Supporting Information for

Original article

Gut commensal metabolite rhamnose promotes macrophages phagocytosis by activating SLC12A4 and protects against sepsis in mice

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1. Supporting materials and methods

1.1. Antibiotics post-treatment sepsis model

Experimental for antibiotics post-treatment sepsis model, it was performed as previously described[1]. Briefly, mice were pretreated with PBS or rhamnose 2 h prior to CLP surgery by using an 18-gauge needle to induce sepsis, and 6 h after the cecum was punctured, Imipenem/Cilastatin (Ipm/Cln, 14 mg/kg, i.p.) was treated to mice. Tissue was collected at 12 h after CLP surgery.

1.2. *E. coli* growth curve

For the *E. coli* growth assay, peritoneal lavage fluid (PLF) was collected from *E. coli* injected (i.p.) ABX and no-ABX pretreated mice and then 10% PLF was added into the bacterial culture medium. *E. coli* growth was monitored at OD600 spectrophotometrically.

1.3. Histological analysis

Lung tissues were fixed in 10% neutral buffered formalin (Cat# DF0110, Leagene Biotechnology, China) for 24 h, embedded in paraffin, sliced into 4 μm-thick sections and stained with hematoxylin and eosin. The mean histological score for organ injury was calculated by taking an average of six random fields of view per slide. The histopathological scoring analysis was performed as described previously[2]. The change in histological grading on a scale of 0 to 3 was evaluated by the parameters for neutrophil infiltration, mean septal thickening, vascular congestion, and patchy hemorrhage.

1.4. Cell isolation and culture

Experiment for human blood monocyte-derived macrophages (MDMs) differentiation. PBMC were isolated from human blood samples by Ficoll-Paque Plus (Cat# 45-001-751, GE Health Care, Sweden) density-gradient centrifugation, followed by $CD14^+$ magnetic bead selection (Cat# 130-042-401, Miltenyi Biotec, Bergisch, Gladbach, Germany). Magnetic cell sorting of epidermal and dermal cell suspensions was performed according to manufacturer recommendations. MDMs were cultured for 7 days in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Cat# 10099-141, Gibco, USA) and 1% penicillin–streptomycin-glutamine (Cat# 15140-122, Gibco, USA) with 10 ng/mL of

human macrophage colony-stimulating factor (hMCSF) from Novoprotein (Cat# C003, Suzhou, China)[3]. The human acute monocytic leukemia cell line (THP-1) was maintained in RPMI 1640 medium (Cat# C118775500BT, Gibco, USA) supplemented with 10% FBS and 1% penicillin–streptomycin and cultured in a 37 °C incubator. Human macrophages were differentiated from THP-1 monocytes by stimulating them with phorbol 12-myristate 13-acetate (PMA, 100 ng/mL, Cat# P1585, Sigma, USA) for 48 h. After removing the PMA-supplemented media, the cells were cultivated under normal conditions for 24 h before using them for further studies.

Murine bone marrow-derived macrophages (BMDMs) were harvested as previously described[4]. Briefly, bone marrow cells were isolated from C57BL/6 mice between the age of 6–8 weeks and cultured in DMEM (Cat# C11995500BT, Gibco, USA) containing 10% FBS, 1% penicillin–streptomycin and 20 ng/mL of macrophage colony-stimulating factor (M-CSF, Cat# 416-ML, R&D Systems, USA) until they attain complete differentiation stage.

Mouse bone marrow-derived neutrophils (BMDNs) were purified by a Histopaque (1077/1119, Sigma, USA) gradient centrifugation according to the manufacturer's instructions, and then cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin–streptomycin for 2 h at 37 °C in a humidified incubator with 5% CO_2 before using them for further experiments.

1.5. Western blot analysis

For activated Rac1 and Cdc42 assay, BMDMs and THP-1 derived macrophages were pretreated with or without rhamnose (100 μmol/L) for 18 h and then incubated with *E. coli* at a ratio of 60:1 for 5 min, next, cells were harvested for the detection. For the detection of GTP-Rac1 and GTP-Cdc42 in mice, peritoneal lavage fluid (PLF) cells were collected at 3 h after CLP. Anti-F4/80 MicroBeads UltraPure (Cat# 130-110-443, Miltenyi Biotec, Bergisch, Gladbach, Germany) were used for the enrichment of peritoneal macrophages from peritoneal lavage fluid (PLF) cells. Total protein was extracted from the cells with cell lysis buffer (Part# CLB01, Cytoskeleton, USA) containing Protease Inhibitor Cocktail (Cat# PIC02, Cytoskeleton, USA) and extracted protein was quantified with a Pierce™ BCA protein assay kit (Cat# 23225, Thermo Fisher Scientific, USA). Proteins (300–800

μg) from the cell lysates were collected into each tube and added with 10 μg of PAK-PBD beads (Cat# PAK02, Cytoskeleton, USA) and then incubated at 4 °C on a rotator for 1 h. After centrifugation at 5000×*g* at 4 °C for 1 min, beads were washed once with a wash buffer (Cat# WB01, Cytoskeleton, USA), GTP-Rac1 and GTP-Cdc42 were eluted with 30 μL of 2× loading buffer (Cat# FD006, Fdbio, China) and boiling for 5 min. Extracted protein was used for Western blot analysis.

