, Germantown, MD) as described by the manufacturer, and converted into cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystem). qPCR amplification of cDNA samples was performed using the Roche 480 LightCycler SYBR Green (Roche) run template settings (hot start 95°C, 10min, 40 amplification cycles; 95°C, 15s; 60°C, 30s; 72°C, 30s); 18S was used as a reference gene.

16S rRNA gene microbiome analysis

Following DNA extraction, microbiome amplification for the 16s rRNA gene V4 regions, and high-throughput sequencing were conducted at the Beijing Genomics Institute, using Illumina Sequencing chemistry and platforms. In Study 1, DNA from end point fecal samples after 4 weeks of treatment were used for PCR-amplification of the V4 region using primers Forward-S-D-Bact0564-a-S-15 (5′-AYT GGG YDT AAA GNG) and Reverse-S-D-Bact-0785-b-A-18 (5′-TAC NVG GGT ATC TAA TCC). PCR products were then evaluated by electrophoresis in 2% agarose gel and purified with the Agencourt AMPure XP system. Jagged ends of DNA fragments were converted into blunt ends by using T4 DNA polymerase, Klenow Fragment and T4 Polynucleotide Kinase. Then, an 'A' base was added to each 3' end to facilitate the incorporation of the adapters. Short fragments were removed using AMPure beads. High Quality libraries were used for 250bp pair-end sequencing. The raw sequencing data were filtered to eliminate the adapter pollution and low quality to obtain clean reads. Paired-end reads with overlap were merged to tags, which were then clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity. Taxonomic ranks were assigned to OTU representative sequence using the Ribosomal Database Project (RDP) and Bayesian Classifier v.2.2. Alpha diversity, beta diversity and the different species screening were analyzed based on OTU and taxonomic ranks. To ensure rigor and reproducibility, the raw data were pre-processed to obtain clean high-quality data by using an in-house procedure to remove: i) truncate sequence reads not having an average quality of 20 over a 25 bp sliding window based on the phred algorithm, and trimmed reads having less than 75% of their original length, as well as its paired reads; ii) reads contaminated by adapter (default: 15 bases overlapped by reads and adapter with maximal 3 bases mismatch allowed); iii) reads with ambiguous basa (N base), and its paired reads; and iv) reads with low complexity (default: reads with 10 consecutive same base). Then, the selected barcoded-pooled clean reads were decoded and assigned to corresponding samples by allowing 0 base mismatch between the sequenced and expected barcode sequences. The reads with sequencing adapters, N base, poly base, and low quality were filtered out with default parameters. Summary data processing results indicated that the sequencing was of utmost quality. For instance, Study 2, had an average of 14,620 readsx2 clean reads per sample with a rate of clean:raw data utilization greater than 99.5% (min, 97.97; max, 99.79%) through the experiment. For merging overlapping clean reads, two associated paired-end reads needed to overlapped, the generate the consensus sequence using FLASH (Fast Length Adjustment of Short reads, v1.2.11; minimal overlapping length, 15 bp; Mismatching ratio of overlapped region: <= 0.1). All paired end reads that did not overlapped were removed from the analysis. In total, Study 2, (104 samples) had 3’490.475 tags associated with high quality paired-end overlapping (connecting ration >95.19%; average, 14074 tags per sample; average length, 252 bp). Instead of subsampling for normalization, we normalized the OTU tables by rescaling the abundances of all samples to the fecal sample having the lowest total sequence abundance in the study.

