

LETTER

Pasteurized *Akkermansia muciniphila* promotes GP2 expression in microfold cells and facilitates *Salmonella* infectionWenhui Zhang^{1,2,†}, Gan Xi^{1,2,†}, Huaiwu Zhang^{1,2}, Jinmiao Bi^{2,3,4,5,6}, Tongtong Zhou^{1,2}, Junhao Zhu^{1,2}, Zhan Zhang⁷, Shuo Wang^{1,2}, Moshi Song^{2,3,4,5,6,*}, Jun Wang^{1,2,*}¹CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China²University of Chinese Academy of Sciences, Beijing 100049, China³Key Laboratory of Organ Regeneration and Reconstruction, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China⁴State Key Laboratory of Membrane Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China⁵Beijing Institute for Stem Cell and Regenerative Medicine, Beijing 100101, China⁶Institute for Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing 100101, China⁷Center for Global Health, Nanjing Medical University, Nanjing 210029, China[†]These authors contributed equally to this work.^{*}Correspondence: songmoshi@ioz.ac.cn (M. Song), junwang@im.ac.cn (J. Wang)

Dear Editor,

In the intestine of humans and other animals, the commensal microbiome has complex roles in shaping infections (Khan et al., 2021). Commensal microbiome contains opportunistic pathogens that are usually suppressed but can cause infection under microbiome dysbiosis or environmental disturbances (Dey and Ray Chaudhuri, 2023). The commensal gut microbiome can directly outcompete and exclude pathogens in the gut, providing a line of defense against infections (Pickard et al., 2017). Indirectly, gut microbiome modulates the metabolic processes of the intestine and other organs through production of diverse metabolites, such as short-chain fatty acids that promote innate immune responses; and complex immune responses by interaction with immune cells, including macrophages, innate lymphoid cells (ILCs), and natural killer (NK) cells, which are essential for the clearance of pathogens (Pickard et al., 2017; Yoo et al., 2020).

Based on the above findings, the gut commensal *Akkermansia muciniphila* (AKK) is a promising probiotic in relieving metabolic diseases, infections, and inflammation (Bian et al., 2019; Chen et al., 2020; Everard et al., 2013). AKK was first investigated in clinical trials

showing that both live and pasteurized AKK had significant effects in attenuating metabolic disorders and low-grade inflammation; in particular, pasteurized AKK had higher efficacy due to a higher exposure of a pili-like protein Amuc_1100 that promotes GLP-1 production from the epithelia cells (Cani et al., 2022; Everard et al., 2013). More recently, research shows that AKK also displays protective effects in sepsis, virus infections like H7N9 influenza virus, and enteric infection, such as *Clostridioides difficile* infection (Li et al., 2024). The latter is attributed to that AKK enhances the gut barrier function by degradation of mucins, which is facilitated by that AKK selectively binds to O-glycan structures found on colonic mucins, promoting tight junction gene expression, and reducing local and systemic immune response (Cani et al., 2022; Shuoker et al., 2023; Elzinga et al., 2024; Li et al., 2024). Despite its benefits, AKK is also increasingly associated with a number of autoimmune and neurological disorders including Parkinson's disease (PD) and multiple sclerosis (MS) (Fang et al., 2021; Vidal-Martinez et al., 2020). Thus, AKK's role in fending off infections might be context-dependent and might vary according to the pathogen, calling for case-by-case studies.

Accepted 9 February 2025.

© The Author(s) 2025. Published by Oxford University Press on behalf of Higher Education Press.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

Salmonella enterica serotype Typhimurium (STm) and *Citrobacter rodentium* (Cr) are two common pathogens used in mouse models to mimic enteric infections in humans (Collins et al., 2014; Galan, 2021). Here, we investigated the role of pasteurized AKK (pAKK), a form that has been established to outperform live bacteria in treating metabolic disorders, against enteric infections by *Salmonella* and *Citrobacter*. We started by gavaging mice with a daily dose of 10^9 CFU of pAKK, after infection with 10^7 CFU STm or 2×10^9 CFU Cr (Fig. 1A). In STm infectious model, we first found that pAKK-treated mice exhibited significantly higher weight loss 4 days post-infection (p.i.), and significantly increased titers of STm were detected in the feces compared to PBS-control mice from 1 day p.i. to 4 days p.i. (Fig. 1B and 1C). We additionally found significantly higher titers of STm in mesenteric lymph nodes (MLN) and spleen in pAKK group (Fig. 1D and 1E). In addition, pAKK treated mice displayed significantly more severe inflammation, epithelial damage and colitis in the cecum compared to the control group (Fig. 1F and 1G). These results demonstrate that pAKK gavage enhances the invasion and pathogenesis of *S. Typhimurium*.

