Supplemental Information

IL-22 Regulates the Complement System to Promote Resistance against Pathobionts after Pathogen-Induced Intestinal Damage

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Supplemental Experimental Procedures

Reagents

Recombinant mouse and human IL-22 were obtained from R&D Systems (Minneapolis, MN). Cobra venom factor (CVF) was obtained from Fisher Scientific.

Post-*C. difficile* Infection Analyses

Eight-week old mice were infected with 10^8 CFU of *C. difficile* VPI10463 after antibiotic treatment as described (Hasegawa *et. al,* 2011). Briefly, mice were pre-treated with a cocktail of seven antibiotics before *C. difficile* infection. Bacterial number was determined by counting CFU in feces and tissue homogenates on selective taurocholate-cycloserine-cefoxitin-fructose agar (TCCFA) and BHI) plates after 24hr-incubation under anaerobic conditions (Hasegawa *et. al,* 2011). To determine bacterial numbers by a culture-independent method, bacterial DNA was extracted from feces using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen, San Diego, CA) and quantitative PCR (qPCR) was performed using *TcdB* gene primers, GAAAGTCCAAGTTTACGCTCAAT and GCTGCACCTAAACTTACACCA, for various

cycles at 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min on an ABI Prism 7900HT system (Applied Biosystems, Foster City, CA). DNA amounts were determined from standard curves established by qPCR of serial dilutions of reference bacteria DNA as previously described (Hasegawa *et al.*, 2010). Tissues harvested from mice 3 days post-infection were fixed and stained with haematoxylin and eosin, and histological assessment of pathology was performed using a numerical scoring system (van den Berg *et al.*, 2006). Tissues harvested from mice on day 0 or day 1 post-infection were homogenized in T-PER tissue protein extraction reagent (Thermo Scientific) and chemokine amounts were determined by ELISA (BD Biosciences, San Jose, CA). The concentrations of AST and ALT in serum samples were examined by AST and ALT activity assay kits (BioVision).

Gene expression in cecum were determined by quantitative RT-PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA) with various cycles at 94°C for 1 min, 60 °C for 1 min, and 72 °C for 1 min on an ABI Prism 7900HT system (Applied Biosystems Foster City, CA). Primer sequences used were GGGCTGTTAAATGGTTGATTCTG and GATGAGGACGAAGGCTGTG for *C3*, CGGATGACGACTCTGTGCAG and TCATACCATCGCTTGTAGGGT for *Cfd*, ACCAGGCTTCAGTCATGAGGAT and ATTTGGGTAAAGGCTGCAAGTG for *Defb4*, GTGGCCGGTGTGCTGTACTT and CGACCTATTTGTTCTTCCTTTCCA for *Defb14*, GCTGACCAGGAAGCCAACAG and CAGGCAGTCCAGGAGGTCTG for *Saa1*, GGCTGCTGAATCTCTTCCAC and TAAGGCTGCAGGTCAGAGGT for *Lbp*, TGTCCTCAGTTTGTGCAGAATATAAA and TCACCATCGCAAGGAACTCC for *S100a8*, ATACTCTAGGAAGGAAGGACACC and TCCATGATGTCATTTATGAGGGC for *S100a9*, and TGCGACTTCAACAGCAACTC and GCCTCTCTTGCTCAGTGTCC for *Gapdh*. The relative ratio of a tested gene to control

GAPDH relative expression was calculated using the 2(−Ct) method, and the fold induction ratio was calculated by comparing the values to those obtained on day 0 (unstimulated).

Apoptosis Assay

Cecal tissues were harvested and fixed with formalin 3 d after infection with 10^8 CFU *C.difficile* as described (Hasegawa *et al*., 2010). Apoptotic cells in tissues were detected by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and counter-stained with DAPI 4',6-diamidino-2-phenylindole (DAPI) (DeadEND Fluorometric TUNEL assay kit, Promega, Madison, WI).

***In vitro* C3 Induction Assay**

HepG2 cells were cultured in MEM (Invitrogen) supplemented with 10% FBS, 1% penicillin/streptomycin/amphotericin, 1 mM sodium pyruvate, and non-essential amino acids.

2 x 10^5 HepG2 cells were treated with 10 ng/ml human recombinant IL-22 with or without indicated doses of WP1066 (Cayman, Ann Arbor, MI) 24 hr. After incubation, *C3* RNA expression in ceca was determined by quantitative RT-PCR and normalized using GAPDH expression. Primer sequences are GAGCCAGGAGTGGACTATGTGTA and CAATGGCCATGATGTACTCG for *C3*, and TGCACCACCAACTGCTTAGC and GGCATGGACTGTGGTCATGAG for *GAPDH*.

Chromatographic Fractionation of Serum Proteins

Mice were i.p. injected with 0.2 ml of 25 μg cobra venom factor with or without 1 μg of IL-22 or with phosphate-buffered saline as control. 24 hr post-injection, blood from each mouse was

collected and immediately treated with EDTA at a final concentration of greater than 10 mM. The serum from each blood sample was collected as the supernatant from each sample after centrifugation at 13,000 x g for 15 min. 50 µl of serum was diluted 20 folds by Buffer A (10 mM potassium phosphate, 5 mM disodium EDTA, 1 mM benzamidine, pH 7.8) and subjected to UnoQ (BioRad, 1.3 ml bed volume) column high performance liquid chromatography (BioRad Biologic DuoFlow system). After a 12 ml wash with Buffer A, proteins were eluted using a 0 to 250 mM linear gradient of NaCl (13.9 mM/ml, 1.5 ml/min) in Buffer A with monitoring eluate absorbance at 280 nm. The proportionality between absorbance at 280 nm and protein concentrations of the input (total serum protein), flow-through and eluted fractions was verified using a commercial protein assay kit (BioRad). 5 µl of indicated fractions (20 % v/v final) were added to 25 µl of the reaction mixture containing 0.5 % (v/v) serum prepared from untreated WT mice and used in neutrophil bacterial killing assay as described in Experimental Procedures.

In vivo C3 Depletion by Cobra Venom Factor (CVF)

Mice received 25 µg/ml CVF i.p. one day before bacterial infection (Belzer *et al*., 2011). 3 days after infection, the sera were collected and C3 expression was determined by immunoblotting using anti-C3 antibody.

