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Supplementary Information for

Morphine tolerance is attenuated in germ-free mice and reversed by probiotics, implicating the role of gut microbiome

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Supplementary Information Text

Supplementary methods

Animal treatment: Morphine sulfate (National Institutes of Health [NIH]/National Institute on Drug Abuse [NIDA], Bethesda, MD) was dissolved in sterile saline. Mice received constant dose of 15mg/kg or escalating doses of (5, 10, 15, 20, 25, 30, 35, 40mg/kg) morphine injection b.i.d. On the ninth day, 25mg/kg morphine sulfate was used to test the anti-nociceptive response. According to established protocol, the non-absorbable pan-antibiotics cocktail (ABX) was prepared every day freshly in drinking water(1). The ABX treatment was given 7-10 days before morphine treatment and continued throughout the following eight days of morphine treatment and behavioral measurement. Mice were orally gavaged with freshly prepared $5x10^{10}$ CFU of the probiotics VSL#3 in 200μL water daily for 21 days before morphine treatment and maintained throughout the following eight days(2).

FMT: Twenty donor mice (10 mice for each batch) were injected with either morphine or saline b.i.d. as previously described. Their fecal contents were collected and pooled after sacrifice. The fecal content was processed according to the established protocol with modification(3). Briefly, 200mg of the fecal extract was suspended in 1mL sterile PBS, filtered through 70μM cell strainer, and centrifuged at 6000Xg for 20min. About $10^{\wedge 10}$ CFU/mL fecal bacteria were suspended in 6% $NaHCO_3$ buffer with 20% sucrose. Each recipient mouse was treated with ABX as previously described(1) and then orally gavaged with 200μL of freshly prepared fecal suspension on seven consecutive days before any downstream experiment or analysis.

Behavioral study: The thermal nociceptive thresholds were assessed by both tail flick and hot plate assays as reference(4). The hot plate and tail flick analgesic responses were calculated as the percentage the maximum possible effect (%MPE). %MPE = (post-drug latency – pre-drug latency)/ (cut-off – pre-drug latency) × 100% for tail flick analgesia and hot plate.

Intestinal permeability and bacterial translocation: To access *in vivo* intestinal permeability, 600 mg/kg of FITC-dextran (wt 4000; Sigma-Aldrich) was orally gavaged into mice 4 hour prior to blood collection. After sacrifice, serum FITC-dextran fluorescence intensity was measured by SpectraMax® i3x. MLN and liver tissue were collected, homogenized and cultured on BD™ Trypticase™ Soy Agar plates with 5% Sheep Blood (TSA II™) to determine bacterial translocation.

Real-Time PCR: Total RNA from spinal cord and ileum was extracted using TRIzol (Invitrogen). cDNA was synthesized using the M-MLV Reverse Transcription kit (Promega) following manufacturer's protocol. Primers for IL-6, IL-1β, TNF-α, and GAPDH were purchased from Invitrogen. Quantitative real-time polymerase chain reaction was performed using LightCycler® 480 SYBR Green I Master (Roche). GAPDH was used to normalize values. The results were analyzed by the relative quantity (ΔΔCt) method. Primer sequences: GAPDH: 5'-ACGGCAAATTCAACGGCACAGTCA-3', 5'-TGGGGGCATCGGCAGAAGG-3'; IL-6: 5'-TGGCTAAGGACCAAGACCATCCAA-3', 5'-AACGCACTAGGTTTGCCGAGTAGA-3'; TNF-α: 5'-CCTCCCTCTCATCAGTTCTATGG-3', 5'-CGTGGGCTACAGGCTTGTC-3'; IL-1β: 5'-GGCAGGCAGTATCACTCATT-3', IL-1β: 5'-AAGGTGCTCATGTCCTCATC-3'.

PCR: Bacterial DNA was isolated from mouse tissues as previously described. The 16S ribosomal RNA (rRNA) gene was amplified by 5'-TTGGAGAGTTTGATCCTGGCTC-3', and 5'-ACGTCATCCCCACCTTCCTC-3'. Gram-positive and gram-negative bacterial DNA was amplified by NF with N6R, and NF with NR and P2F, respectively. NF: 5'-GGCGGCAKGCCTAAYACATGCAAGT-3', NR: 5'- GACGACAGCCATGCASCACCTGT-3', P2F: 5'-GCGRCTCTCTGGTCTGTA-3', N6R: 5'-GGTGCCTTCGGGAAC-3'. Amplified DNA was visualized using 1% agarose electrophoresis gel(5).

Intestinal cell isolation and FACS: Intestinal cell isolation was performed as previously described(6). Single cell suspensions were incubated with a mixture of antibodies: Pan-Keratin (C11) Mouse mAb (Alexa Fluor® 488 Conjugate, cell signaling), redFluor™ 710 Anti-Mouse CD45 (30-F11, Tonbo bioscience), Ghost Dye™ Red 780 (Tonbo bioscience), and either BV421 Rat Anti-Mouse CD282 (TLR2, BD bioscience) or BV421 Rat Anti-Mouse CD284 (TLR4, BD bioscience). The data were collected and analyzed by cytoExpert software with cytoFlex S (Beckman Coulter). The figures were made by Kaluza.

ELISA: The concentrations of IL-6, IL-1β and TNF-α in brain, liver and MLN were quantified using enzyme-linked immunosorbent assay kits (Fisher Scientific).

Histology: Sections of formalin-fixed paraffin-embedded gut tissues were subjected to H&E staining for the evaluation of morphine-induced morphological disruption of gut epithelial integrity as previously described(7).

Statistical analysis: Experimental data were analyzed using Prism 6 (GraphPad Software, Inc.). Parametric data were compared using Student's t-test. For multiple-group comparison, data were analyzed by ANOVA one-way analysis, followed by Bonferroni correction or two-way analysis, followed by Tukey's multiple comparison method.