In contrast to the STm-infected mice, we found that pAKK gavage had no detectable effects on the pathogenesis of Cr. We found no significant difference in weight loss between the pAKK and PBS group (Fig. 1H). In addition, mice consuming pAKK showed a reduced fecal bacteria burden 8 days and 12 days p.i., although not significantly (Fig. 1I and 1J). Furthermore, mice displayed similar pathogenesis with or without pAKK treatment, as indicated by the decrease of colonic length or pathology score in the gastrointestinal tract of mice (Fig. 1K–M). In summary, we found that the effects of pAKK gavage differ between STm and Cr infection, and more specifically, gavage of pAKK significantly promotes STm infections in mice.

Supplementation of pAKK did not increase STm titers in LB or M9 medium, indicating that pAKK may instead promote STm indirectly (Fig. 2A). We then began to dissect the difference in pAKK's effect on STm and Cr infections. STm differs from Cr mainly in the infection cycle: STm depends on intracellular replication within the membrane-bound compartment (*Salmonella*-containing vacuole), while Cr is an extracellular intestinal pathogen (Collins et al., 2014; Galan, 2021). More specifically, STm has a preference for microfold cells (M cells), which are specialized intestinal epithelial cells primarily residing in Peyer's patches and process pathogens and antigens to underlying organized lymphoid follicles (Hase et al., 2009). Additionally, we did not detect STm in the liver (data not shown). The increased STm load in spleen but not liver suggests that STm travels from PPs to mesenteric lymph nodes and then spread to spleen but not easily via the hepatoportal vein to liver. To check this

hypothesis, we conducted a ligated loop assay (adapted from (Fukuda et al., 2011)) in which we directly exposed Peyer's patches (PPs) to 10^6 CFU of STm for 1 h, with or without addition of pAKK (Fig. 2B). Results indicated that the addition of pAKK significantly increased STm titers in Peyer's patches compared to the control group (Fig. 2C). We then detected the recognized M cell marker gene expressions in two groups, while among these markers, *Ccl20* is the special follicle-associated epithelium (FAE) related gene, *Anxa5* and *Marcks1* are early M cell markers that may determine the early differentiation of M cells, *Sox8* and *Spib* are essential to the M cell maturation to directly bind to *Gp2* promoter and activate the transcription, *Tnfaip2* is a SPIB-dependent immature M cell marker, and finally, *Gp2* is a gene encoding an uptake receptor to FimH⁺ bacteria including STm and only expressed by mature M cells in the intestine (Hase et al., 2009; Kobayashi et al., 2019) (Fig. 2D). It is also worth noting that pAKK enhanced the expression of M cell related genes *Ccl20* and *Gp2* (Fig. 2D). In summary, pAKK promotes microfold cells and consequently facilitates the STm infection.

We then examined the roles of pAKK on M cells in mice without STm infection, by gavaging pAKK daily to specific-pathogen-free (SPF) mice for 2 weeks. We found a significant increase in the number of Peyer's patches in the pAKK-gavaged group compared with PBS control (Fig. S1B). We also observed an elevated mRNA level of M cell marker genes including *Spib*, *Tnfaip2* and *Gp2* in Peyer's patches of mice treated with pAKK (Fig. S1C). To further elucidate the effect of pAKK pretreatment on STm infection, we applied the ligated loop assay on mice after a 2-week gavage of pAKK. We found that the number of PPs after 2 weeks of pAKK treatment had significantly increased compared to the PBS group, and the PPs had significantly higher levels of GP2 and an increased number of microfold cells as revealed by immunostaining; correspondingly (Figs. 2E and S1D), pAKK-treated mice also had significantly higher STm load in the ligation assay where individual PPs were exposed to STm (Fig. 2F). We also aimed to determine whether the adhesion of STm and Cr to GP2 and especially the glycans on this surface protein differs, acting via their type I fimbriae (FimH). Thus, we investigated the binding capabilities of STm and Cr in both pAKK-induced M-like cells and Caco-2 cells. The results indicate that Cr exhibits significantly lower binding to M-like cells compared to STm, suggesting a potential mechanism for their differential ability to interact with GP2 glycosylation (Fig. S1E).