For phosphorylated SLC12A4 (p-SLC12A4) assay, BMDMs were treated with rhamnose (100 μmol/L) for 5 min and then cells were lysed in RIPA buffer (Cat# P0013B, Beyotime, China). *In vivo*, peritoneal macrophages were obtained from PLF cells and were lysed in lysis buffer. The phosphorylated SLC12A4 was detected using Phos-Tag SDS-PAGE. Briefly, samples were separated by 8% SDS-PAGE gel containing 50 μmol/L phosbind acrylamide (Cat# F4002, ApexBio, USA) and 100 μmol/L $MnCl_2$ and the SDS-PAGE gel was washed in a transfer solution containing ethylene diamine tetraacetic acid (EDTA, Cat# ND0081, Leagene Biotechnology, China) (5 mmol/L) for 20 min (two times). Then, the gel was incubated in transfer solution for 10 min, and proteins were transferred to PVDF membranes.

Subsequently, the membranes were blocked with 5% milk for 1 h at room temperature, then incubated with specific primary antibodies diluted in Antibody Diluent (Cat# WB500D, New Cell & Molecular Biotech, China), including anti-Cdc42 mouse monoclonal antibodies (1:250, Cat# ACD03, Cytoskeleton, USA), anti-Rac1 mouse monoclonal antibodies (1:500, Cat# ARC03, Cytoskeleton, USA) and SLC12A4 polyclonal antibody (1:1000, Cat# 15927-1-AP, Proteintech, USA) overnight at 4 °C. Membranes were incubated for 1 h with secondary antibodies: anti-mouse IgG-HRP (1:2000, Cat# 7076s) and anti-Rabbit IgG-HRP (1:2000, Cat# 7074s) from Cell Signaling Technology (MA, USA). Finally, the membranes were developed with ECL (Cat# P0018AM, Beyotime, China) substrate.

2. Supporting figures

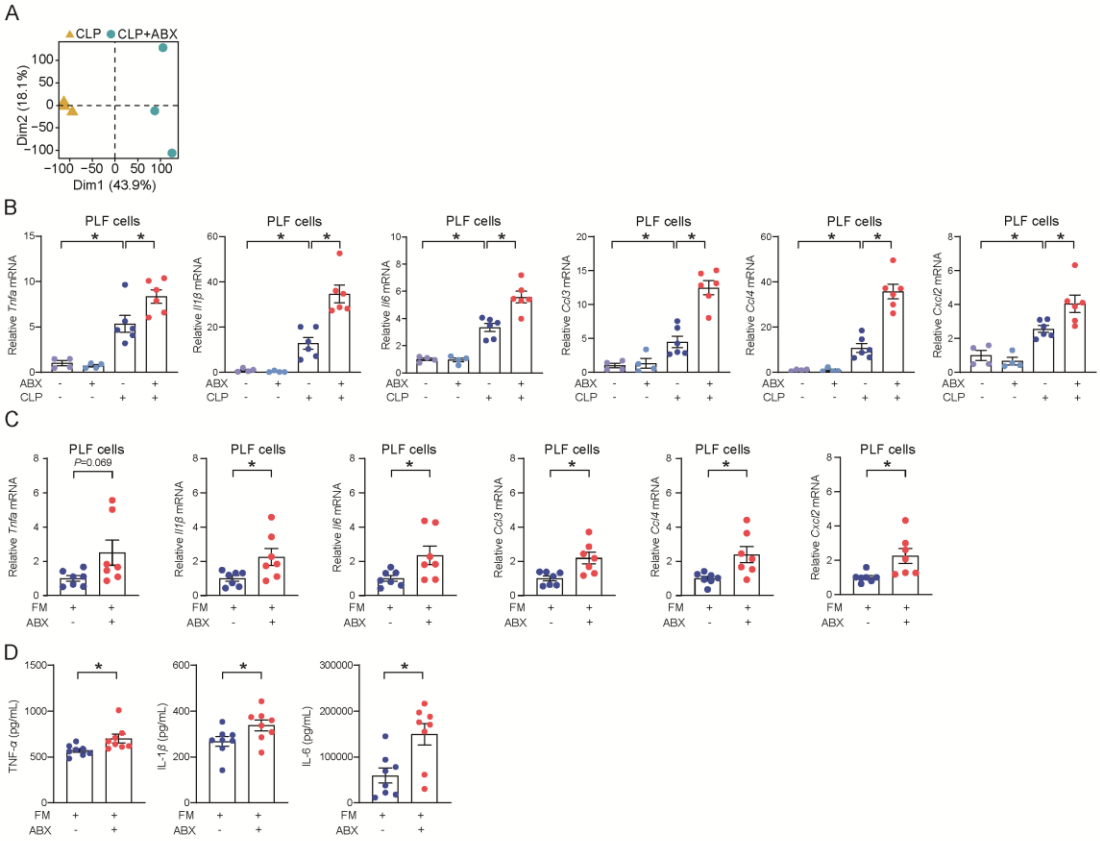

Figure S1 The depletion of gut microbiota increased the inflammation in septic mice.