16S rRNA gene sequencing: Contents of ileum were collected under aseptic conditions from all mice after sacrificing. Sequencing and bioinformatics were performed by the University of Minnesota Genomic Center, MN, United States, and Microbiome Insights, Vancouver, BC, Canada. DNA was isolated using DNeasy PowerSoil® kits (Qiagen, Germantown, Maryland) modified to include a bead-beating step. At University of Minnesota, after DNA isolation, 16S ribosomal DNA hypervariable regions V5 and V6 were polymerase chain reaction amplified using primers with the V5F RGGATTAGATACCC and V6R CGACRRCCATGCANCACCT gene-specific sequences, Illumina adaptors, and molecular barcodes as described to produce 427 base pair (bp) amplicons. Samples were sequenced on an Illumina MiSeq (Illumina, San Diego, California) using MiSeq 600 cycle v3 kit(8). In Microbiome Insights, the V4 region was amplified with adapter-barcode-pad/linker-16S primer as shown below: AATGATACGGCGACCACCGAGTCTACACCTACTATATATGGTAATTGTGTGCCA GCMGCCGCGGTAA and CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHV GGGTWTCTAAT. Samples were sequenced by Illumina MiSeq.

Metagenomic data analysis: Primer sequences were removed from raw sequence reads and low-quality bases (Phred score<20) were trimmed from 3' end using cutadapt(9). Microbial taxonomy assignment abundance quantification was analyzed with Greengenes database (ver.08/13) using dada2 pipeline. Microbial diversity between samples (β-diversity) was quantified by Bray-Curtis dissimilarity using R package "vegan"(10). The dissimilarity between pairs of treatment groups was assessed using permutation multivariate analysis of ANOVA (PERMANOVA) adjusting for the batch difference, and the significance of pair-wise comparison was adjusted for multiple comparison using Bonferroni correction. Individual differential taxa were identified accounting for batch difference using DESeq2 with significance determined based on a false discovery rate of 0.05 (FDR)(11). The raw sequencing data that support the findings of this study have been submitted to Sequence Read Archive (SRA) of the National Center for Biotechnology Information under accession number PRJNA531200. The source data that generated Fig.5 and Fig.6 C, D, E, F are available in Supplementary Dataset S1. All other data that support the findings of this study are available upon request.

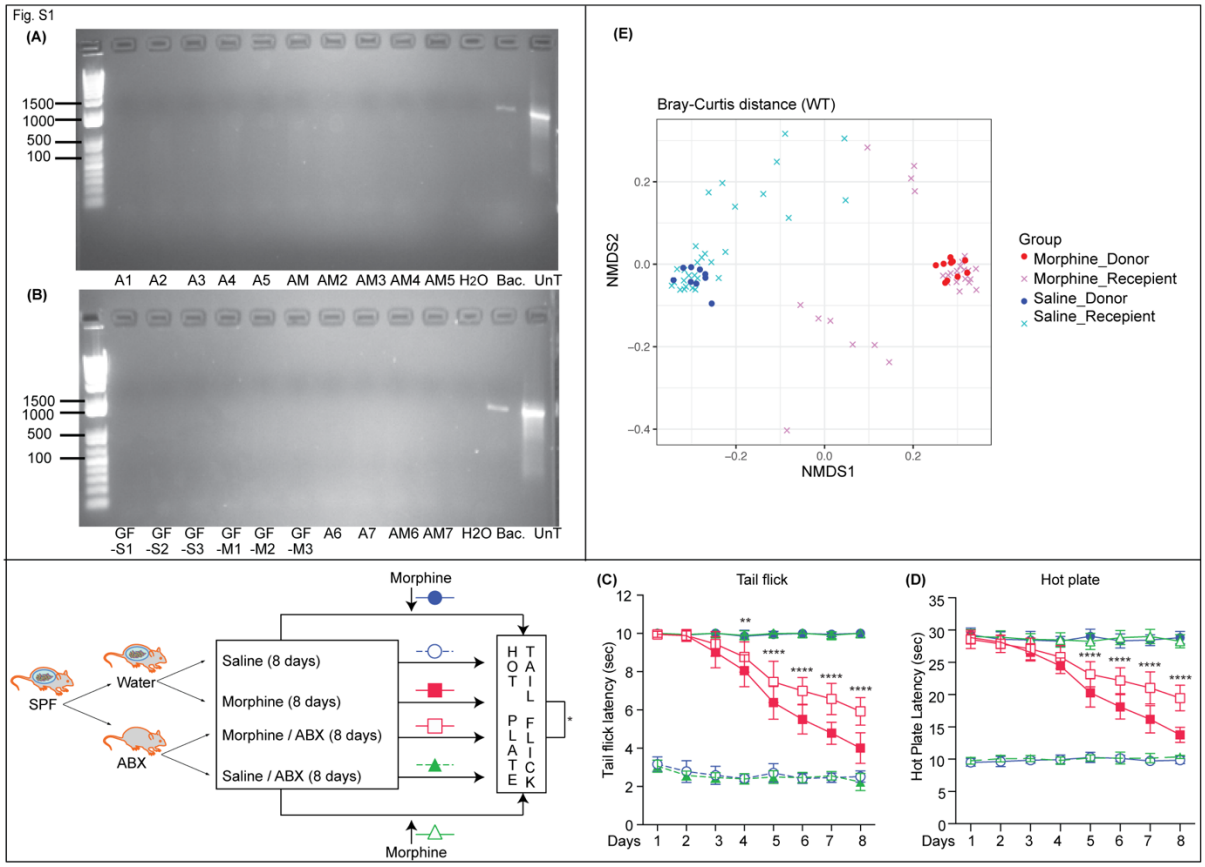

Fig. S1: Gut microbiota are essential for morphine-induced analgesic tolerance. (*A*, *B*) Representative image of PCR product of bacterial 16S rRNA gene on electrophoresis gel, GF: germ-free saline-treated mice; GF-M: germ-free morphine-treated mice; n=3 for each group. A: ABX + Saline-treated mice; AM: ABX + Morphine-treated mice; n=7 for each group. Bac., bacterial DNA; Untreated, ABX untreated mice as positive controls. (*C*, *D*) Time course of ABX treatment on morphine tolerance. n=10-16, presented as Mean ± SD. $F_{Treatment\ x\ time}$ (35, 434) = 52.46 for tail flick. $F_{Treatment\ x\ time}$ (35, 434) = 60 for hot plate. Significance was assessed by two-way ANOVA followed by Tukey's multiple comparisons method. **, p<0.01, ****, p<0.0001. (*E*) Multidimensional scaling analysis plot of stool samples based on Bray-Curtis distance. The microbiome of recipient mice was similar to the donor mice after FMT (Saline_Recepient vs Saline_Donor, p=0.282; Morphine_Recepient vs Morphine_Donor, p=0.33612, Permutation ANOVA test). NMDS: non-metric multidimensional scaling.