We next investigated the potential mechanism of pAKK in promoting M cells. M cells are considered as highly differentiated enterocytes and it has been reported that *in vivo* they arise from crypt stem cells after induction by the receptor activator of NF- κ B ligand (RANKL), which then activates non-canonical NF- κ B pathway (Kobayashi

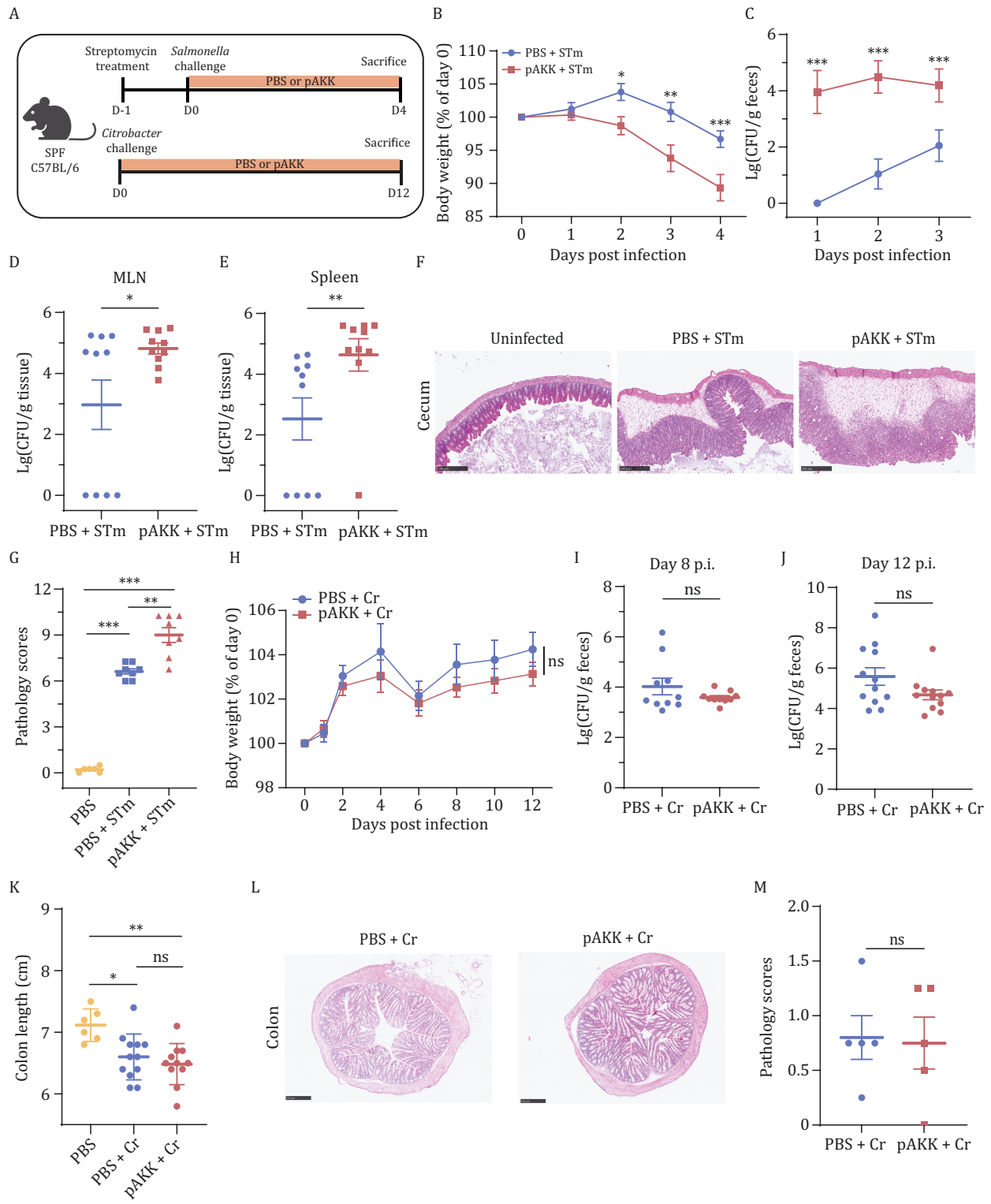


Figure 1. pAKK promotes STm but not Cr infection in a murine model. (A) Experimental design. 1) C57BL/6 mice were orally gavaged with streptomycin before treated with 10^7 colony-forming units (CFU) STm, supplemented with a daily dose of 100 μL PBS or PBS containing 10^9 CFU (pAKK) before sacrifice. Feces and tissue were collected for the determination of bacterial burdens and histopathology analysis. 2) C57BL/6 mice were orally gavaged with a daily dose of 100 μL PBS or PBS containing 10^9 CFU pAKK after treated with 2×10^9 CFU Cr. Created with BioRender.com. (B) Body weight change of mice post STm infection; $n = 10$ per group. (C) Daily change of STm load in feces, measured by plating after serial-dilution, $n = 10$ per group. (D and E) STm burden 4 days post-infection (p.i.) in mesentery lymph nodes (D) and spleen (E), $n = 10$ per group. (F) Representative images of hematoxylin and eosin (H&E) staining of cecum sections; scale bar: 250 μm. (G) Pathology scores of samples in (F), $n = 6$ for PBS group, $n = 8$ for PBS + STm and pAKK + STm group. (H) Body weight change of mice post Cr infection; $n = 12$ per group. (I and J) Cr load in feces 8 days p.i. (I),

et al., 2019) (Fig. 2H). We employed an *in vitro* transwell system to study M cell differentiation using Caco-2 cells (a human colorectal cancer cell line) (Beloqui et al., 2017) (Fig. 2G). By adding pAKK or RANKL protein to culture medium of Caco-2 monolayers and culturing for 6 days, we found that pAKK treatment led to a significant elevation of Gp2 expression; and different from RANKL, this effect could not be ablated by RANKL antibodies, suggesting an independent pathway that promotes Gp2 expression (Figs. 2I, 2J and S1A).