(A) Principal component analysis (PCA) of the full transcriptomes in the peritoneal lavage fluid (PLF) cells (*n*=3/group). Mice were pretreated with PBS or ABX for three days followed by moderate CLP surgery, and then PLF cells were collected at 16 h for further Bulk RNA-seq analysis.

(B) The gene expression level of *Tnfα*, *Il1β*, *Il6*, *Ccl3*, *Ccl4* and *Cxcl2* in peritoneal lavage fluid (PLF) cells. Mice were pretreated with PBS or ABX for three days and then performed moderate CLP or sham surgery. Peritoneal lavage fluid (PLF) cells were collected at 16 h after surgery (Sham, *n*=4/group; CLP, *n*=6/group).

(C) The gene expression level of *Tnfα*, *Il1β*, *Il6*, *Ccl3*, *Ccl4* and *Cxcl2* in PLF cells. Mice were pretreated with PBS or ABX for three days and then mice were injected intraperitoneally (i.p.) with a single dose of fecal material (FM, cecal content) supernatant (100 μL). Peritoneal lavage fluid (PLF) cells were collected at 16 h after injection (*n*=7/group).

(D) TNF-α, IL-1β and IL-6 protein levels in plasma at 16 h after fecal material (FM, cecal content) supernatant injection. Experimental design as in Fig. S1C (n=8/group).

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) analysis was used for three or more groups. Two groups were determined by a two-tailed unpaired Student's t-test. *P<0.05 was considered statistically significant. n: indicates number of samples.