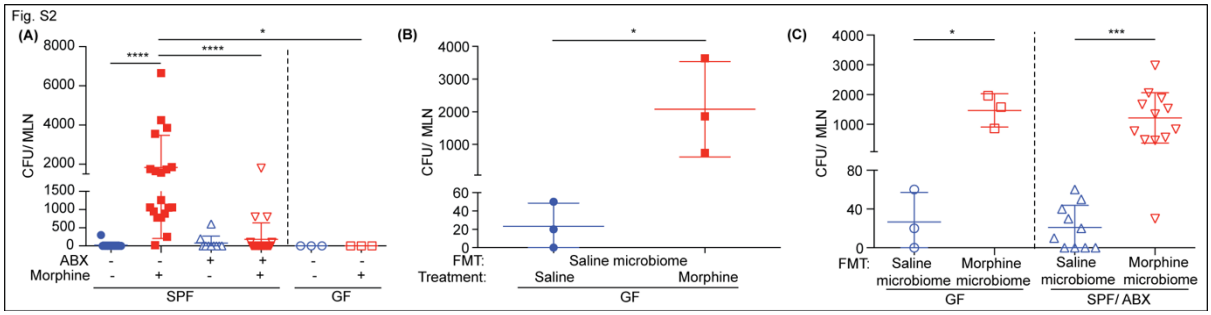

Fig. S2: The MLNs were collected from mice and their homogenates were cultured on blood agar plate as previously described. CFU refers to total Colony Forming Unit. Mean ± SD. (A) Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (B, C) Student's t-test was used for statistical comparison. *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$ and ****, $p<0.0001$.