Pasteurized AKK and its outer membrane protein Amuc_1100 are suggested to be capable of activating the canonical NF- κ B pathway through TLR2-MyD88 signaling, which may activate the differentiation to M cell indirectly (Cani et al., 2022) (Fig. 2H). To verify this hypothesis, we first used siRNA to knock down the expression of RelA, an essential component for canonical NF- κ B pathway, in Caco-2 cells treated with pAKK. Results showed that Gp2 mRNA levels were significantly decreased compared to the Caco-2 cells treated with pAKK, indicating that canonical NF- κ B pathway was required for pAKK-induced M cell differentiation (Fig. 2K). Furthermore, supplementation with TLR2 inhibitors C29 or MyD88 inhibitors T6167923 led to the ablation of Gp2-upregulating effect of pAKK (Fig. 2K). Finally, we tested the addition of Amuc_1100 protein, the identified TLR2 ligand from pAKK, and found that this protein alone was sufficient to upregulate Gp2 expression (Fig. 2K). Thus, we demonstrated that pAKK bypasses RANKL signaling by activating TLR2-MyD88-NF- κ B pathway and promotes the differentiation of M cells.

In conclusion, our study presents a cautionary tale for the application of probiotics, in this case the commonly called “next-generation” probiotic *Akkermansia muciniphila* that is noted for its ability to alleviate metabolic disorders in the pasteurize form. We found that its capacity to promote the specific M cells in the intestine can be exploited by STm to aggravate its infection in the murine model. We demonstrated that pAKK increases the number of M cells and even Peyer’s patches, a gut-associated lymphoid tissue where M cells reside, in uninfected mice; as well as in an *in vitro* culture system utilizing Caco-2 cells. While research shows that RANKL is essential for M cell formation *in vivo* and in gut organoids (Kobayashi et al., 2019; Luna Velez et al., 2023), here in our study, we provided evidence for a novel, gut-microbial-originated M cell differentiation and maturation signal, that pAKK can induce M cell formation