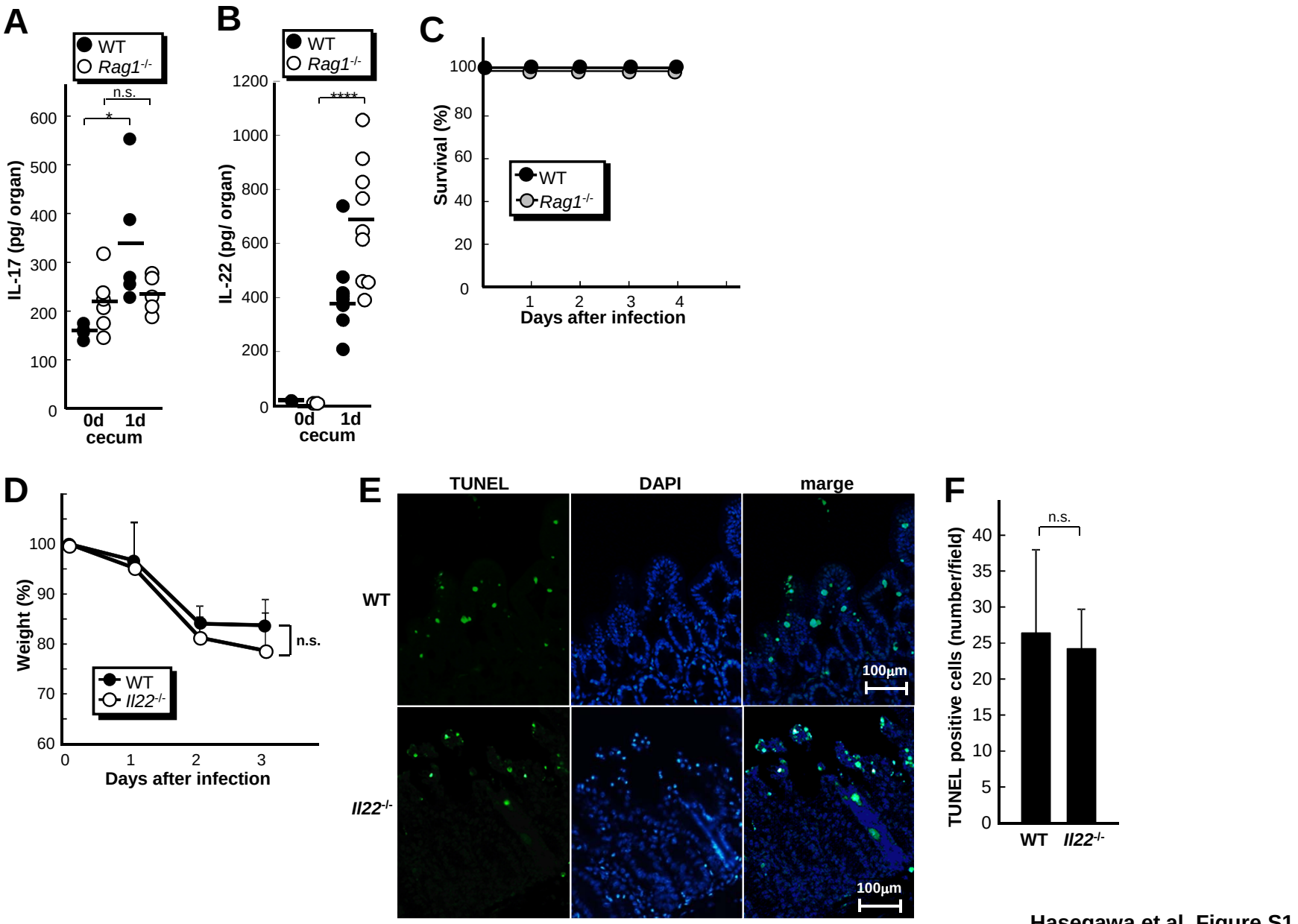
Salmonella Infection

Mice were pretreated by oral gavage with 20 mg of streptomycin 1 day before bacterial infection. The mice were inoculated with 5 x 10^5 CFU *Salmonella enterica* serovar Typhimurium strain SL1344 via the orogastric route.