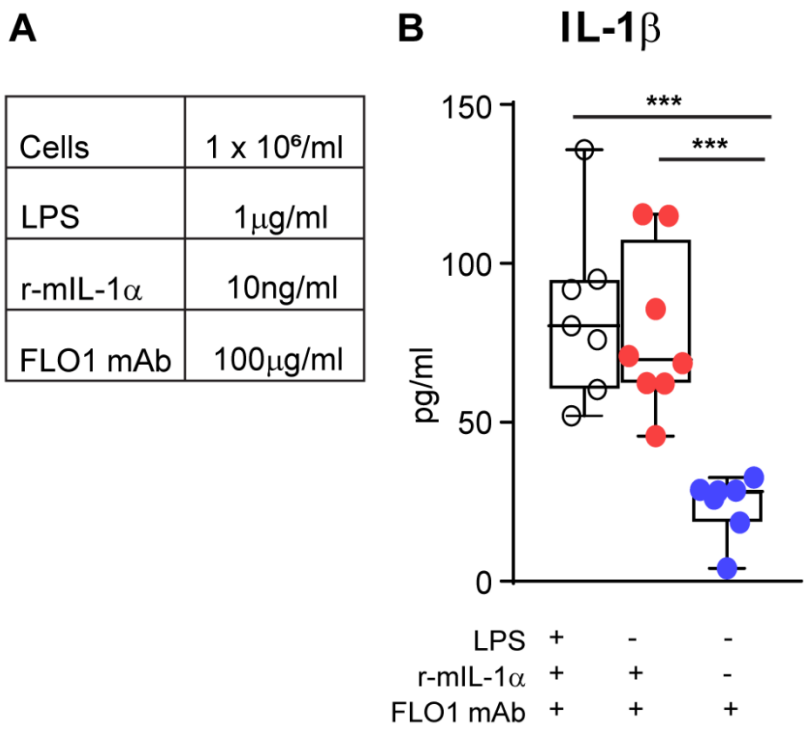

Fig. S1. FLO1 mAb in vitro administration leads to a remarkable reduction of IL-1β production. (**A**) Experimental design. (**B**) MLN cells were cultured with a combination of LPS and r-mIL-1α, with or without FLO1 for 48 hrs. Supernatants were collected and analyzed by ELISA assay for production of IL-1β which displayed a remarkable reduction in the presence of FLO1 mAb.