and maturation *in vitro* by promoting TLR2-MyD88-dependent signaling that activates canonical NF- κ B pathway. We expect this to hold *in vivo*, which remain to be investigated using RANKL blocking antibody in the future experiments; and more importantly in human if feasible. M cells serve as antigen-sampling cells that initiate mucosal immune response but are also portals of infection for a series of pathogens, which include known bacterial pathogens include *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Mycobacterium avium* in addition to STm, and viruses including HIV type 1, influenza, polio and reovirus (Fujimura et al., 2004; Gonzalez-Hernandez et al., 2014). Thus, the activation of M cell by pAKK can be beneficial or detrimental depending on the contexts, and application of pAKK in the presence of pathogens that exploit M cells thus has considerable risk and demands particular caution.

Supplementary data

Supplementary data is available at *Protein & Cell* online <https://doi.org/10.1093/procel/pwaf017>.

Footnotes

This work is supported by the National Key Research and Development Program of China (2022YFC2303200, 2020YFA0113400), the National Natural Science Foundation of China (92368112), Beijing Natural Science Foundation (JQ22017), CAS Project for Young Scientists in Basic Research (YSBR-076), Initiative Scientific Research Program of the Institute of Zoology, Chinese Academy of Sciences (2024IOZ0103, 2023IOZ0202), and State Key Laboratory of Membrane Biology and Key Laboratory of Organ Regeneration and Reconstruction of the Chinese Academy of Sciences. We appreciate the support from the CAS Key Laboratory of Pathogenic Microbiology and Immunology at Institute of Microbiology. We also thank the BioRender platform for schematic diagram.

All animal procedures were performed in accordance with the standards of the Department of Health and Human Services and under protocols approved by the Ethics Committee of Institute of Microbiology, Chinese Academy of Sciences (IMCAS) (permit APIMCAS2021002).

WZ, GX, HZ, and JW conceptualized the study. WZ, GX, JB, HZ, ZZ, SW, MS, and JW developed the methodology, conducted experiments, and interpreted data. WZ, GX, and JW prepared the initial manuscript draft and visualizations. MS and JW revised the manuscript and provided

n = 10 per group, and 12 days p.i. (J), n = 12 per group. (K) Colon length of mice 12 days p.i. (PBS n = 6, PBS + Cr n = 12, AKK + Cr n = 12). (L) Representative images of hematoxylin and eosin (H&E) staining of cecum sections; scale bar: 250 μ m. (M) Pathology scores of samples in (L), n = 5 for each group. Statistical significance was assessed by Mann-Whitney U test or unpaired Student’s t-test, ns denotes no significance, *P < 0.05, **P < 0.01, ***P < 0.001. All values are presented by mean \pm SEM. pAKK: pasteurized *Akkermansia muciniphila*, STm: *Salmonella enterica* serotype Typhimurium, Cr: *Citrobacter rodentium*.