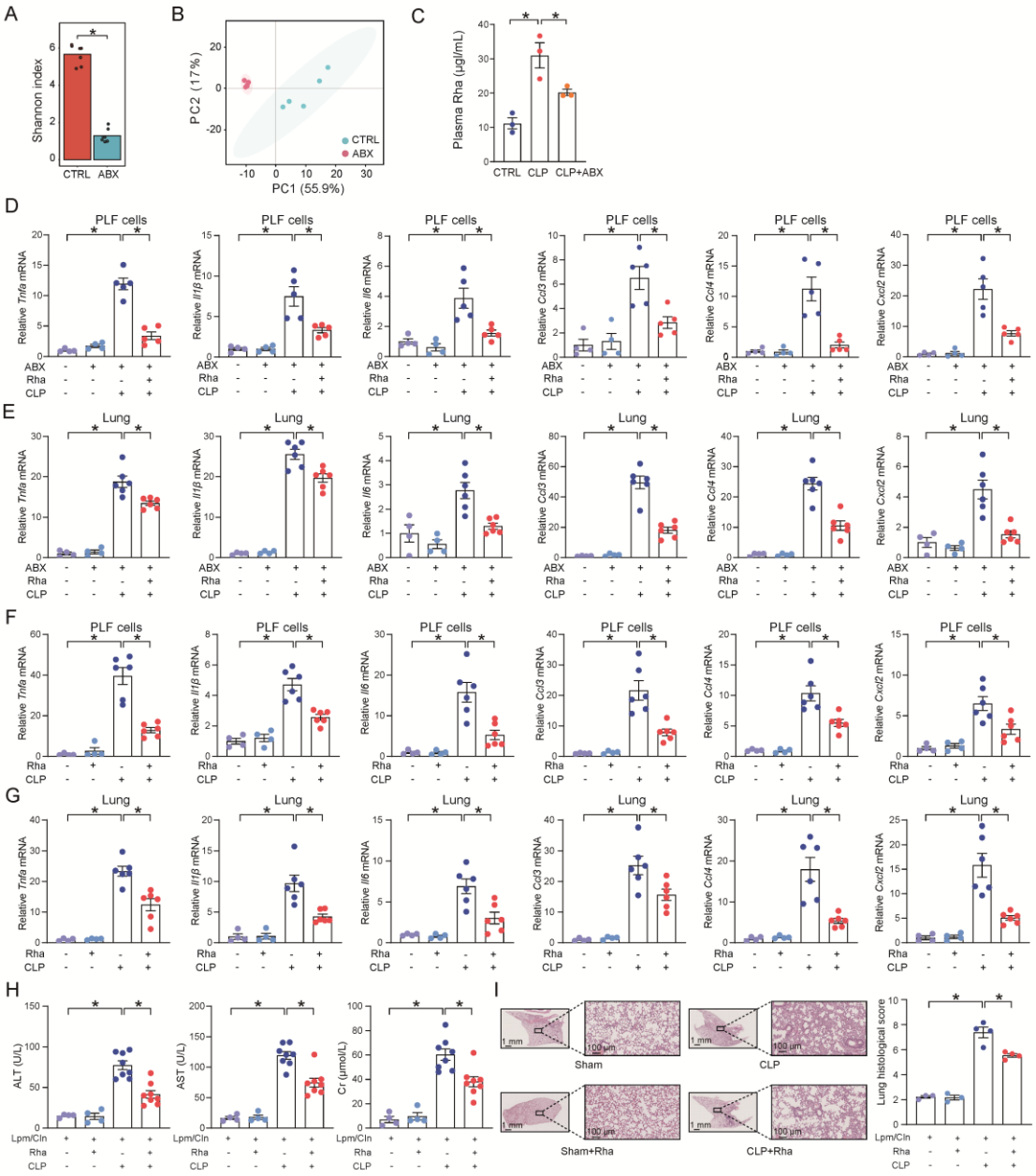

Figure S2 Rhamnose reduces the expression level of chemokines and cytokines in septic mice.

(A) A histogram showing the change in Shannon diversity index. Mice were pretreated with ABX or PBS for three days and stool samples were collected for 16S rDNA sequencing (*n*=7/group).

(B) A PCA plot for metabolomics analysis. Mice were pretreated with ABX or PBS for three days and stool samples were collected for metabolomics analysis (*n*=5/group).

(C) Rhamnose concentration in plasma. Mice were pretreated with ABX(CLP+ABX) or PBS (CLP) for three days followed by moderate CLP or sham surgery (CTRL) (*n*=3/group).

(D, E) The gene expression level of *Tnfα*, *Il1β*, *Il6*, *Ccl3*, *Ccl4* and *Cxcl2* in the PLF cells (D) and lung tissues(E). Mice were pretreated with PBS or ABX for three days and then orally administrated with PBS or rhamnose 2 h prior to moderate CLP or sham surgery and tissue samples were collected at 16 h (Sham, *n*=4/group; CLP, *n*=5-6/group).

(F, G) The gene expression level of *Tnfα*, *Il1β*, *Il6*, *Ccl3*, *Ccl4* and *Cxcl2* in the PLF cells (F) and lung tissues (G). Mice were pretreated with PBS or rhamnose for 2 h followed by lethal CLP or sham surgery and tissue samples were collected at 12 h (Sham, *n*=4/group; *n*=6/group).

(H) Plasma ALT, AST, and Cr levels. Mice were pretreated with PBS or rhamnose 2 h prior to CLP surgery by using an 18-gauge needle to induce sepsis, and 6 h later the cecum was punctured, Imipenem/Cilastatin (Ipm/Cln,14 mg/kg) was treated to mice. Tissue was collected at 12 h after CLP surgery (Sham, *n*=4/group; CLP, *n*=8/group).

(I) Representative H&E images and the histological scores of lung tissues from mice (Sham, *n*=3; CLP, *n*=4/group). Insets are low magnification images, Scale bar=1 mm; high magnification view (200×), Scale bar=100 μm. Experimental design as in Fig. S2H.

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) analysis was used for three or more groups. Two groups were determined by a two-tailed unpaired Student's *t*-test. **P*<0.05 was considered statistically significant. *n*: indicates number of samples.