Fig. S3

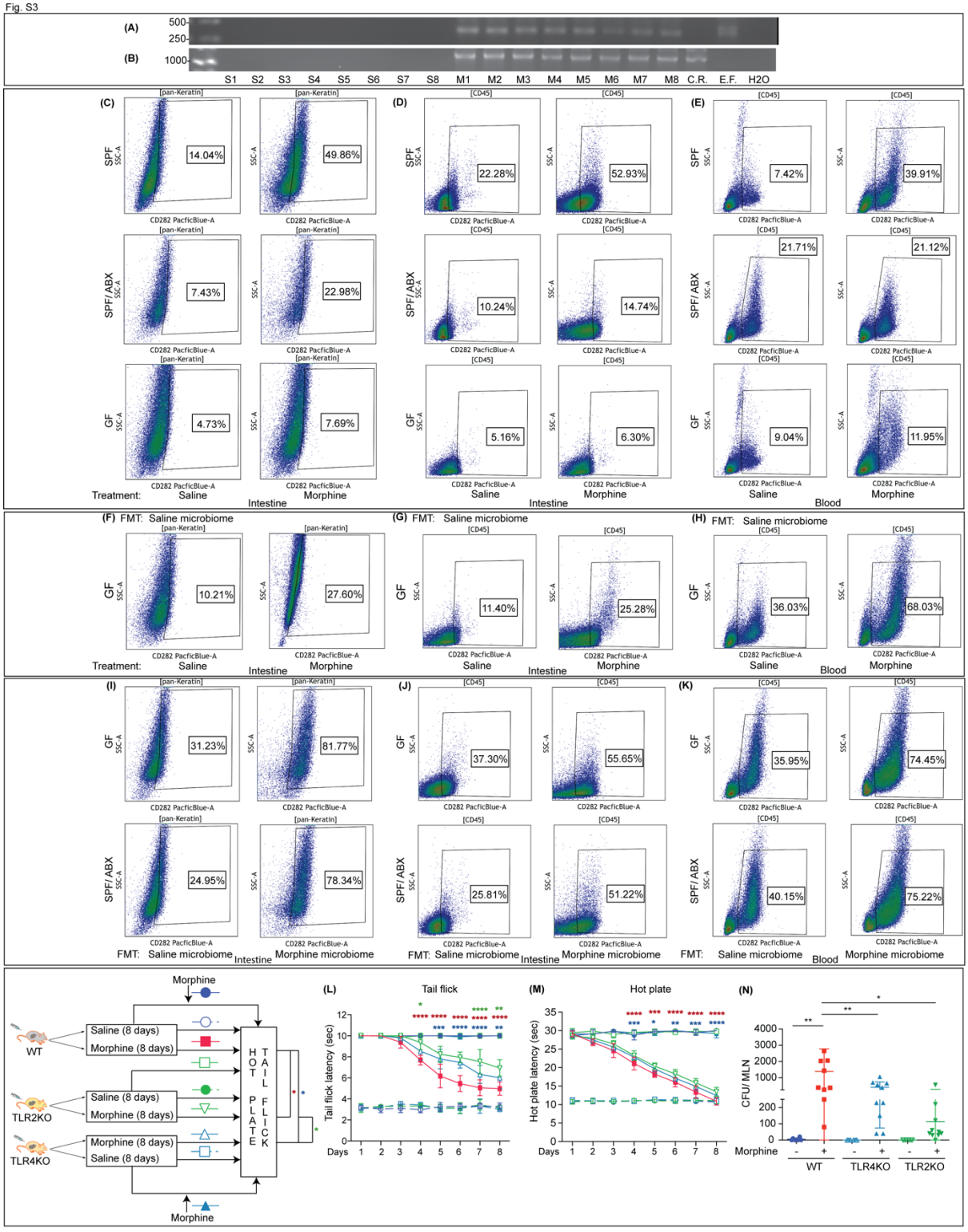

Fig S3: Increased TLR2 expression in gut tissue and circulating immune cells in morphine tolerant animals is induced by altered gut microbiota. (*A*) The 355-bp amplicon is specific for gram-positive bacteria, and (*B*) The 985-bp amplicon is specific for gram-negative bacteria. Lane 1 is DNA ladder. Lane 2-9 is liver homogenates from saline-treated mice; lane 10-17 is liver homogenates from morphine-treated mice; lane 18 is *C. Rodentium* for gram-negative bacteria as a positive control; lane 19 is *E. Faecalis* for gram-positive bacteria as a positive control; lane 20 is purified water for negative control. (*C*, *D*, *E*) Representative images of epithelial or immune cells stained

with CD282, n_{GF}=3, n_{SPF}=9-15. (*F*, *G*, *H*) Representative images of epithelial or immune cells stained with CD282 after FMT from naïve mice. n_{GF}=3. (*I*, *J*, *K*) Representative images of epithelial or immune cells stained with CD282 after FMT from saline or morphine-tolerant mice. n_{GF}=3, n_{SPF}=5. One-way ANOVA followed by Bonferroni's multiple comparisons test was used for data analysis. (*L*, *M*) WT, TLR2KO and TLR4KO mice were treated with constant doses of morphine twice daily. $F_{Treatment\ x\ time}$ (56, 329) = 23.09 for tail flick. $F_{Treatment\ x\ time}$ (56, 329) = 103.5 for hot plate. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison method. *WT Morphine compared to TLR2KO Morphine, *WT Morphine compared to TLR4KO Morphine, *TLR2KO Morphine compared to TLR4KO Morphine. (*N*) Bacterial colonies from liver homogenates were counted as CFU as previously described. Mean ± SD. Statistical analyses were carried out by one-way ANOVA followed by Bonferroni's multiple comparisons test. *, $p<0.05$; **, $p< 0.01$.