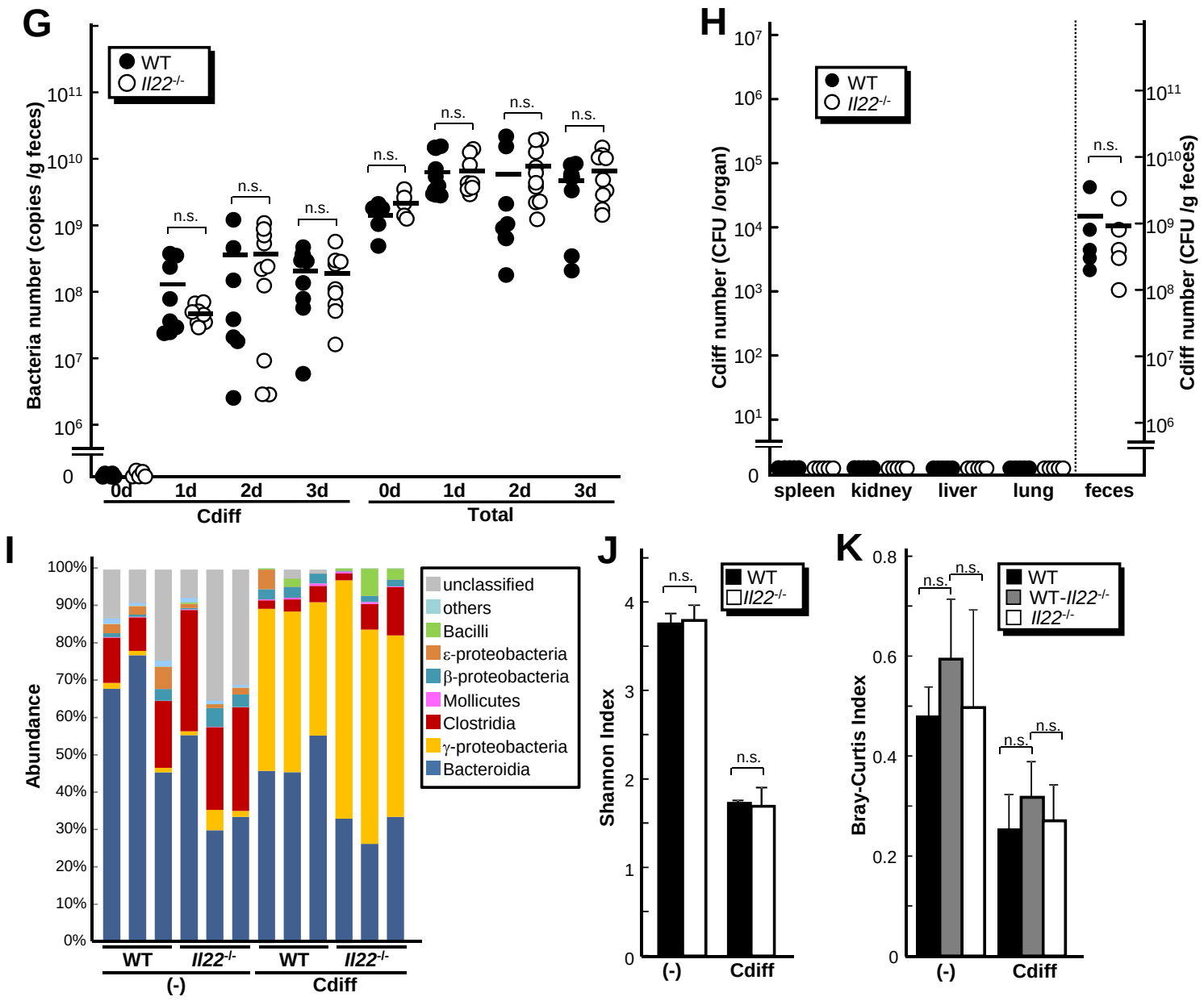
Supplemental Reference

Belzer, C., Liu, Q., Carroll. M.C. and Bry, L. (2011) THE ROLE OF SPECIFIC IgG AND COMPLEMENT IN COMBATING A PRIMARY MUCOSAL INFECTION OF THE GUT EPITHELIUM. Eur J Microbiol Immunol (Bp). 1:311-318

van den Berg R.J., Kuijper, E.J., van Coppenraet, L.E., and Claas, E.C. (2006). Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR. Clin. Microbiol. Infect. *12*:184-186.



Hasegawa et al. Figure S1



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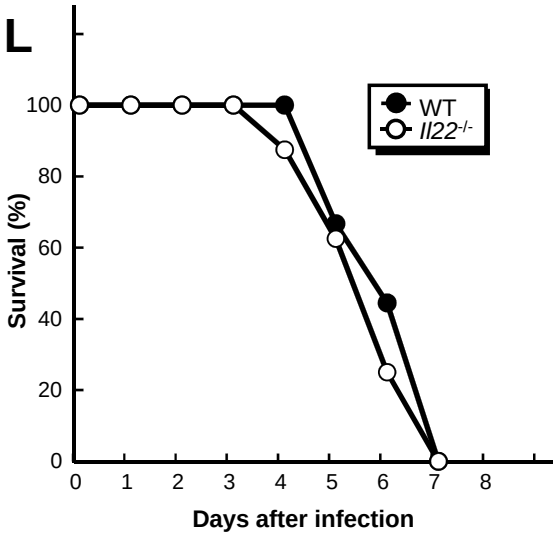
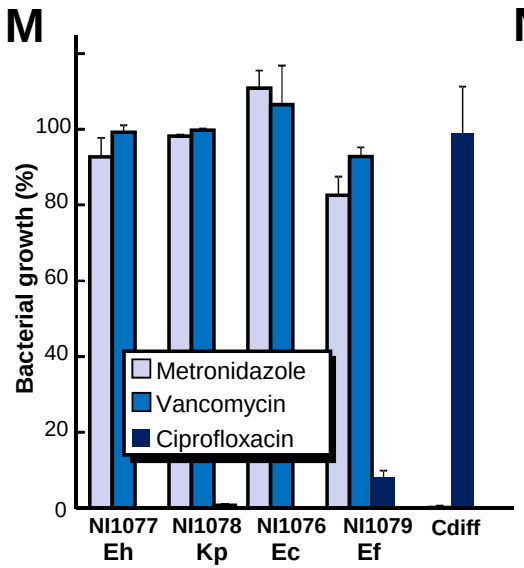
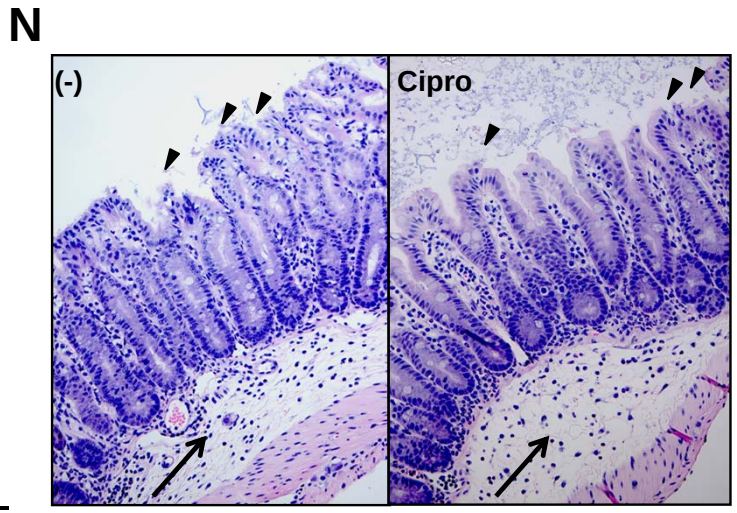