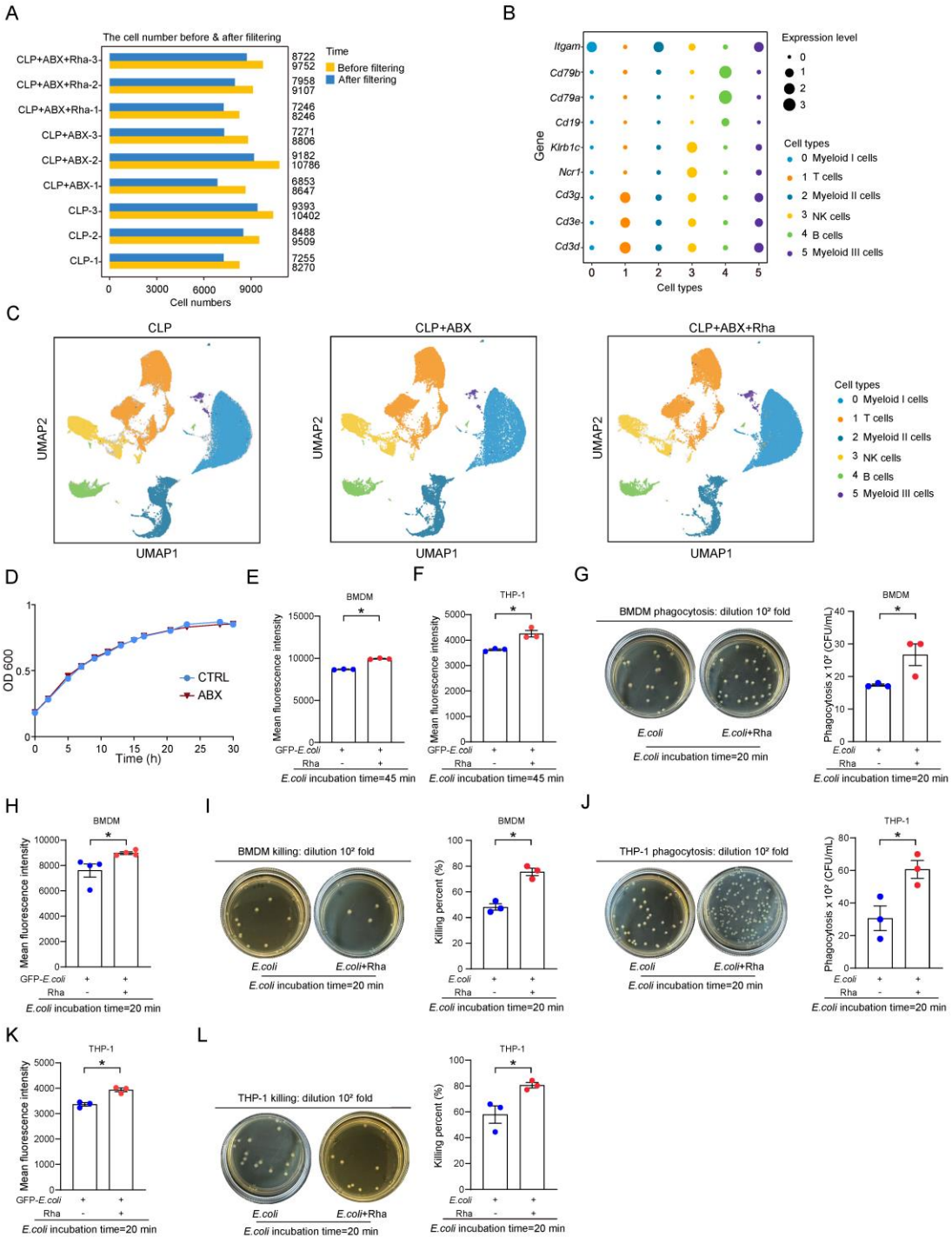

Figure S3 Rhamnose could enhance bacterial clearance in macrophages.

(A) A histogram showing the number of cells per sample before and after filtering (*n*=3/group).

(B) A bubble chart showing the expression of major genes in each cluster.

(C) Unsupervised clustering demonstrates 6 distinct clusters, as shown in UMAP map.

(D) The growth curve of *E. coli*. Peritoneal lavage fluid (PLF) was collected from ABX-pretreated mice and no-ABX pretreated mice and a 10% PLF was added into the bacterial culture medium. *E. coli* growth was monitored at OD600 spectrophotometrically (*n*=4/group).

(E) Phagocytosis assay by flow cytometry, BMDMs were pretreated rhamnose (100 μmol/L) or PBS for 18 h and then incubated with GFP-*E. coli* for 45 min at a MOI of 60 (*n*=3/group).

(F) Phagocytosis assay by flow cytometry, THP-1 cells were pretreated rhamnose (100 μmol/L) or PBS for 18 h and then incubated with GFP-*E. coli* for 45 min at a MOI of 60 (*n*=3/group).

(G) Phagocytosis of *E. coli* in BMDMs. BMDMs were pretreated with rhamnose (100 μmol/L) or PBS for 18 h and then incubated with *E. coli* at 37 °C in 5% CO_2 for 20 min with a MOI of 60 (*n*=3/group).

(H) Phagocytosis assay by flow cytometry, BMDMs were pretreated rhamnose (100 μmol/L) or PBS for 18 h and then incubated with GFP-*E. coli* for 20 min at a MOI of 60 (*n*=4/group).

(I) Percentage of *E. coli* killing by BMDMs in control (CTRL) and rhamnose (Rha) groups (*n*=3/group). Cells were lysed with 0.1% TritonX-100 and plated on LB agar for overnight. The bacterial load as CFUs were assessed by manual counting.

(J) Phagocytosis of *E. coli* in THP-1 cells. THP-1 cells were pretreated with rhamnose (100 μmol/L) or PBS for 18 h and then incubated with *E. coli* at 37 °C in 5% CO_2 for 20 min with a MOI of 60 (*n*=3/group).

(K) Phagocytosis assay by flow cytometry, THP-1 cells were pretreated rhamnose or PBS for 18 h and then incubated with GFP-*E. coli* for 20 min at a MOI of 60 (*n*=3/group).

(L) Percentage of *E. coli* killing by THP-1 cells in control (CTRL) and rhamnose (Rha) groups (*n*=3/group). Cells were lysed with 0.1% TritonX-100 and plated on LB agar overnight. The bacterial load as CFUs were assessed by manual counting.