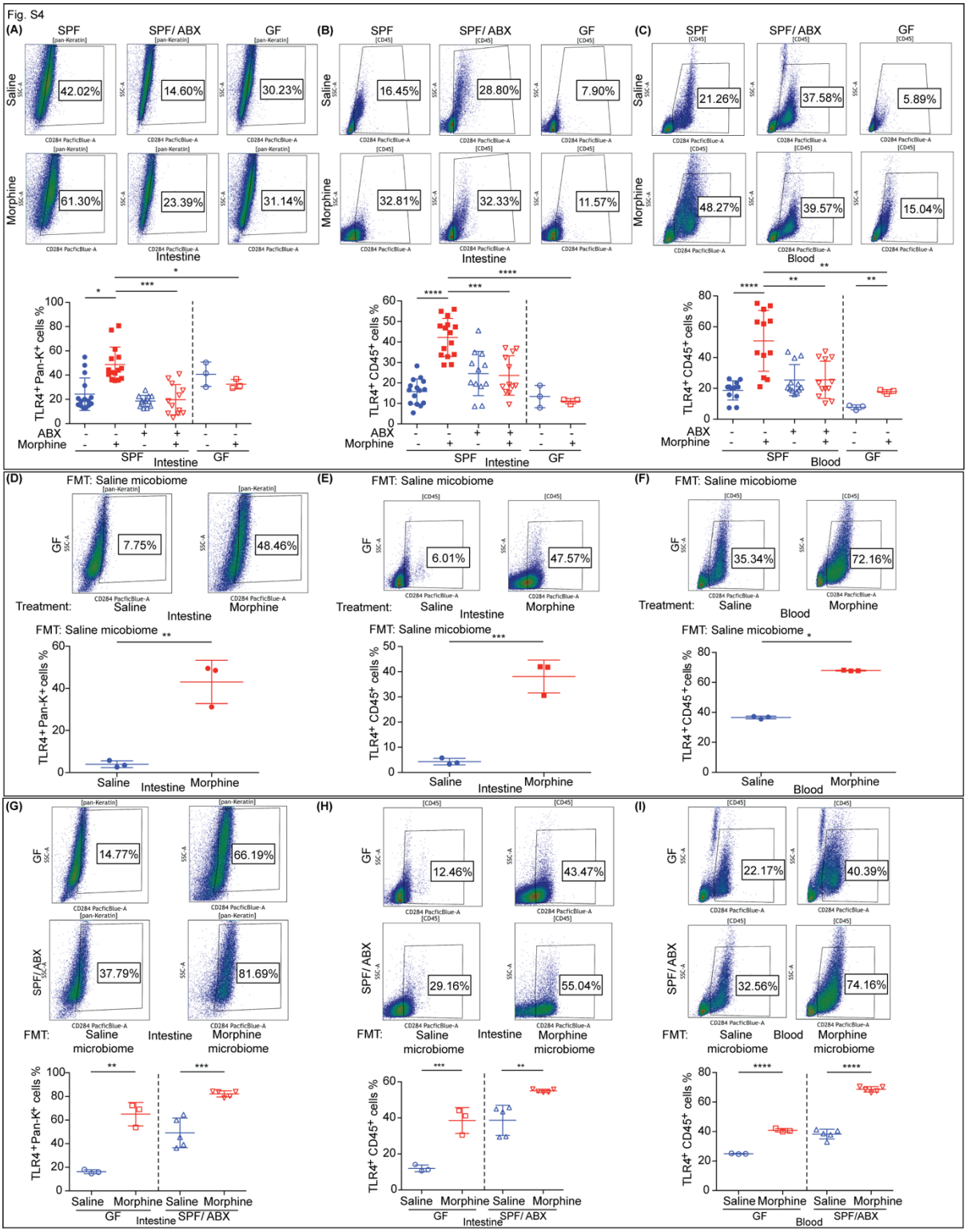

Fig. S4: Gut and systemic TLR4 expression was induced in morphine tolerant animals by gut microbiota. (*A*, *B*, *C*) Representative images and summary of epithelial or immune cells staining with CD284 for TLR4 expression. n_{GF}=3, n_{SPF}=12-15. (*D*, *E*, *F*) Morphine induced TLR4 expression following gut microbiome reconstitution in GF mice. n_{GF}=3. The data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (*G*, *H*, *I*) FMT from morphine-tolerant mice elevated TLR4 expression in GF mice and ABX mice. n_{GF}=3. n_{ABX}=5. Two-tailed student t-test was used. *, p<0.05; **, p<0.01; ***, p< 0.001; ****, p<0.0001. Mean ± SD.

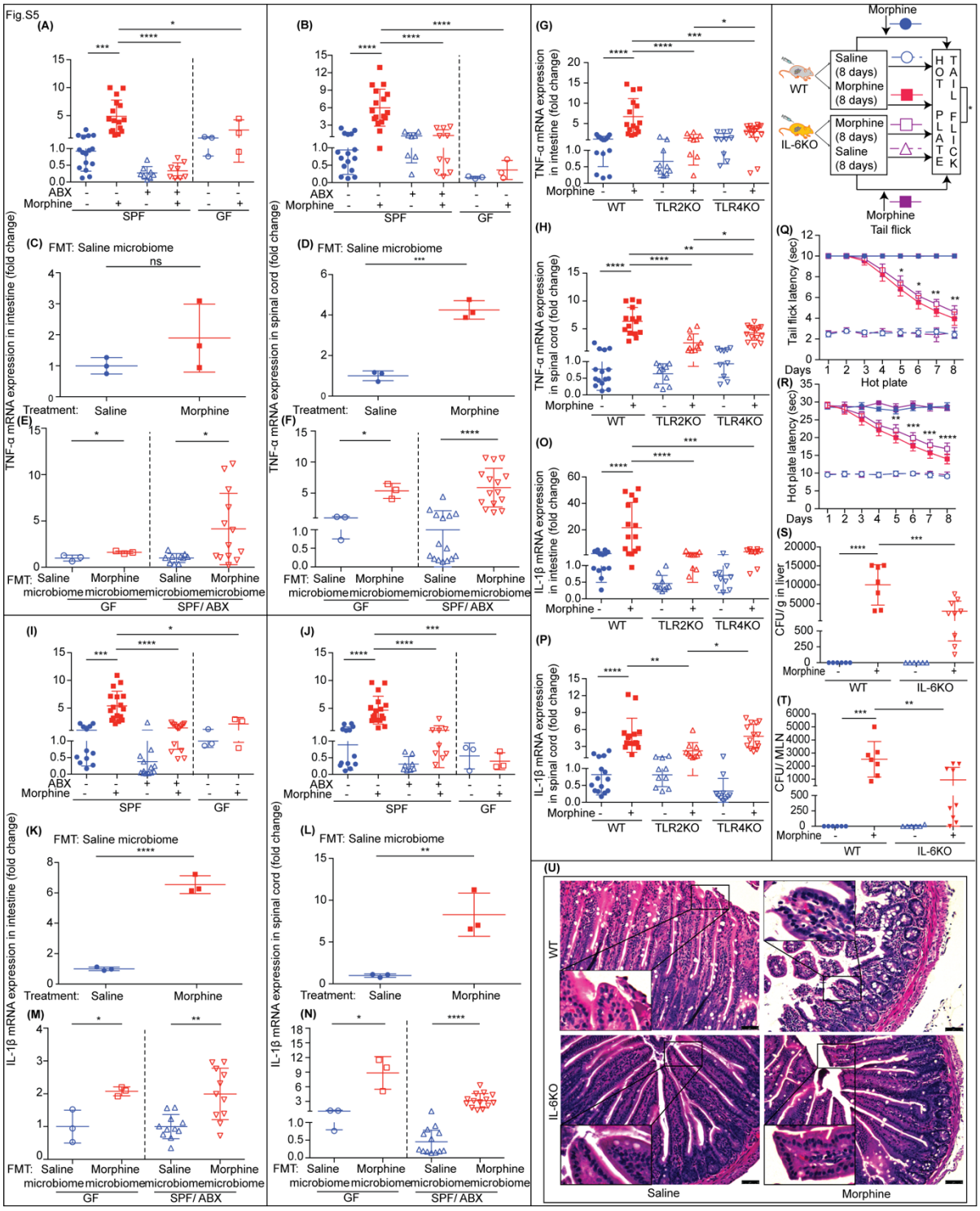

Fig. S5: Gut microbiome is essential for TNF-α and IL-1β induction in spinal cord and intestine of morphine tolerant animals. (*A*, *B*, *I*, *J*) After escalating doses of morphine treatment, TNF-α and IL-1β expression in GF mice remained at low levels compared to WT controls. n_{GF}=3, n_{SPF}=10-17. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (*C*, *D*, *K*, *L*) Intestinal TNF-α and IL-1β expression was elevated after gut microbiota were restored in GF mice. n_{GF}=3. (*E*, *F*, *M*, *N*) TNF-α and IL-1β levels were up-regulated by microbiome from morphine-tolerant mice (FMT) without previous exposure to morphine. n_{GF}=3. $n_{SPF/ABX}$ =11-14. Two-tailed student's t-test was used for statistical analysis. (*G*, *H*, *O*, *P*)

Proinflammatory cytokines in TLR2KO and TLR4KO were not up-regulated by chronic morphine treatment when compared to WT. n=10-15. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (*Q*, *R*) WT and IL-6KO mice were treated with constant doses of morphine for 8 days. n=4-10. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison method. $F_{Treatment\ x\ time}$ (35, 321) = 83.33 for tail flick. $F_{Treatment\ x\ time}$ (35, 321) = 79.3 for hot plate. (*S*, *T*) The liver and MLN homogenates from WT and IL6KO mice were plated on blood agar plates overnight. Bacterial colonies were counted (colony forming unit). n=6-9. One-way ANOVA followed by Bonferroni's multiple comparisons test was used for statistical analysis. *, $p<0.05$; **, $p<0.01$; ***$p<0.001$; ****$p<0.0001$. Mean $\pm$ SD. (*U*) Representative H&E stained intestinal sections from morphine treated- WT and IL-6KO mice. n=6.