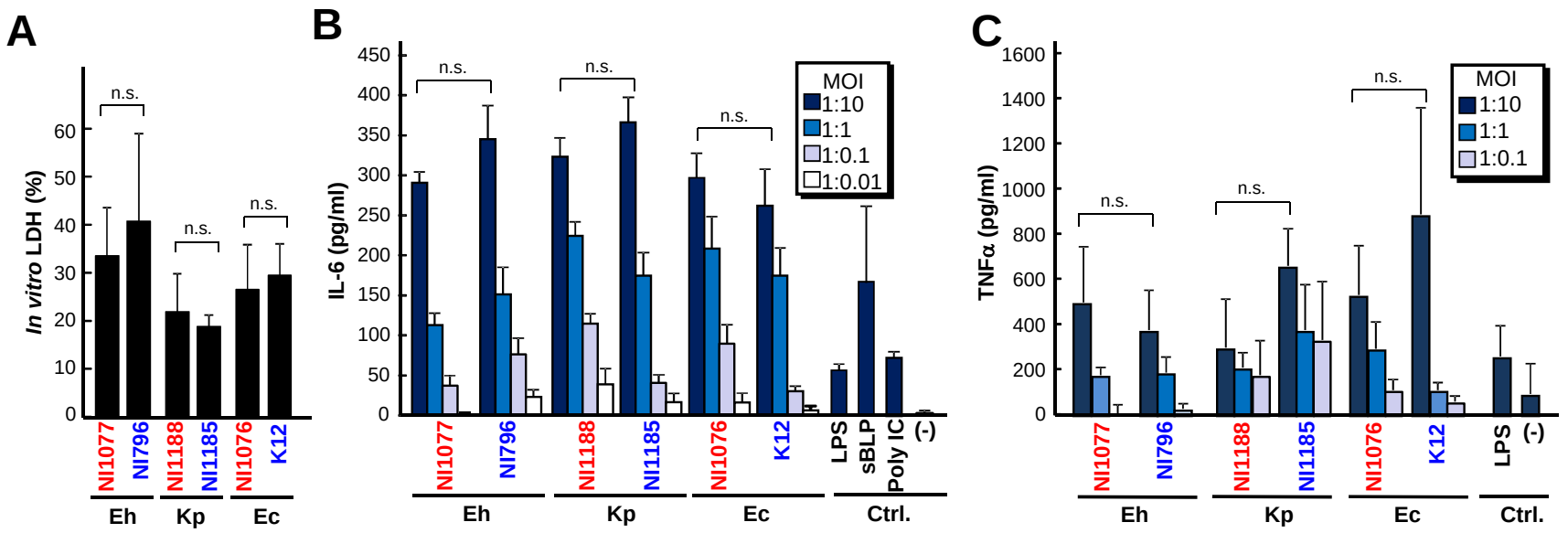


Hasegawa et al. Figure S1

Figure S1, Innate induction of IL-22 after CDI, comparison of *Il22*$^{-/-}$ and WT mice on CDI-induced weight loss and apoptosis, composition of intestinal bacteria, *C. difficile* colonization, growth of bacterial isolates and pathology, and Salmonella susceptibility (related to Figure 1)

A, *Rag1*$^{-/-}$ and WT mice (n=10 per group) were infected with 10^8 CFU of *C.difficile* as described in Experimental Procedures. The amounts of IL-17 (**A**) and IL-22 (**B**) in ceca from uninfected (0 d) and mice infected for 24 hr (1 d) were determined by ELISA. **C,** Survival of *Rag1*$^{-/-}$ and WT mice infected with *C.difficile* was monitored for 14 days (n=10 per group). No deaths were observed beyond 5 days after infection. **D,** The body weights of *Il22*$^{-/-}$ and WT mice infected with *C.difficile* (n=10 per group) were monitored. **E,** *Il22*$^{-/-}$ and WT mice were infected with *C.difficile* and TUNEL positive cells (left panels) in the cecum were measured as described in Supplemental Experimental Procedures. The cells were also stained by DAPI as a control (middle panels). Results are representative of four mice per group. **F,** The number of TUNEL$^+$ cells per optical field from four mice per group were counted and the means were shown with S.D. **G,** The number of *C.difficile* and total bacteria in feces from *I-22*$^{-/-}$ and WT mice (n=7-9 per group) on day 0, 1, 2 and 3 post-infection were determined by qPCR. Results are representative of three independent experiments. **H,** The number of *C.difficile* in spleen, kidney, liver, lung and feces from *Il22*$^{-/-}$ (n=9) and WT mice (n=8) infected with 10^8 CFU of *C.difficile.* Bacterial loads were determined on day 3 post-infection by plating on TCCFA plates. **I,** *Il22*$^{-/-}$ and WT mice (n=3 per group) were co-housed for 2 weeks and infected with 10^8 CFU of *C.difficile.* Bacterial composition in feces before antibiotics treatment (-) and on day 3 post-infection were determined by Illumina sequencing and Mothur analysis. **J and K**, The α-

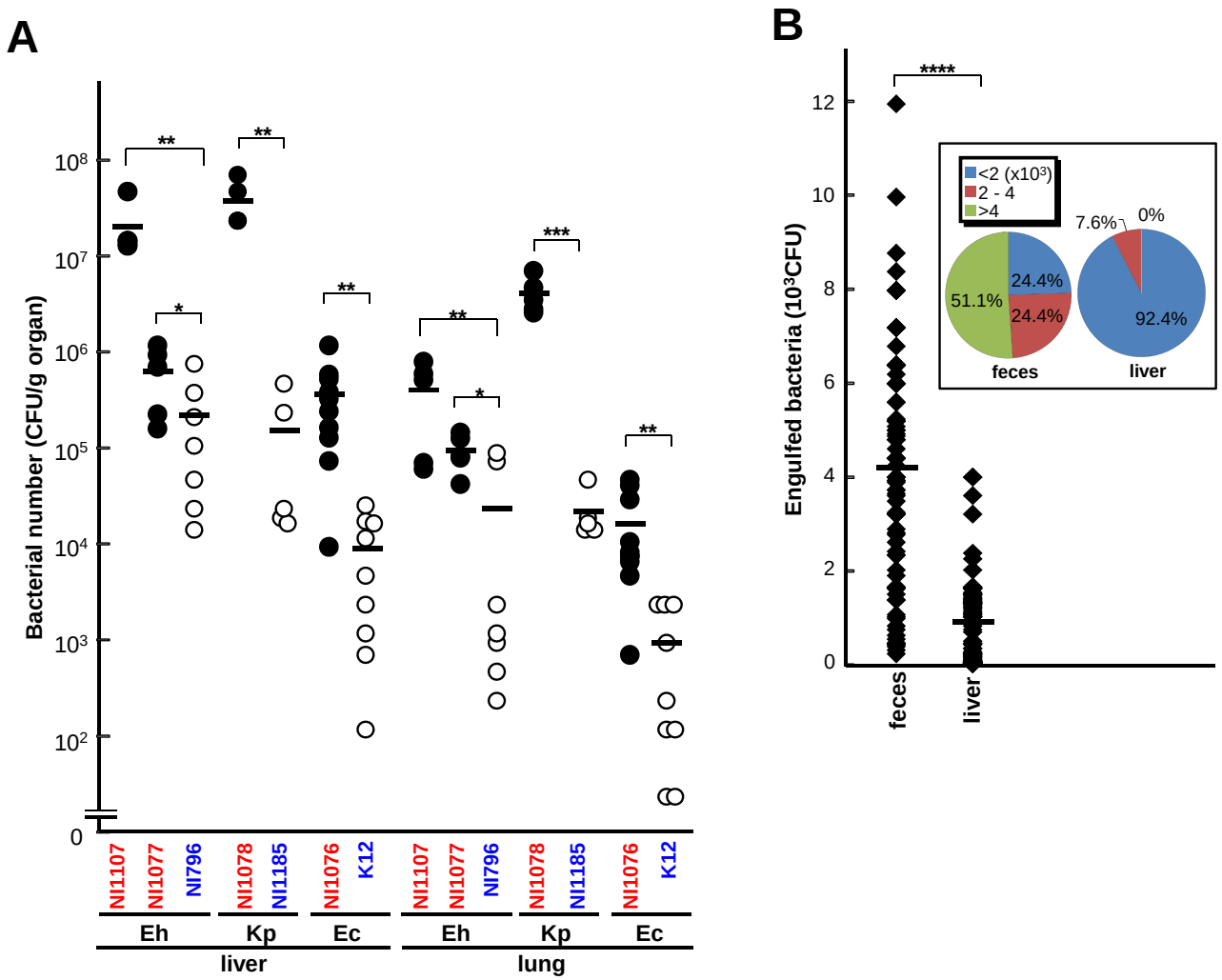
diversity (Shannon index, **J**) and the β–diversity (Bray-Curtis index, **K**) of the indicated microbiota from *Il22*$^{-/-}$ and WT mice were determined as described (Schloss *et al.*, 2009). **L,** Survival of *Il22*$^{-/-}$ (n=9) and WT and mice (n=9) infected orally with *Salmonella enterica* Typhimurium was monitored. Results are representative of two independent experiments. **M,** Indicated bacterial strains were cultured in liquid BHI medium containing 1 μg/ml metronidazole, vancomycin, or ciprofloxacin. Bacteria growth was monitored by absorbance at 600 nm after 24 hr. The relative bacterial growth rates were calculated, compared to the growth rates in antibiotic-free medium which was considered as 100%. The bacterial numbers of NI1077, NI1078, NI1076, NI1079 and *C.difficile* were 1.0×10^9, 8.5×10^8, 1.1×10^9, 5.7×10^8 and 2.0×10^8 CFU/ml, respectively, in antibiotic-free media. **N,** *Il22*$^{-/-}$ mice were treated with 417 mg/kg ciprofloxacin in the drinking water after CDI or left alone. Representative histology of ceca from the mice on day 3 post-infection stained with haematoxylin and eosin. The arrows and arrow heads indicate submucosal edema and epithelial damage, respectively (200 × magnification). Bars indicate means. **, $p < 0.01$; ****, $p<0.0001$; n.s., not significant ($p > 0.05$) Results are representative of three independent experiments.