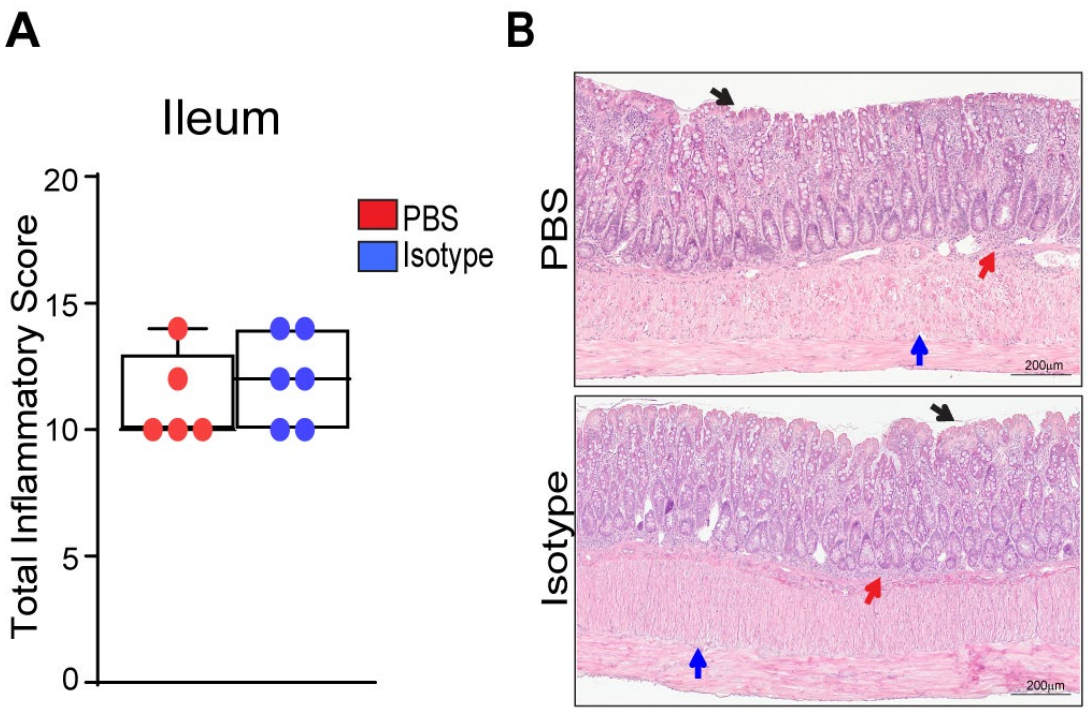

Fig. S2. Effects of IgG2ak-isotype antibody administration on SAMP CD-like ileitis. (**A**) Histologic analysis shows that the total inflammatory scores of ileal tissue in IgG2ak-Isotype-treated SAMP mice is comparable to PBS-treated SAMP mice (n≥5). (**B**) Representative photomicrographs of full-thickness H&E-stained ileal tissue from SPF SAMP mice treated with either PBS (top panel) or IgG2ak-Isotype control (bottom panel) show similar cellular infiltration (red arrows), mucosal thickening (blue arrows) and villous architecture alterations (black arrows); scale bar=200μm.