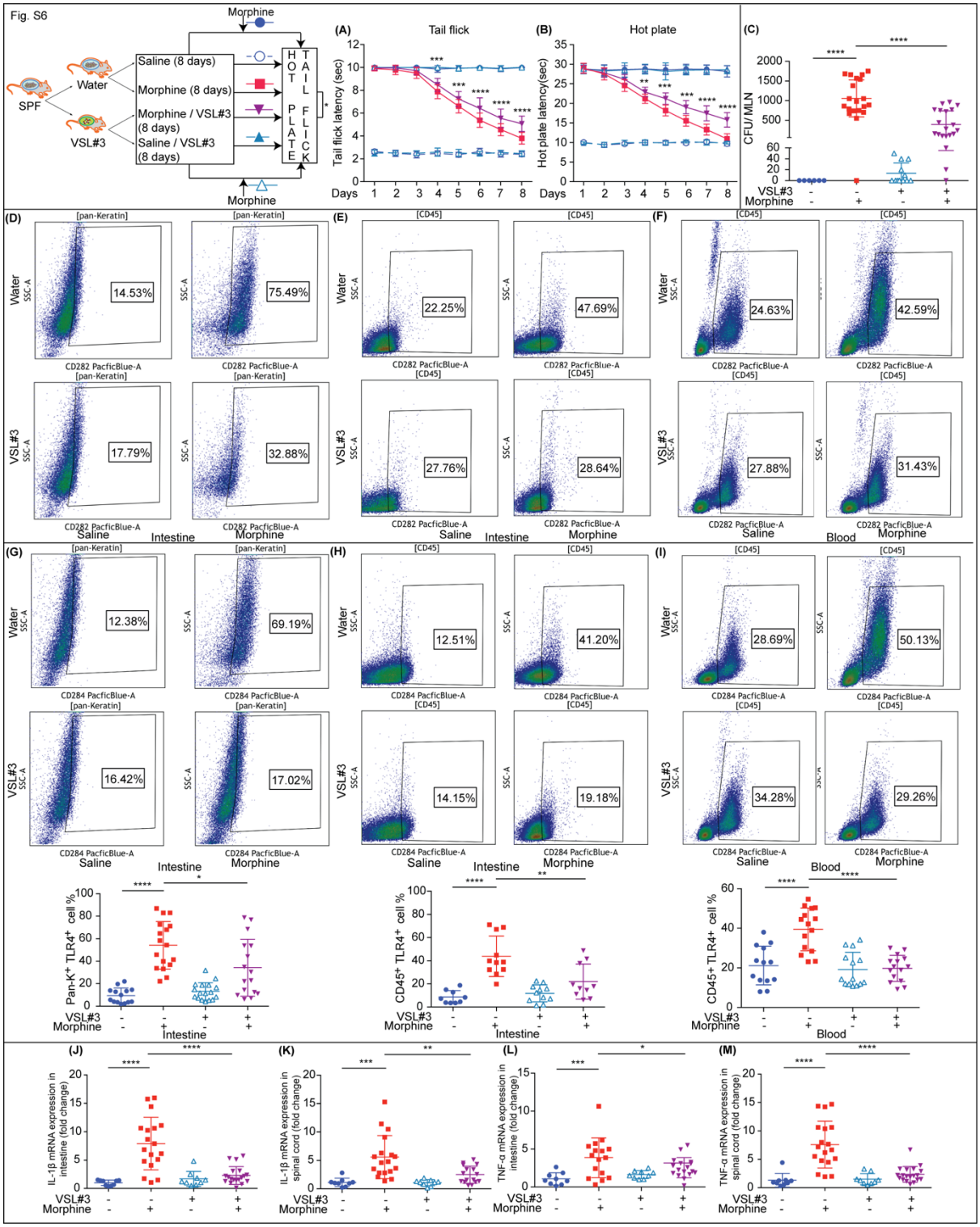

Fig. S6: Probiotics pretreatment attenuates morphine analgesic tolerance and prevents morphine-induced gut microbiota alterations. (*A*, *B*) Time course of morphine tolerance was assessed by tail flick and hot plate tests. Mice were gavaged with probiotics and treated with constant doses of morphine. Data represent mean ± SD from 10-20 mice. Statistical analyses were carried out by two-way ANOVA followed by Tukey's multiple comparison method. $F_{Treatment\ x\ time}$ (35, 490) = 143.0 for tail flick. $F_{Treatment\ x\ time}$ (35, 490) = 126.2 for hot plate. (*C*) Bacterial colonies from MLN homogenate of each mouse in different treatment groups. n=6-20. (*D*, *E*, *F*) Representative figures of TLR2 expression in different groups. n=9-15. (*G*, *H*, *I*)

Probiotics pre-treatment inhibits the up-regulation of TLR2 by morphine. Dissociated cells from intestine and circulating immune cells were stained with CD282 and pan-keratin or CD45. n=9-15. (*J*, *K*, *L, M*) Real-time quantitative RT-PCR of IL1-β and TNF-α gene expressions in mouse intestine and spinal cord. Each dot represents one mouse. n=9-19. (*A-M*) One-way ANOVA followed by Bonferroni's multiple comparisons test was used to analyze the data. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p< 0.0001$. Mean ± SD.