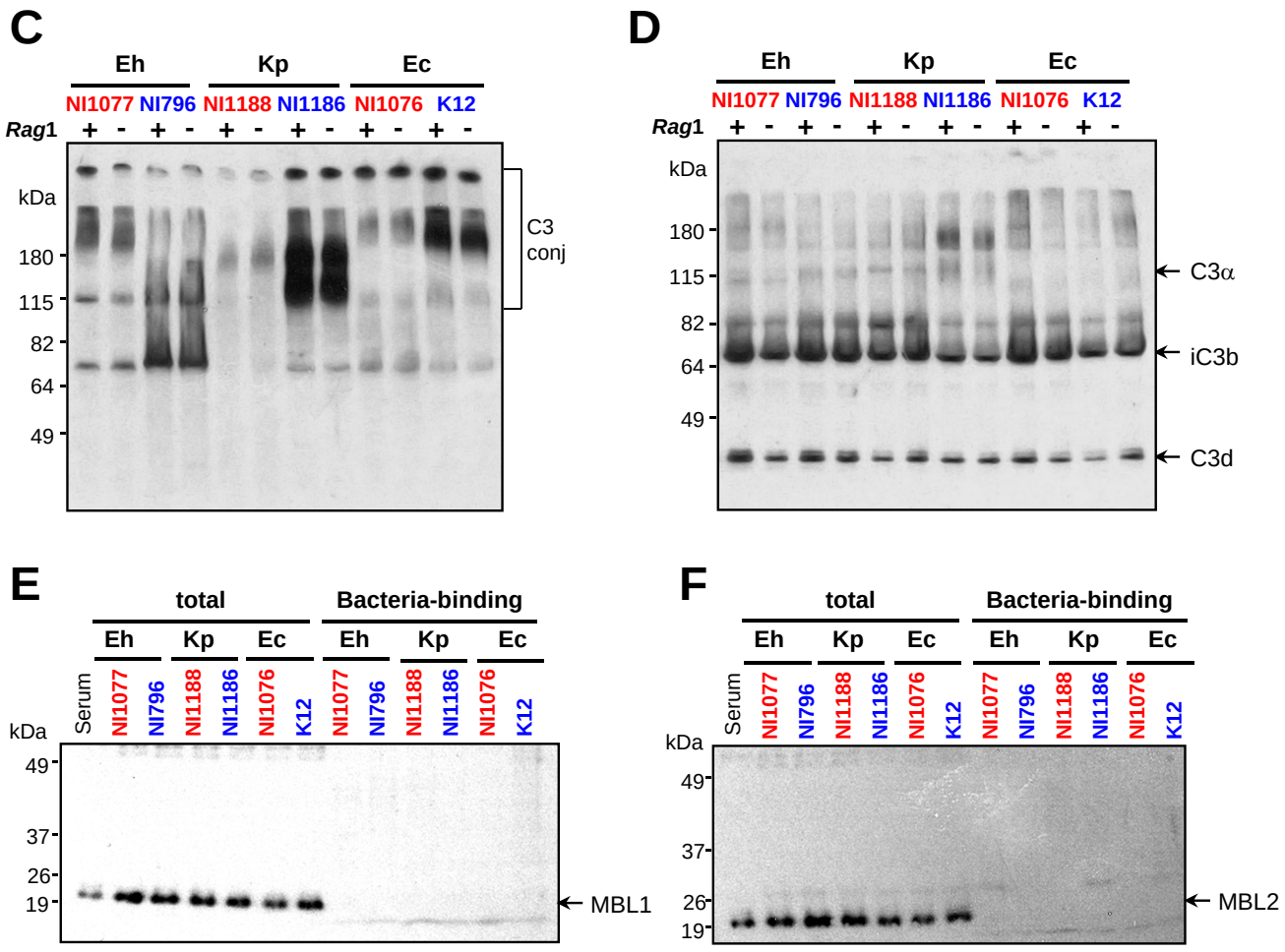
Hasegawa et al. Figure S2

Figure S2, *In vitro* cytotoxicity and cytokine production induced by isolated commensals (related to Figure 2)

10^5 human kidney embryonic 293T cells (**A**) and mouse bone marrow-derived macrophages (**B and C**) were cultured with indicated strains at various MOI for 24 hr. The cytotoxicity was determined by using LDH cytotoxicity assay kit (Promega) **(A)**. The production of IL-6 (**B**) and TNF-α (**C**) was determined by ELISA**.** Bars indicate means ± SD. n.s., not significant ($p > 0.05$). Results are representative of three independent experiments. The strains that were isolated from liver after CDI are indicated by red, whereas intestinal strains are indicated by blue.



