SUPPLEMENTARY MATERIALS FOR

Pasteurized *Akkermansia muciniphila* promotes GP2 expression in Microfold cells and facilitates *Salmonella* infection

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Mouse experiments

All mouse experiments were approved by local authorities. Eight-week years old C57BL/6J female mice were maintained in Biological Safety Level 2 (P2) animal facility. For STm infection, mice were oral administrated 20 mg/per mouse streptomycin, 24 hours before colonization. All mice were taken off food and water for 6 hours, and then gavaged either with 10^7 colony-forming units (CFU) STm or 2 x 10^9 CFU *Citrobacter rodentium* (Cr) in 100 µl PBS, supplemented with a daily dose of 100 µl PBS or PBS containing 10^9 CFU pasteurized *Akkermansia muciniphila* (pAKK) before sacrifice. Feces were collected daily and homogenized in sterile PBS in a TissueLyzer (JINGXIN) for 1 min at 30 Hz and then plating serial dilutions on the Salmonella-Shigella agar to determine CFU. Feces in Cr group were collected every 4 days and bacteria load were measured by quantitative PCR (See quantitative PCR for details). Mice were sacrificed by cervical dislocation after starving for 6 hours at 4 days p.i. for STm and 12 days p.i. for Cr. STm burdens in mesenteric lymph nodes (MLN) and spleen were determined using the same procedure of that in fecal samples. Ceca of mice were fixed in 4% paraformaldehyde (PFA, Servicebio, #G1101), embedded in paraffin and stained with hematoxylin and eosin (H&E) according to standard protocols, histology score is determined from four aspects: depletion of goblet cells, inflammatory cell infiltration, the extent of submucosal edema and epithelial integrity, and each category scores from 0 to 3 (Pedicord et al., 2016).

Ileum ligated loop assays were performed as previously described.(Fukuda et al., 2011) Briefly, mice were anaesthetized and injected with 10^6 CFU STm in the ileum ligated loop centered around a Peyer's patch. After 1 hour, PPs are washed by PBS three times and then homogenized for STm load analysis. Additional SPF mice were treated by daily gavage with 10^9 CFU pAKK for 2 weeks, and then number of PPs were measured and collected for further RNA expression analysis.

Bacteria

Salmonella enterica serotype Typhimurium TA 1537(Cat#1.1194) and *Citrobacter rodentium* DBS100 (ATCC 51459) were used in this study. Both strains were cultured in LB (Luria Broth) overnight at 37 °C, 200 rpm, followed by 5 hours sub-culture to log-phase growth. Both were washed once and resuspended in sterile PBS and diluted into indicated concentrations for colonization. *Akkermansia muciniphila* DSM 26127 was cultured in anaerobic GAM overnight at 37 °C, washed and resuspended in sterile PBS, and pasteurized at 70 °C for 30 min. For STm growth experiments, 0.1% STm were transferred into fresh LB or M9 medium in 96-well plates with or

without 1% pAKK and cultured in 37 °C, 200 rpm. OD_{600} was measured every two hours using Multiskan Spectrum (Bio Tek).

Cellular experiments

In vitro M cell culture was adopted from Beloqui *et al* (Beloqui et al., 2017). Briefly, Caco-2 cells (ATCC HTB-37) was cultured in DMEM (Gibco, #C11995500BT) supplemented with 10% FBS (Gemini, #900-108), 1% Glutamax (Gibco, #35050061) and 1% NEAA (Gibco, #11140050) on 6 transwell inserts (Corning, #3420) or 12 transwell inserts (LABSELECT, #14222) for 2 weeks to stimulate enterocyte-like cells, and then the cell monolayers were treated by medium with 10% pAKK, 50 ng/mL RANKL (MCE) or 100 μg/mL Amuc_1100 for 6 days. 10 μg/mL Denosumab (anti-TNFSF11, MCE, #HY-P9958A) was added to block RANKL signals in pAKK and RANKL group. Cells incubated with pAKK were also supplemented with 25 μM C29 (TLR2 inhibitor, MCE, #HY-100461), 10μM T6167923 (MyD88 inhibitor, MCE, #HY-19744) or transfected with 16nM NF-κB siRNA (targeting RelA, Sangon Biotech) using jetPRIME mix (Polyplus-transfection, #101000046).