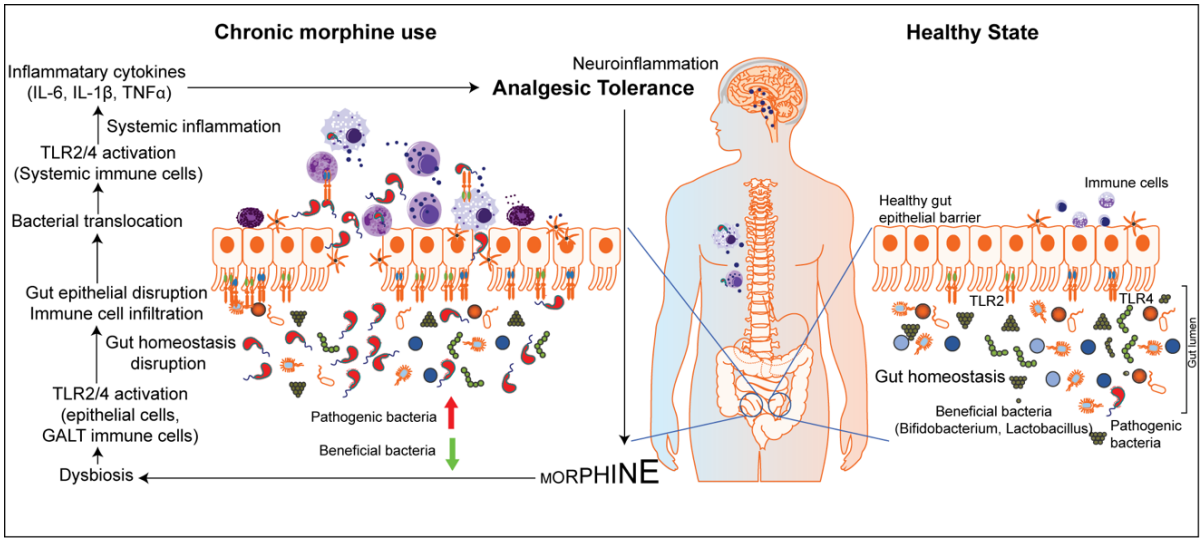

Fig. S7: Schematic diagram shows the role of the gut-immune-brain axis in morphine tolerance. Chronic morphine use induces gut dysbiosis and initiates local inflammation through TLR2 and TLR4 activation. This results in increased expression of pro-inflammatory cytokines, contributes to morphine tolerance, and acts as a feed-forward loop in aggravating dysbiosis, impairing gut integrity, leading to further bacterial translocation, thus exacerbating inflammation and sustaining morphine tolerance.

Table S1: Inflammation induced by morphine tolerance was alleviated in SPF/ABX and GF mice.

Mice		SPF mice				GF mice	
Tissue		Treatment					
	Pro-inflammatory cytokines (pg/μg)	Water + Saline	ABX + Saline	Water + Morphine	ABX + Morphine	Saline	Morphine
Brain	IL-6	0.0755±0.0293***	0.0583±0.0364***	0.3743±0.1707	0.1499±0.0826**	0.0088±0.0055**	0.039±0.0019**
	IL-1β	0.0721±0.0616***	0.0463±0.0315****	0.3921±0.2102	0.0789±0.0541***	0.0194±0.0078****	0.0252±0.0098****
	TNFα	0.1222±0.0540****	0.1298±0.0439****	0.4263±0.1091	0.1790±0.0949****	0.0188±0.0036****	0.0234±0.0059****
Liver	IL-6	0.0420±0.0263***	0.0075±0.0074****	0.1112±0.0552	0.1076±0.0781***	0.0301±0.0206**	0.0393±0.0322*
	IL-1β	0.0373±0.0310**	0.0241±0.0169***	0.1180±0.0463	0.0621±0.0496*	0.0204±0.0286	0.0442±0.0365
	TNFα	0.0748±0.0388**	0.0422±0.0324****	0.2804±0.01909	0.1252±0.1252***	0.058.8±0.0346**	0.1138±0.0765*
MLN	IL-6	0.0158±0.0126****	0.0682±0.065.6****	0.3625±0.1913	0.0327±0.0353****	0.0204±0.0044***	0.0123±0.0035***
	IL-1β	0.0402±0.0183***	0.0404±0.0258****	0.1668±0.0598	0.0589±0.0455****	0.0404±0.0055**	0.0272±0.0055**
	TNFα	0.0440±0.0348***	0.0382±0.0205****	0.1393±0.0643	0.028.1±0.0226****	0.0364±0.0088***	0.0388±0.0081***

IL-6, IL-1β and TNFα concentrations in the homogenates of brain, liver and MLN from SPF and GF mice were determined by ELISA. n_{WT}=6-10, n_{GF}=3. Data show Mean±SD. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. Water+Morphine treatment group was used for statistical comparison. Significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparisons test.

Table S2: After FMT with naïve SPF microbiota, GF mice displayed higher expression of cytokines following morphine treatment.

Mice		GF mice	
		Treatment	
	Pro-inflammatory cytokines (pg/μg)	Saline microbiome -Saline	Saline microbiome -Morphine
Brain	IL-6	0.0197±0.0107	0.1349±0.0694*
	IL-1β	0.0452±0.0164	0.2992±0.1081*
	TNFα	0.0238±0.0052	0.3132±0.1444*
Liver	IL-6	0.0566±0.0098	0.4967±0.2318*
	IL-1β	0.0756±0.0635	0.8375±0.4089*
	TNFα	0.1071±19.8	0.654±0.3056*
MLN	IL-6	0.0483±0.0310	0.3020±0.0945*
	IL-1β	0.1094±0.0724	0.5588±0.2297*
	TNFα	0.1283±0.0656	0.2132±0.0609***

After GF mice were transplanted with control microbiome (FMT), inflammatory cytokine levels were measured and compared to morphine-treated mice. Data show Mean ± SD. n_{GF}=3. The data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. *, $p<0.05$; ***, $p<0.001$.