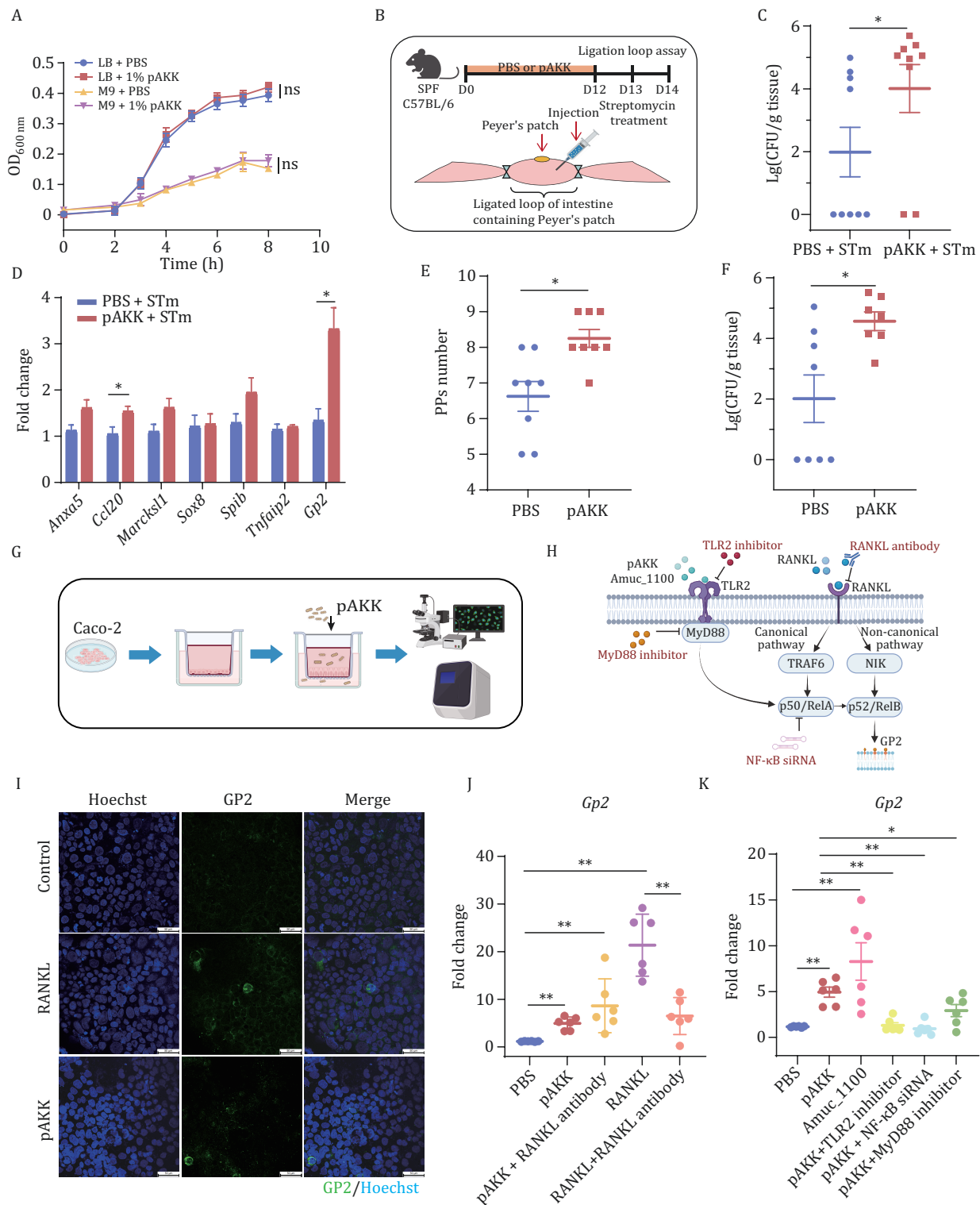


Figure 2. pAKK increases the number of microfold cells in vivo and in vitro. (A) Growth curve of STm incubated with pAKK and control; each time point was measured in triplicates. (B) Schema of ligated loop assay, adopted from (Fukuda et al., 2011). (C) STm load in PPs in the ligated loop assay, $n = 9$ per group. (D) Relative expression of M cell marker genes in ligated loop assay ($n = 5$ per group) determined by fold change compared to PBS group using *Gapdh* as endogenous control. (E) The number of PPs in the small intestine in 2-week pAKK treatment to uninfected mice, $n = 8$ per group. (F) STm load in PPs in the ligated loop assay on mice after 2-week gavage of pAKK, $n = 8$ per group. (G) Schematic graph of transwell experiment. Created with BioRender.com. (H) Proposed mechanism of the pAKK or Amuc_1100 promotes GP2 expression in vivo. TRAF6: tumor necrosis factor (TNF) receptor-associated factor 6, NIK: NF- κ B-inducing kinase, adopted from (Kobayashi et al., 2019). Created with BioRender.com. (I) Representative images of fluorescence staining of GP2 in M-like cells; scale bar: 50 μ m. (J and K) *Gp2* expression levels in each group ($n = 6$). Statistical significance was assessed by Mann-Whitney U test or unpaired Student's t -test, ns denotes no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Values are mean \pm SEM.

research supervision. JW was responsible for overall project administration and supervision.

All authors declare no conflict of interest. All of them confirm their willingness to participate and publish.

All data associated with this study are present in the main text or the [Supplementary Materials](#).