Data are expressed as mean ± SEM. Two groups were determined by a two-tailed unpaired Student's *t*-test. **P*<0.05 was considered statistically significant. *n*: indicates number of samples.

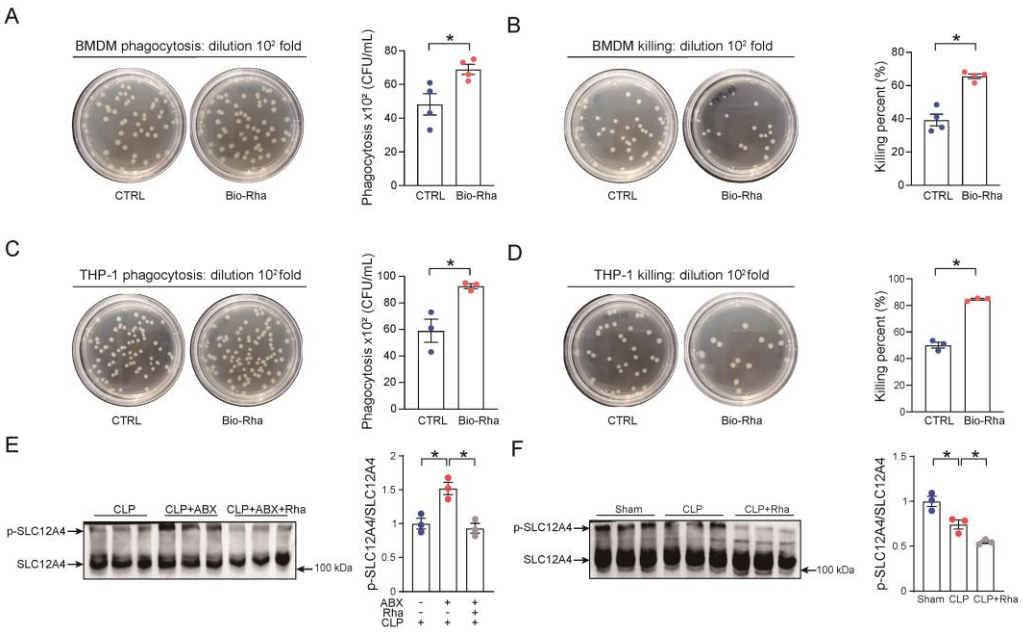

Figure S4 Biotinylated-Rhamnose promotes *E. coli* phagocytosis.

(A) Phagocytosis of *E. coli* in BMDMs. BMDMs were pretreated with biotin labeled rhamnose (Bio-Rha) or PBS (CTRL) for 18 h and then incubated with *E. coli* at 37 °C in 5% CO_2 for 45 min with a MOI of 60 (*n*=4/group).

(B) Percentage of *E. coli* killing by BMDMs cells in PBS (CTRL) and biotin labeled rhamnose (Bio-Rha) groups. BMDMs were lysed with TritonX-100 after incubated with culture medium containing 25 μg/mL gentamycin to remove extracellular bacteria for 4 h (*n*=4/group).

(C) Phagocytosis of *E. coli* in THP-1 cells. THP-1 cells were pretreated with biotin labeled rhamnose (Bio-Rha) or PBS (CTRL) for 18 h and then incubated with *E. coli* at 37 °C in 5% CO_2 for 45 min with a MOI of 60 (*n*=3/group).

(D) Percentage of *E. coli* killing by THP-1 cells. in PBS (CTRL) and biotin labeled rhamnose (Bio-Rha) groups. THP-1 cells were lysed with TritonX-100 after incubated with culture medium containing 25 μg/mL gentamycin to remove extracellular bacteria for 4 h (*n*=3/group).

(E) Western blot image and quantification of p-SLC12A4 in peritoneal macrophage. Mice were pretreated with ABX or PBS for three days followed by rhamnose or PBS administration and then moderate CLP model was performed to induce sepsis and then PLF cells were collected at 3 h after CLP model. Peritoneal macrophages were obtained by

using Anti-F4/80 MicroBeads from PLF cells (*n*=3/group).

(F) Western blot image and quantification of p-SLC12A4 levels in peritoneal macrophage. Mice were pretreated with rhamnose or PBS for 2 hours followed by sham or lethal CLP surgery. At 3 h after CLP or sham surgery, peritoneal macrophages were obtained by using Anti-F4/80 MicroBeads from PLF cells (*n*=3/group).

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) analysis was used for three or more groups. Two groups were determined by a two-tailed unpaired Student's *t*-test. **P*<0.05 was considered statistically significant. *n*: indicates number of samples.