Table S3: Morphine microbiome FMT alone induced inflammation without exposure to morphine.

Mice		SPF mice		GF mice	
		Treatment			
	Pro-inflammatory cytokines (pg/μg)	Saline microbiome	Morphine microbiome	Saline microbiome	Morphine microbiome
Brain	IL-6	0.0164±0.0102	0.1561±0.0612****	0.0187±0.0115	0.1893±0.0754$^{\#}$
	IL-1β	0.0461±0.0221	0.1789±0.0657****	0.0465±0.0327	0.4851±0.1374$^{\#\#}$
	TNFα	0.0508±0.0290	0.3148±0.095.2****	0.0721±0.0611	0.3945±0.0541$^{\#\#}$
Liver	IL-6	0.0304±0.0197	0.2281±0.1279***	0.0359±0.0081	0.2868±0.1354$^{\#}$
	IL-1β	0.026.9±0.0138	0.3248±0.1191****	0.0671±0.0293	0.3521±0.1260$^{\#}$
	TNFα	0.0369±0.0109	0.3241±0.1146****	0.0758±0.0165	0.03633±0.1558$^{\#}$
MLN	IL-6	0.0526±0.0329	0.292.2±0.1315****	0.0204±0.0079	0.1493±0.0219$^{\#\#\#}$
	IL-1β	0.1272±0.1119	0.7111±0.3607****	0.0646±0.0308	0.1653±0.0255$^{\#}$
	TNFα	0.0462±0.0279	0.5247±0.2448****	0.0531±0.0116	0.2828±0.0402$^{\#\#\#}$

Saline microbiome group (FMT) was compared with morphine microbiome (FMT) in either SPF or GF mice. Data show Mean ± SD. n_{SPF}=10, n_{GF}=3. The data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. *, $^{\#}$, p<0.05; **, $^{\#\#}$, p<0.01; ***, $^{\#\#\#\#}$, p<0.001; ****, $^{\#\#\#\#}$, p<0.0001

Table S4: Inflammation induced by morphine tolerance was attenuated in TLR2KO and TLR4KO mice.

Mice		WT mice		TLR2KO mice		TLR4KO mice	
Tissue		Treatment					
	Pro-inflammatory cytokines (pg/μg)	Saline	Morphine	Saline	Morphine	Saline	Morphine
Brain	IL-6	0.0192±0.0091****	0.1992±0.0638	0.0178±0.0041****	0.0210±0.0079****	0.0112±0.0055****	0.0159±0.0053****
	IL-1β	0.0403±0.0105****	0.2445±0.1150	0.0464±0.0135****	0.0596±0.0297****	0.0200±0.0119****	0.0376±0.0310****
	TNFα	0.0364±0.0134****	0.3071±0.0333	0.0430±0.0173 ****	0.0555±0.0269****	0.0070±0.0049****	0.0476±0.0168****
Liver	IL-6	0.0110±0.0044****	0.0623±0.0315	0.0114±0.0039****	0.0180±0.0093****	0.0136±0.0066****	0.0236±0.0145***
	IL-1β	0.0351±0.0175****	0.1515±0.0490	0.0160±0.0101****	0.0209±0.0063****	0.0298±0.0502****	0.0873±0.0520
	TNFα	0.0217±0.0180***	0.0873±0.0467	0.0080±0.0029****	0.0291±0.0215***	0.0300±0.0283****	0.0500±0.0221
MLN	IL-6	0.0177±0.0067****	0.1172±0.0493	0.0069±0.0051****	0.0322±0.0189****	0.0101±0.0055****	0.0189±0.0114****
	IL-1β	0.0170±0.0055****	0.0959±0.0217	0.0182±0.0089****	0.0459±0.0319**	0.0187±0.0101****	0.0682±0.0526*
	TNFα	0.0267±0.0158****	0.2458±0.1212	0.0147±0.0160****	0.0542±0.0310****	0.0406±0.0775****	0.0473±0.0196****

Inflammatory cytokine levels were measured in tissue homogenates. Data are represented as Mean ± SD. n_{WT}=7-10, n_{TLR2KO}=8-12, n_{TLR4KO}=8-12. One-way ANOVA followed by Bonferroni's multiple comparisons test was used for statistical analyses. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Table S5: Probiotics pre-treatment attenuates morphine tolerance-induced inflammation.

Tissue		Treatment			
	Pro-inflammatory cytokines (pg/μg)	Sham + Saline	VSL#3 + Saline	Sham + Morphine	VSL#3 + Morphine
Brain	IL-6	0.0202±0.0094****	0.239±0.0075****	0.1680±0.0896	0.0502±0.0725****
	IL-1β	0.0477±0.0119****	0.0389±0.0176****	0.2953±0.1089	0.1161±0.0980****
	TNFα	0.0343±0.0178****	0.0313±0.0160****	0.1969±0.0829	0.0622±0.0539****
Liver	IL-6	0.0144±0.0118**	0.0099±0.0039***	0.0438±0.0238	0.0228±0.0093*
	IL-1β	0.0981±0.0365****	0.0851±0.0236****	0.4084±0.1384	0.1422±0.1382****
	TNFα	0.0271±0.0229****	0.0256±0.0160****	0.1026±0.0305	0.0994±0.0723**#
MLN	IL-6	0.0196±0.0114****	0.0331±0.0314****	0.1110±0.0645	0.0279±0.0132****
	IL-1β	0.0280±0.0118***	0.0281±0.0134***	0.1212±0.0408	0.0739±0.0499*
	TNFα	0.0318±0.0236***	0.0345±0.0212***	0.1306±0.0626	0.0579±0.0325**

Cytokine levels from control treated animals (gavaged with water) and morphine treated animals were compared to all the other treatment groups. Data show Mean ± SD. n=5-12. One-way ANOVA followed by Bonferroni's multiple comparisons test was used. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. Cytokine levels from control treated animals (gavaged with water) and treated with saline were compared to VSL#3 + Morphine. #$p<0.05$.

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