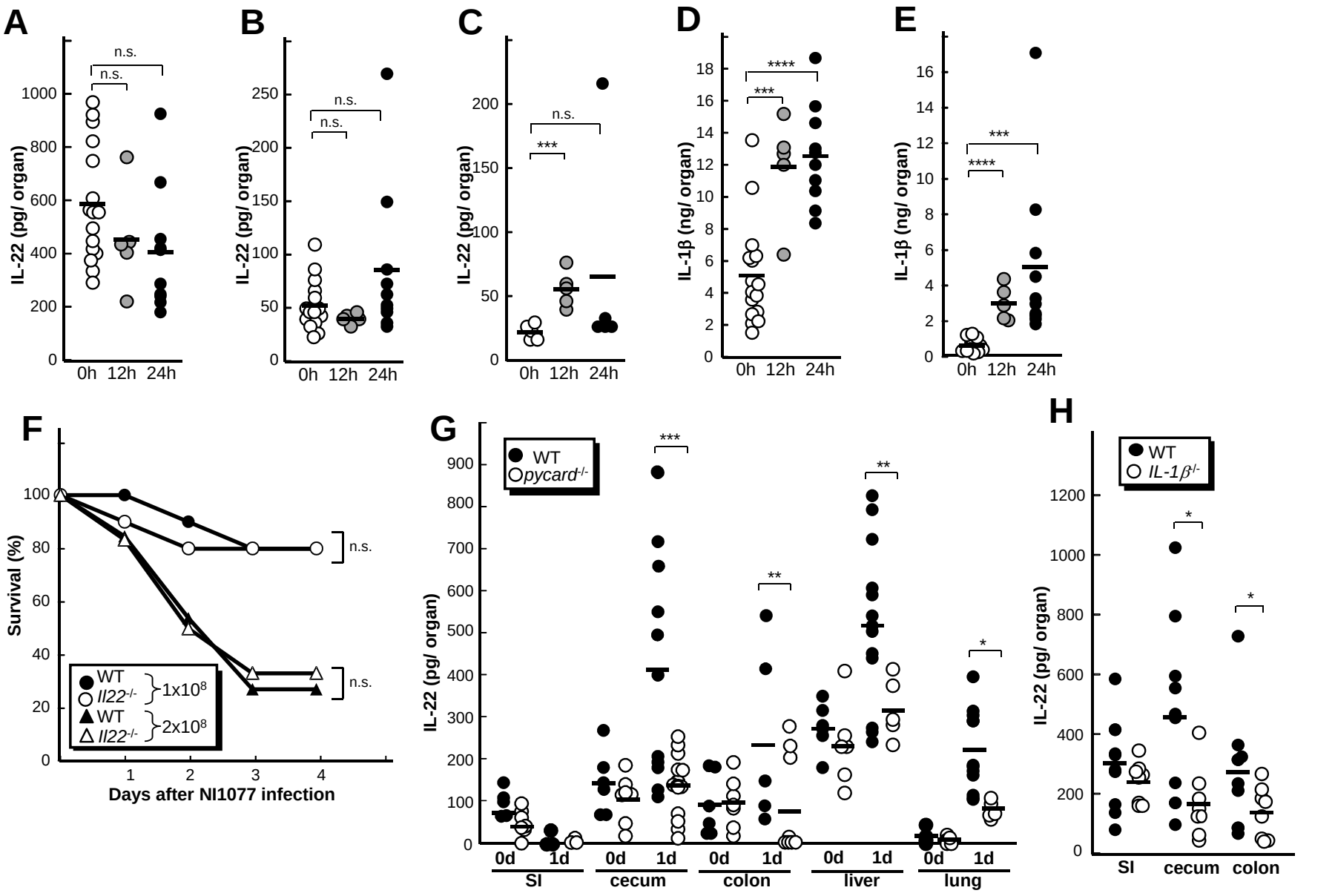
Hasegawa et al. Figure S3



Hasegawa et al. Figure S3

Figure S3, Characterization of host immune responses to bacteria isolates from *C.difficile* -infected mice. (related to Figure 4)

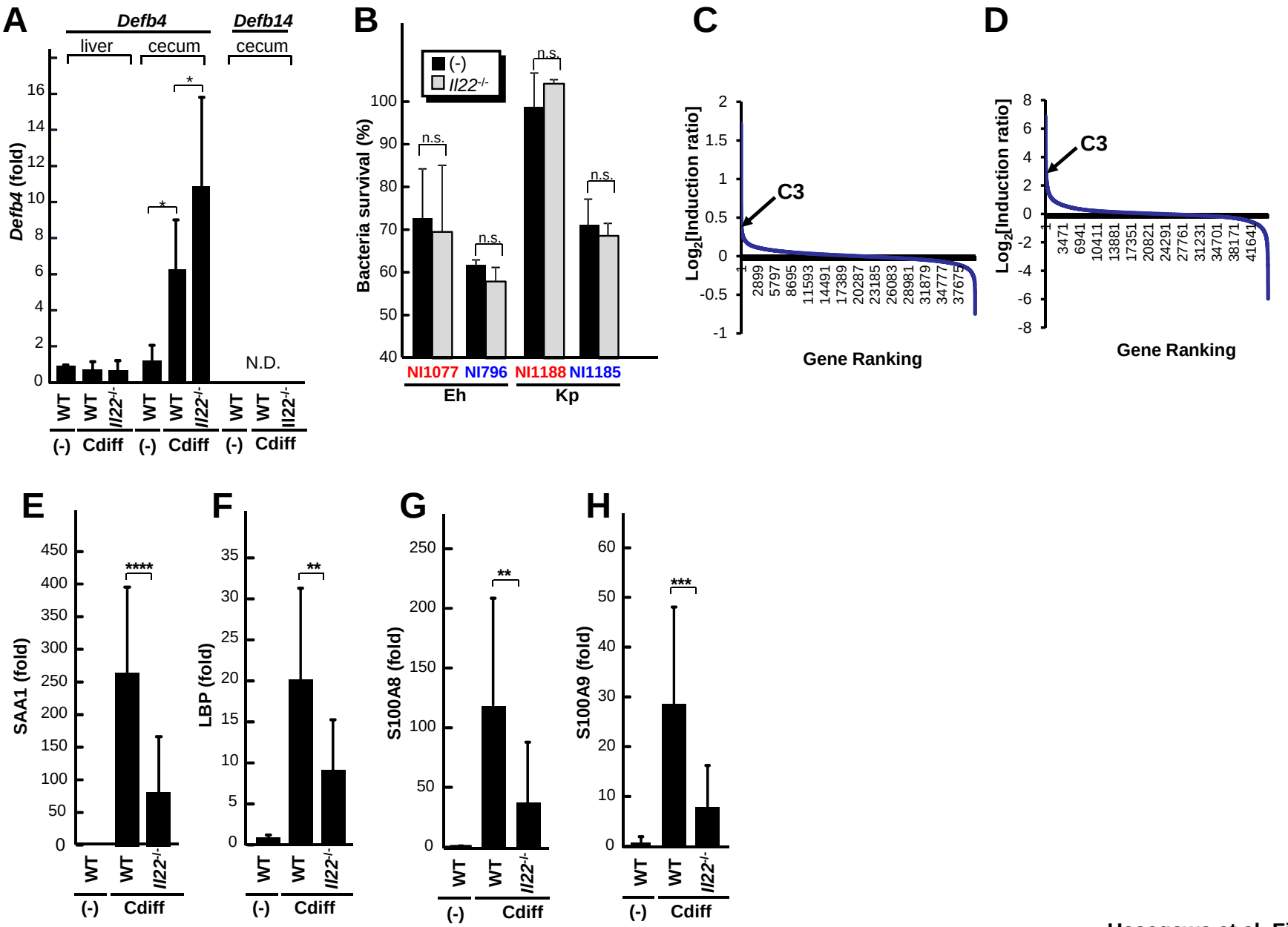
A, WT mice were infected with 10^8 CFU of indicated strains *i.v.* as described in Fig.4. The numbers of bacteria in the liver and lung (n= 5 to 9) on day 2 post-infection were determined by plating on BHI. **B,** One hundred bacterial clones were isolated randomly from feces and liver of *Il22*$^{-/-}$ mice infected with 10^8 CFU of *C.difficile* on day 3 post-infection. The phagocytotic rates of the bacterial clones were determined after incubation with bone marrow-derived macrophages at MOI 1:1 for 20 min. The percentage of bacterial clones with different uptake by macrophages: sensitive (>4x10^3 CFU), intermediate (2-4x10^3 CFU) and resistant (<2x10^3 CFU) in the population are shown in inset. **C and D**, C3 deposition on indicated bacterial strains after incubation with sera from *Rag1*$^{-/-}$ (-) and control WT (+) mice was detected as described in Fig. 4. The bacteria-conjugated (**C**) and total (**D**) C3 subfragments were detected by immunoblotting with anti-C3 antibody. **E and F,** Total and bacteria-bound MBL1 **(E)** and MBL2 **(F)** were detected after incubation with sera from WT mice. The MBL proteins are indicated by arrows. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Results are representative of three independent experiments. Bacterial strains isolated from liver after CDI are indicated by red, whereas control strains are indicated by blue.