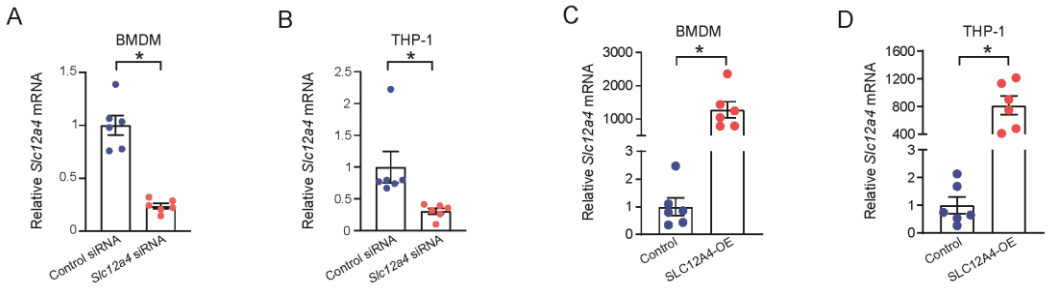

Figure S5 SLC12A4 silencing and overexpression efficiency verification in BMDMs and THP-1 cells.

(A) *Slc12a4* or control siRNA was transfected into BMDMs for 72 h, and the gene expression level of *Slc12a4* in BMDMs were detected by qPCR (*n*=6/group).

(B) *Slc12a4* or control siRNA was transfected into THP-1 cells for 48 h, and the gene expression level of *Slc12a4* in THP-1 cells were detected by qPCR (*n*=6/group).

(C) The control plasmid or SLC12A4-overpression plasmid (SLC12A4-OE) was transfected into BMDMs for 48 h, and the gene expression level of *Slc12a4* in BMDMs were detected by qPCR (*n*=6/group).

(D) The control plasmid or SLC12A4-overpression plasmid (SLC12A4-OE) was transfected into THP-1 for 48 h, and the gene expression level of *Slc12a4* in THP-1 cells were detected by qPCR (*n*=6/group).

Data are expressed as mean ± SEM. Two groups were determined by a two-tailed unpaired Student's *t*-test. **P*<0.05 was considered statistically significant. *n*: indicates number of samples.

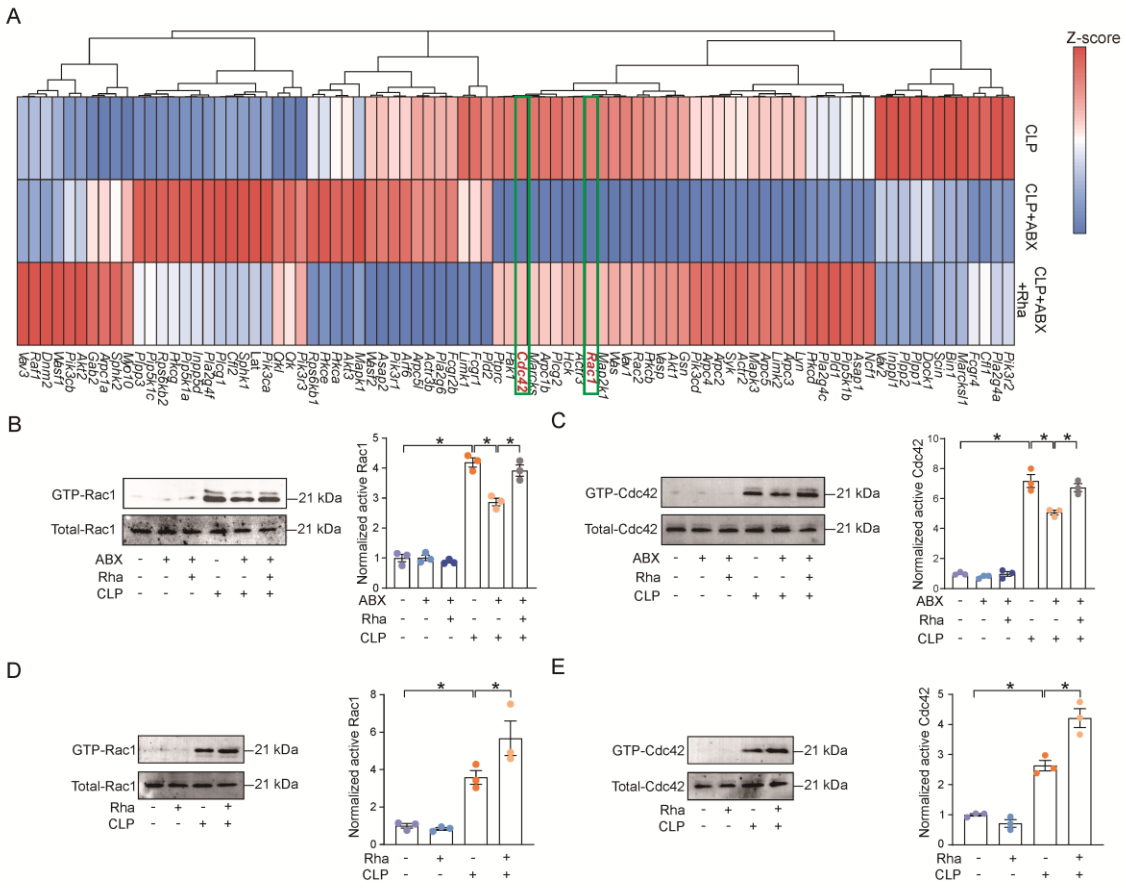

Figure S6 Rhamnose regulates the level of GTP-Cdc42 and GTP-Rac1 in mice.