References

- Beloqui A, Brayden DJ, Artursson P et al. A human intestinal M-cell-like model for investigating particle, antigen and microorganism translocation. *Nat Protoc* 2017;**12**:1387–1399.
- Bian X, Wu W, Yang L et al. Administration of *Akkermansia muciniphila* ameliorates dextran sulfate sodium-induced ulcerative colitis in mice. *Front Microbiol* 2019;**10**:2259.
- Cani PD, Depommier C, Derrien M et al. *Akkermansia muciniphila*: paradigm for next-generation beneficial microorganisms. *Nat Rev Gastroenterol Hepatol* 2022;**19**:625–637.
- Chen Z, Qian X, Chen S et al. *Akkermansia muciniphila* enhances the antitumor effect of cisplatin in Lewis lung cancer mice. *J Immunol Res* 2020;**2020**:2969287.
- Collins JW, Keeney KM, Crepin VF et al. *Citrobacter rodentium*: infection, inflammation and the microbiota. *Nat Rev Microbiol* 2014;**12**:612–623.
- Dey P, Ray Chaudhuri S. The opportunistic nature of gut commensal microbiota. *Crit Rev Microbiol* 2023;**49**:739–763.
- Elzinga J, Narimatsu Y, de Haan N et al. Binding of *Akkermansia muciniphila* to mucin is O-glycan specific. *Nat Commun* 2024;**15**:4582.
- Everard A, Belzer C, Geurts L et al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci U S A* 2013;**110**:9066–9071.
- Fang X, Li FJ, Hong DJ. Potential role of *Akkermansia muciniphila* in Parkinson's disease and other neurological/auto-immune diseases. *Curr Med Sci* 2021;**41**:1172–1177.
- Fujimura Y, Takeda M, Ikai H et al. The role of M cells of human nasopharyngeal lymphoid tissue in influenza virus sampling. *Virchows Arch* 2004;**444**:36–42.
- Fukuda S, Hase K, Ohno H. Application of a mouse ligated Peyer's patch intestinal loop assay to evaluate bacterial uptake by M cells. *J Vis Exp* 2011;**58**:3225.
- Galan JE. Salmonella Typhimurium and inflammation: a pathogen-centric affair. *Nat Rev Microbiol* 2021;**19**:716–725.
- Gonzalez-Hernandez MB, Liu T, Payne HC et al. Efficient norovirus and reovirus replication in the mouse intestine requires microfold (M) cells. *J Virol* 2014;**88**:6934–6943.
- Hase K, Kawano K, Nochi T et al. Uptake through glycoprotein 2 of FimH⁺ bacteria by M cells initiates mucosal immune response. *Nature* 2009;**462**:226–230.
- Khan I, Bai Y, Zha L et al. Mechanism of the gut microbiota colonization resistance and enteric pathogen infection. *Front Cell Infect Microbiol* 2021;**11**:716299.
- Kobayashi N, Takahashi D, Takano S et al. The roles of Peyer's patches and microfold cells in the gut immune system: relevance to autoimmune diseases. *Front Immunol* 2019;**10**:2345.
- Li L, Li M, Chen Y et al. Function and therapeutic prospects of next-generation probiotic *Akkermansia muciniphila* in infectious diseases. *Front Microbiol* 2024;**15**:1354447.
- Luna Velez MV, Neikes HK, Snabel RR et al. ONECUT2 regulates RANKL-dependent enterocyte and microfold cell differentiation in the small intestine; a multi-omics study. *Nucleic Acids Res* 2023;**51**:1277–1296.
- Pickard JM, Zeng MY, Caruso R et al. Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunol Rev* 2017;**279**:70–89.
- Shuoker B, Pichler MJ, Jin C et al. Sialidases and fucosidases of *Akkermansia muciniphila* are crucial for growth on mucin and nutrient sharing with mucus-associated gut bacteria. *Nat Commun* 2023;**14**:1833.
- Vidal-Martinez G, Chin B, Camarillo C et al. A Pilot Microbiota Study in Parkinson's disease patients versus control subjects, and effects of FTY720 and FTY720-mitoxoy therapies in Parkinsonian and multiple system atrophy mouse models. *J Parkinsons Dis* 2020;**10**:185–192.
- Yoo JY, Groer M, Dutra SVO et al. Gut microbiota and immune system interactions. *Microorganisms* 2020;**8**:2046.