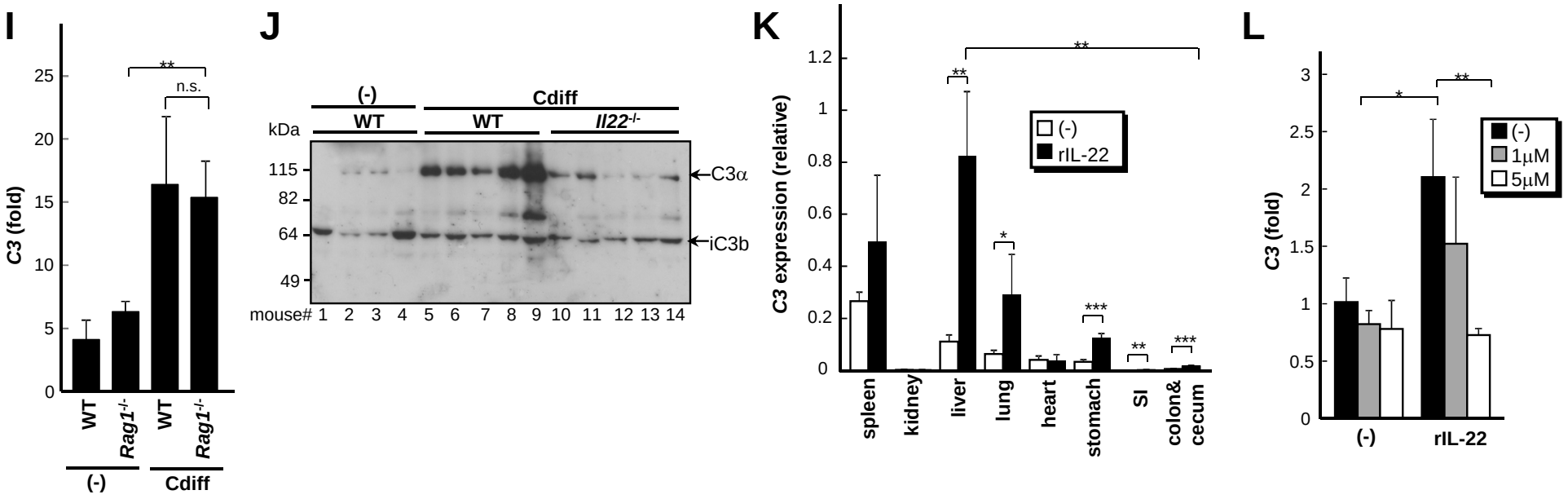
Hasegawa et al. Figure S4

Figure S4. IL-22 production is impaired in *Pycard*- and *Il1b*-deficient mice after CDI (related to Figure 5)