Immunofluorescence staining

Cells on transwell inserts were washed by phosphate-buffered saline (PBS, Solarbio, #P1020) twice, and fixed by 4% PFA (Servicebio, #G1101) at 4°C for 30 min. After incubation with block buffer (Beyotime, #P0260) for 1 h, specimens were incubated with anti-GP2 primary antibody (GP2 Rabbit pAb, ABclonal, #A12438) diluted at 1:200 by dilution buffer (Beyotime, #P0262) overnight in 4 °C, and then specimens were incubated with second antibody (Goat Anti-Rabbit IgG Alexa Fluor® 488, Abcam, #ab150077) with 1:1000 dilution by buffer for 1 h, followed by 5-min Hoechst 33342 (Abcam, #ab285390) staining with 1:1000 dilution by PBS. Cell layers were finally observed by STED microscope (Leica) in 40x objectives.

For immunostaining of Peyer's patches, the Peyer's patches of mice were embedded in Tissue Freezing Medium (SAKURA, #4583), followed by snap-freezing in liquid nitrogen and stored at -80°C. Then the tissue was sliced into 5μm sections using a cryostat microtome (Leica, CM3050S), fixed with 4% PFA for 20 minutes, and washed with PBS for 3 minutes. Specimens were blocked with 3% BSA solution for 2 h, and then incubated with anti-GP2 primary antibody (GP2 Rabbit pAb, ABclonal, #A12438) at a 1:100 dilution overnight at 4°C. After that the specimens were washed 5 times with PBS for 5 minutes each, then incubated with the secondary antibody (Goat Anti-Rabbit IgG Alexa Fluor® 488, Abcam, #ab150077) at a 1:1000 dilution in PBS for 1 hour, followed by a 5-minute staining with Hoechst 33342 (Abcam, #ab285390) at a 1:1000 dilution in PBS. After 4 times washing with PBS for 5 minutes each and a final wash with 0.2% PBST, specimens was ready for observation by STED microscope (Leica) in 100x oil objectives.

Quantitative PCR or RT-PCR

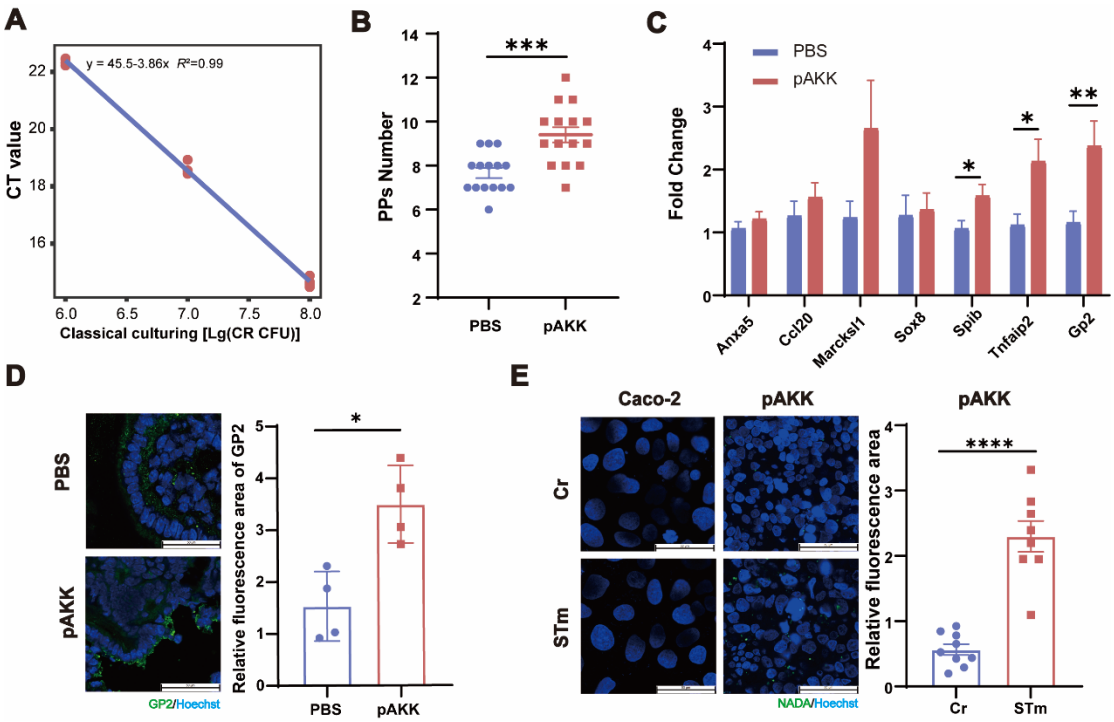
Fecal DNA of Cr group were extracted by DNeasy PowerSoil Pro Kit (Qiagen, #47016) and utilized in following qPCR with Taq Pro Universal SYBR qPCR Master Mix (Vazyme, #Q712) in an QuantStudio 7 system (Life Technologies). RNA from PPs and cells was extracted by TRIzol (Invitrogen, #15596018), and qRT-PCR were performed using HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme, #Q221-01) in QuantStudio 7 system. All primers are listed in Supplementary Table 1.