(A) A heatmap showing significantly expressed genes within FcγR-mediated phagocytosis signal pathway in CLP, CLP+ABX and CLP+ABX+Rha groups. Red color shows Cdc42 and Rac1.

(B, C) Representative western blot image and the quantification of GTP-Cdc42 and GTP-Rac1 levels in peritoneal macrophage. Mice were pretreated with ABX or PBS for three days followed by rhamnose or PBS administration and then moderate CLP model was performed to induce sepsis. Peritoneal macrophages from PLF cells were collected 3 h after CLP surgery by using Anti-F4/80 MicroBeads (*n*=3/group).

(D, E) Representative western blot image and the quantification of GTP-Cdc42 and GTP-Rac1 levels in peritoneal macrophage. Mice were pretreated with rhamnose or PBS for 2 hours followed by lethal CLP or sham surgery. At 3 h after CLP or sham surgery, peritoneal macrophages were obtained by using Anti-F4/80 MicroBeads from PLF cells (*n*=3/group). Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) analysis was

used for three or more groups. *P<0.05 was considered statistically significant. n: indicates number of samples.

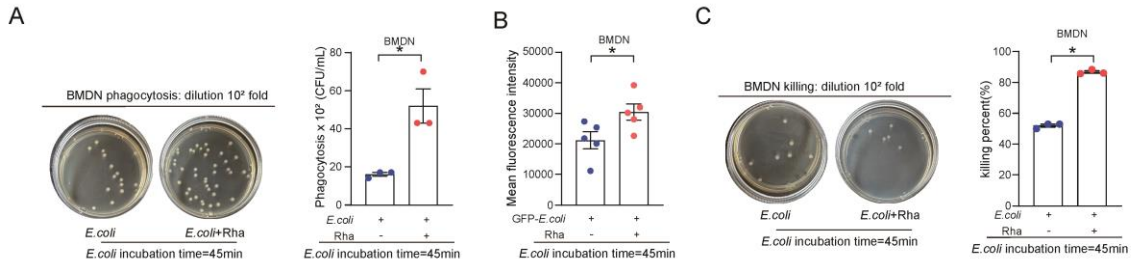

Figure S7 Rhamnose increased the bacterial clearance in neutrophils.

(A) Phagocytosis of *E. coli* in BMDNs. BMDNs were pretreated with rhamnose (100 μmol/L) or PBS for 18 h and then incubated with *E. coli* at 37 °C in 5% CO_2 for 45 min with a MOI of 60 (n=3/group).

(B) Phagocytosis assay by flow cytometry, BMDNs were pretreated rhamnose (100 μmol/L) or PBS for 18 h and then incubated with GFP-*E. coli* for 45 min at a MOI of 60 (n=5/group).

(C) Percentage of *E. coli* killing by BMDNs in control (CTRL) and rhamnose (Rha) groups (n=3/group). BMDNs were pretreated with rhamnose or PBS for 18 h and then incubated with *E. coli* at 37 °C in 5% CO_2 for 45 min with a MOI of 60, and then cells were incubated with culture medium containing 25 μg/mL gentamycin to remove extracellular bacteria for 4 h.

Data are expressed as mean ± SEM. Two groups were determined by a two-tailed unpaired Student's t-test. *P<0.05 was considered statistically significant. n: indicates number of samples.

3. Supporting table

Table S1 List of primer sequences designed for qPCR.

Primer	Forward primers	Reverse primers
16S	GTGTGYCAGCMGCCGCGGTAA	GCGGACTACNVGGGTWTCTAAT
Mouse-*18s*	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
Mouse-*Tnfα*	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
Mouse-*Il1β*	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
Mouse-*Il6*	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
Mouse-*Ccl3*	TGTACCATGACACTCTGCAAC	CAACGATGAATTGGCGTGGAA
Mouse-*Ccl4*	TTCCTGCTGTTTCTCTTACACCT	CTGTCTGCCTCTTTTGGTCAG
Mouse-*Cxcl2*	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
Mouse-*Slc12a4*	TCTACCTGGGGACGACATTTG	CCGATGGGTAAAAGATGGCAG
Human *18s*	AGGAATTCCCAGTAAGTGCG	AGGAATTCCCAGTAAGTGC
Human- *Slc12a4**	TGACACGTCGAATGCCACTTT	GGAAACACGGGAGGGTCAAA

*Human-*Slc12a4* was used for *Slc12a4* overexpression and knockdown experiments.

References

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