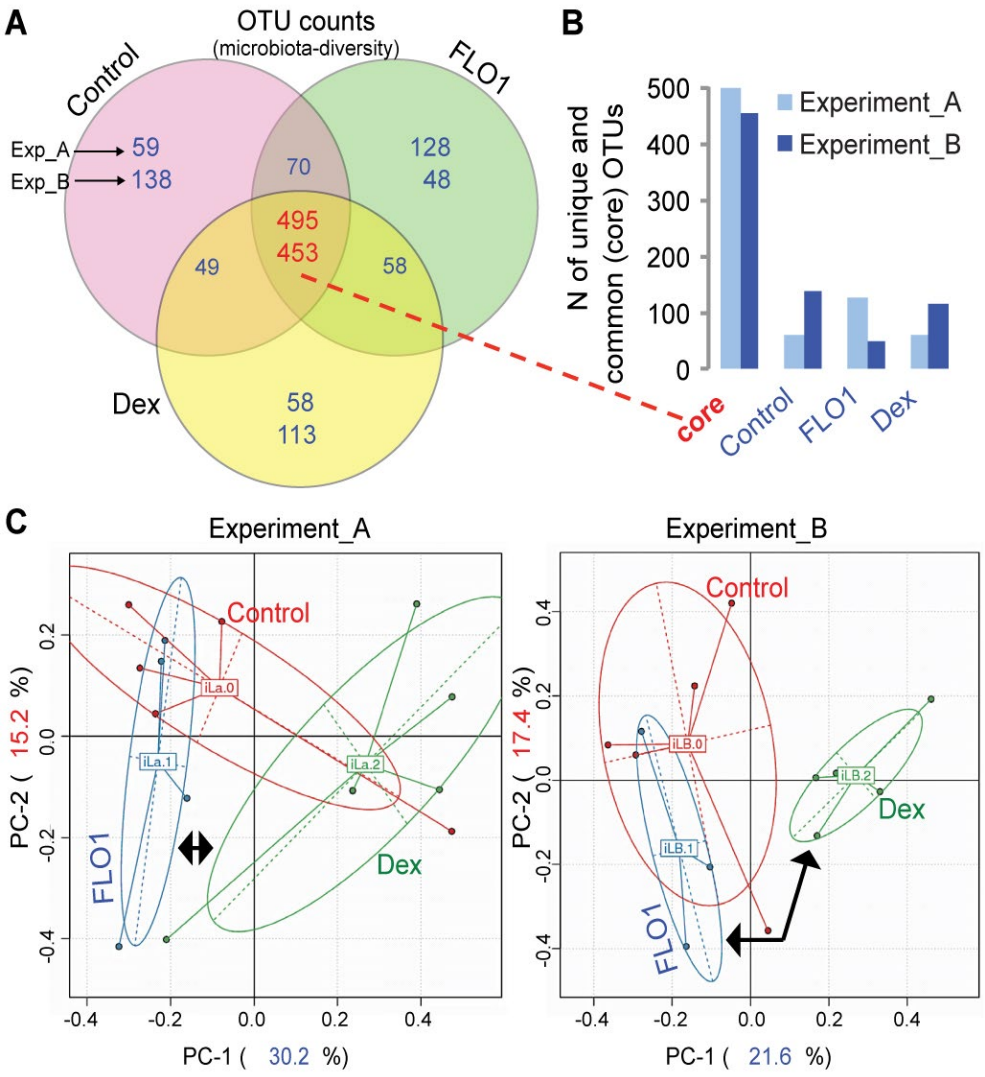

Fig. S3. Bacterial community structure shifts in FLO1- versus Dex-treatment and controls. (**A**) Venn diagram, and (**B**) histogram plot display the number of unique and common (core) OTU taxonomic species across groups in 2 separate experiments (Exp. A (n=14) and B (n=13), respectively). (**C**) Principal Component Analysis (PCA) ordination plot of faecal microbiota profiles of indicated groups (zero overlapping of sample variation; Two-tailed Fisher Exact p= 0.0079 for exp. A, and 0.0179 for exp. B) between the FLO1 and Dex in both experimental replicas, indicating that FLO1 reproducibly induces microbiota composition changes that are distinct from Dex in two independent experiments (Overall Two-tailed Fisher Exact p< 0.0001).

Table S1. Multivariable linear regression coefficients between relevant bacteria

	Parabacteroides distasonis	*Mucispirillum schaedleri*	*Lactobacillus salivarius*	*Lactococcus garvieae*	*Ruminococcus gnavus*
Parabacteroides distasonis		0.040 (0.36)	-0.198 (2.29)*	0.096 (1.53)	-0.282 (3.55)**
Mucispirillum schaedleri	0.036 (0.36)		-0.110 (1.31)	-0.043 (0.72)	0.246 (3.23)**
Lactobacillus salivarius	-0.276 (2.29)*	-0.170 (1.31)		0.200 (2.77)**	0.042 (0.42)
Lactococcus garvieae	0.261 (*1.53*)	-0.131 (*0.72)*	0.389 (2.77)**		0.138 (0.99)
Ruminococcus gnavus	-0.431 (3.55)**	0.417 (3.23)**	0.046 (0.42)	0.077 (0.99)	
Escherichia coli	0.003 (0.03)	0.026 (0.22)	0.204 (2.20)*	0.221 (3.45)**	-0.073 (0.81)
Helicobacter hepaticus	0.170 (1.49)	0.612 (5.92)**	0.241 (2.55)*	-0.077 (1.11)	0.009 (0.10)
[a] Group2(FLO1 treatment)	-0.528 (1.35)	0.952 (2.35)*	0.665 (2.03)*	0.391(1.66)	-0.451 (1.43)
[a] Group3(Dex treatment)	-0.772 (2.09)*	0.236 (0.59)	0.480 (1.52)	0.241(1.06)	-0.538 (1.79)
[b] Phase 1	0.722 (1.23)	-0.001 (0.00)	1.149 (2.36)*	0.037 (0.10)	-0.381 (0.80)
[b] Phase 2	3.084 (5.37)**	0.065 (0.09)	0.270 (0.48)	0.252 (0.63)	0.520 (0.98)
[b] Phase 3	3.049 (4.35)**	1.234 (1.54)	-1.070 (1.67)	0.161 (0.35)	1.335 (2.20)*
[c] Replica B	0.242 (0.71)	-0.459 (1.29)	-0.338 (1.18)	0.046 (0.22)	-0.153 (0.55)
R2	0.59	0.55	0.51	0.37	0.40
N	104	104	104	104	104

Data depicts Coef. and (SE); *p<0.05, **p<0.01. Positive coefficients indicate positive linear correlation between the model outcome (column name on top) and the independent variables in the model (the variable names for all rows in left column). acomparator group is Group1 (control), bcomparator group is Phase 0, ccomparator group is Replica A. Phase=timepoints (0=before IsPreFeH, 1=before FLO1/Dex, 2=after FLO1/Dex, 3=after DSS). Replica code indicates that model includes 2 independent experiments.