Binding assay

All bacteria used for the binding assay were first cultured overnight in LB medium containing 25μM NADA-Green (GLPBIO, # GC50534) to ensure bacterial surfaces were labeled with NADA-green. Caco-2 cells were seeded at 2×10^5 cells per well on coverslips in a 24-well plate, then used for binding assay when the cells reached confluence of over 90% in 36 h. NADA-labeled STm and Cr were added at a concentration of 2×10^7 CFU to Caco-2 cells and M-like cells per well. The bacteria and cells were co-incubated at 37°C in the dark for 2 hours. After incubation, the wells were washed twice with PBS, and the cells were fixed with 4% PFA (Servicebio, #G1101). After two additional washes with PBS, the cells were stained with Hoechst 33342 (Abcam, #ab285390) for 5 minutes. Subsequently, the wells were washed twice with PBS and mounted for observation by STED microscope (Leica) in 100x oil objectives.

Supplementary Table 1 Primers used in qPCR and qRT-PCR assays. *'m' denotes *Mus musculus* and 'h' denotes *Homo sapiens*.

Target gene*	Primers (5'-3')
mGp2	ACAGTGTCAACCATCTTGCTC
	CCCGATTATAGTCAATGGCTGG
mTnfaip2	AGGAGGAGTCTGCGAAGAAGA
	GGCAGTGGACCATCTAACTCG
mSpib	AGGAGTCTTCTACGACCTGGA
	GAAGGCTTCATAGGGAGCGAT
mSox8	CGAGGGGATACTGCTGAGG
	AGCTCTGCGTTATGGAGATGC
mMarcksl1	CAATGGAGACTTAACCCCCAAG
	GGCCACTCAATTTGAAAGGCT
mAnxa5	ATCCTGAACCTGTTGACATCCC
	AGTCGTGAGGGCTTCATCATA
mCcl20	GCCTCTCGTACATACAGACGC
	CCAGTTCTGCTTTGGATCAGC
mGapdh	TGTGTCCGTCGTGGATCTGA
	TTGCTGTTGAAGTCGCAGGAG
hGapdh	GCCTTCCGTGTCCCCACTGC
	GGCTGGTGGTCCAGGGGTCT
*h*Gp2	TTCCCTGAACGTCAGTGTGG
	AGCACGGACTCAACAGACA
espB (for Cr)	ATGCCGCAGATGAGACAGTTG
	CGTCAGCAGCCTTTTCAGCTA



Supplementary Figure 1. (A) The standard curve of the CT value versus lg(CFU) for Cr. (B) The number of PPs in the small intestine in 2-week pAKK treatment to uninfected mice, n=15 per group. (C) Relative expression of M-cell marker genes, n=10 per group, determined by fold change compared to PBS group using *Gapdh* as endogenous control. (D) Representative images (left) and quantitative data (right) of fluorescence staining of GP2 in PPs in the small intestine of uninfected mice after 2-week gavage of pAKK; Green: GP2; Blue: Hoechst, scale bar: 50 μm. *Gp2* expression levels in each group (n=4). (E)Binding assay result of STm and Cr to Caco-2 cell line with or without pAKK treatment. Quantitative data (right) of Caco-2 cell line after pAKK treatment are presented. Green: NADA; Blue: Hoechst, scale bar: 50 μm. Statistical significance was assessed by Mann-Whitney tests, *p<0.05, **p<0.01, ***p<0.001. Values are mean±SEM.

References

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