Table S2. Multinomial Logistic Predictive Model: Relative risk ratio (RRR) and predictive relationship between IL-1α neutralization and specific bacterial species.

	Control vs Dex			Control vs FLO1			FLO1 vs Dex		
Groupcode	**RRR**	**P>z**	**[95%C.I.]**	**RRR**	**P>z**	**[95%C.I.]**	**RRR**	**P>z**	**[95%C.I]**
Parabacteroides distasonis	1.49	0.05**	0.99, 2.24	1.26	0.3	0.80, 1.96	1.18	0.45	0.76, 1.85
Mucispirillum schaedleri	0.94	0.77	0.62, 1.41	0.54	0.007***	0.35, 0.84	1.72	0.01*	1.10, 2.70
Lactobacillus salivarius	0.81	0.38	0.50,1.30	0.54	0.02**	0.31, 0.93	1.49	0.17	0.83, 2.67
Lactococcus garvieae	0.5	0.06*	0.24,1.04	0.57	0.14	0.26, 1.22	0.88	0.74	0.43, 1.80
Ruminococcus gnavus	2.05	0.01***	1.17,3.61	1.33	0.32	0.75, 2.34	1.54	0.11	0.89, 2.67
Escherichia coli	1.1	0.67	0.69, 1.77	1.23	0.44	0.72, 2.09	0.89	0.69	0.52, 1.52
Helicobacter hepaticus	0.89	0.63	0.58, 1.39	2.23	0.003***	1.30, 3.81	0.4	0.001***	0.23, 0.69
Ruminococcus flavefaciens	1.41	0.11	0.91, 2.17	0.68	0.07*	0.45, 1.03	2.07	0.001***	1.32, 3.25
Clostridium methylpentosum	0.84	0.59	0.46, 1.54	1.41	0.29	0.73, 2.69	0.6	0.12	0.31, 1.14
Clostridium aldenense	1.31	0.65	0.39, 4.43	2.45	0.21	0.59, 10.13	0.53	0.4	0.12, 2.33
Blautia obeum	3.36	0.15	0.62, 18.00	7.19	0.06*	0.86, 59.84	0.46	0.5	0.05, 4.36
Phase – 0 vs 1	1.74	0.63	0.18,16.79	1.93	0.58	0.17, 21.06	0.9	0.93	0.09, 9.01
Phase – 0 vs 2	0.16	0.18	0.01,2.32	1.52	0.78	0.08, 26.51	0.11	0.13	0.006, 1.99
Phase – 0 vs 3	0.15	0.22	0.007, 3.16	0.56	0.71	0.02, 12.73	0.27	0.4	0.01, 5.68
2.Replicacode	1.24	0.74	0.32, 4.72	1.27	0.74	0.29, 5.52	0.977	0.97	0.22, 4.16
Costant	0.1	0.43	0.0003, 30.08	12.93	0.42	0.02, 7364.34	0.008	0.15	0.00001, 6.55

RRR of 1.0 indicates risk in each group is the same, RRR>1 suggests an increased risk of that outcome in first group vs. second group, RRR<1 suggests reduced risk in first group vs. second group. Phase=timepoints (0=before flora homogenization, 1=before FLO1/Dex, 2=after FLO1/Dex, 3=after DSS-induced colitis. Replica-code indicates that model includes 2 independent experiments. *p<0.05, **p<0.01, *** p≤0.007.

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