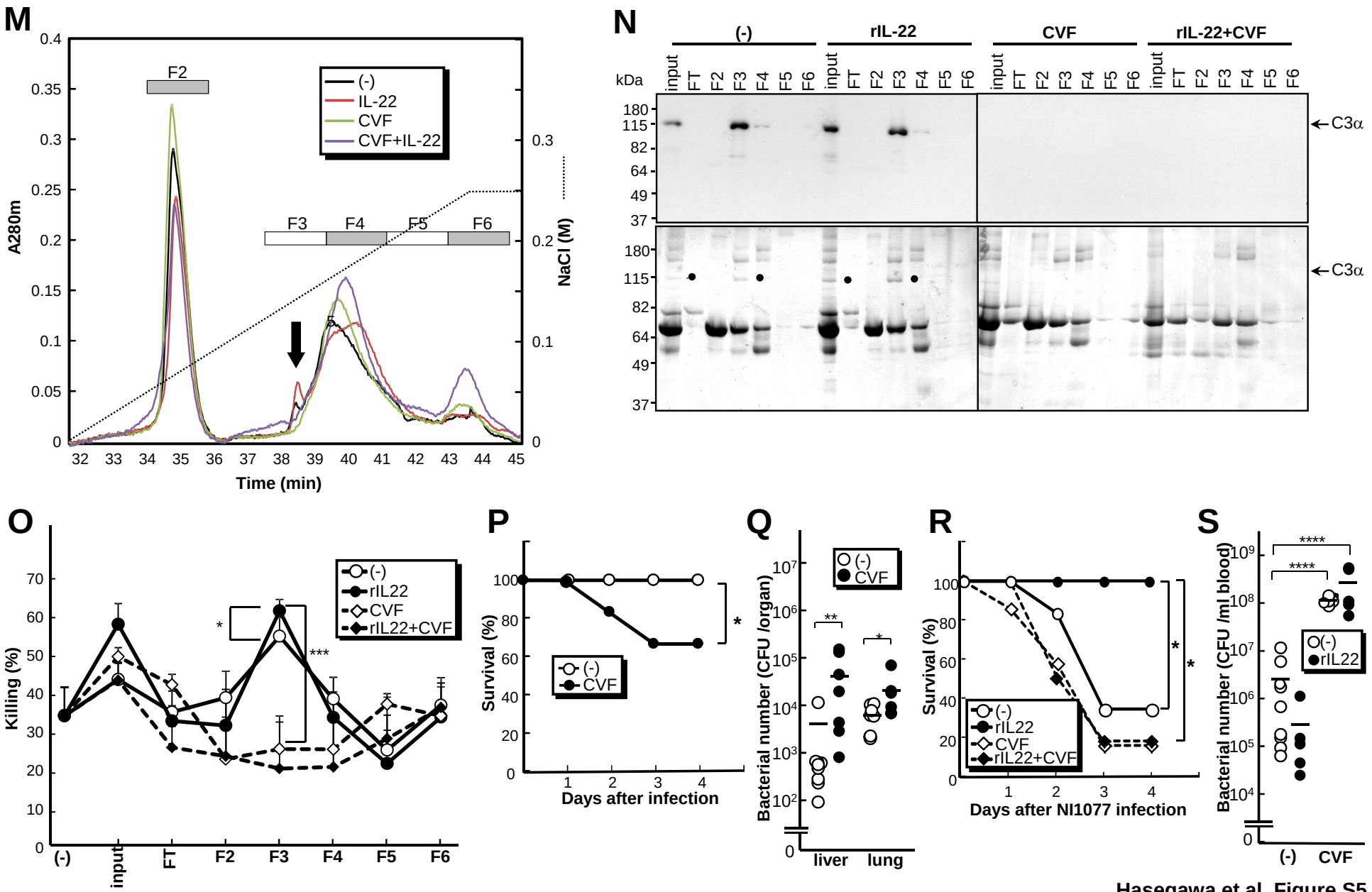
A to E, WT mice were infected with 2 x 10^8 CFU of NI1077 by i.v. injection. The amounts of IL-22 (**A** to **C**) and IL-1β (**D and E**) in liver (**A and D**), lung (**B and E**) and cecum (**C**) 0, 12 and 24 hr after infection were determined by ELISA (n=5 to 17 per group). **F**, 1 x 10^8 CFU or 2 x 10^8 NI1077 were intravenously administered to *Il22*$^{-/-}$ or WT mice (n=10 per group) as performed in Figure 3. Survival of infected mice was monitored for 14 days. No additional deaths were observed beyond 5 days after infection. **G and H,** *Pycard*$^{-/-}$ (**G**), *Il1b*$^{-/-}$ (**H**) and WT mice (n=5-12 per group) were infected with 10^8 CFU *C.difficile*. The amounts of IL-22 in indicated organs from uninfected (0 d) and mice infected for 24 h (1 d) were determined by ELISA. Control WT mice were the same shown in Fig. 1. SI, small intestine. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$. n.s., not significant. Results are representative of three independent experiments.



Hasegawa et al. Figure S5



Hasegawa et al. Figure S5



Hasegawa et al. Figure S5

Figure S5. CDI-induced IL-22 controls expression of specific proteins including complement factor C3 (related to Figure 6)

A, Expression of *Defb4* and *Defb14* in liver and in cecum of *Il22*$^{-/-}$ (n=14) and WT mice (n=12) on day 2 post-infection was determined by quantitative RT-PCR. N.D., not detected. **B,** Comparable bactericidal activity of neutrophils from *Il22*$^{-/-}$ and WT mice. Neutrophils isolated from *Il22*$^{-/-}$ and WT mice were incubated with 5 x 10^3 CFU of the indicated strains in 2.5% fresh mouse serum of WT mice for 2 hr. Surviving bacteria were counted by plating on BHI. **C and D,** *C3* expressions in the intestine of mice treated with recombinant IL-22 **(C)** and TcdA and TcdB (**D**) for 24hr. The average induction of individual gene expression was calculated from the GEO data sets GDS3226 and GSE44091 (Zheng *et al*., 2008; D'Auria *et al*. 2013) and ranked in order. *C3* expression is indicated by arrows. **E to H,** Expression of *SAA1* **(E)** and *LBP* **(F)** in liver and *S100A8* **(G)** and *S100A9* **(H)** in ceca from *Il22*$^{-/-}$ (n=14) and WT mice (n=12) on day 2 post-infection was determined by quantitative RT-PCR. **I,** *C3* gene expression in the livers of *Rag1*$^{-/-}$ (n=10) and WT mice (n=10) on day 2 post-infection was determined by quantitative RT-PCR. **J,** *Il22*$^{-/-}$ and WT mice were infected with 10^8 CFU of *C.difficile*. The amounts of C3 in the sera of *Il22*$^{-/-}$ (n=5) and WT mice (n=5) on day 0 (-) and day 2 post-infection were determined by anti-C3 antibody. **K,** WT mice (n=3 per group) were treated with or without recombinant IL-22 (1μg per mouse) by i.p. injection. Expression of *C3* in 5 μg of total RNA of indicated organs was determined by quantitative RT-PCR. Expression of *C3* was normalized to control GAPDH expression. **L,** STAT3-dependent induction of *C3* in HepG2. 2 x 10^5 HepG2 cells were pretreated with 1 and 5 μM WP1066 (STAT3 inhibitor) or mock (-) 1 hr before stimulation with 10 ng/ml recombinant IL-22. 20 hr after IL-22 stimulation, *C3* expression was determined by quantitative RT-PCR. **M to O,** Mice were i.p. injected with of 25 μg CVF with or without 1 μg

of IL-22 or mock (-). 24 hr post-injection, sera were collected and separated by UnoQ ion exchange column chromatography. Proteins were eluted with 0 to 250 mM NaCl linear gradient while monitoring absorbance at 280 nm (**M**). The arrow in Panel M and dots in Panel N indicate C3. **N,** Proteins in indicated fractions were detected by Coomassie Brilliant Blue R250 staining (lower panel) and immunoblotting with anti-C3 antibody (upper panel). **O,** Indicated fractions were added to the reaction mixture containing 0.5 % serum prepared from untreated WT mice and subjected to neutrophil bacterial killing assay as described in Supplemental Experimental Procedures. **P and Q,** WT mice were treated with 25 μg CVF or mock i.p. one day before CDI (n=9 per group). **P,** The survival of infected mice was monitored for 7 days. No additional deaths were observed beyond 4 days after infection. **Q,** The number of total cultivable bacteria in liver and lung from CVF-treated and -untreated WT mice (n=7 per group). The bacterial number in the tissues on day 3 post-infection was determined by plating on BHI media. Results are representative of three independent experiments. **R and S,** WT mice were pretreated with 25 μg CVF i.p. and 1 μg recombinant IL-22 or mock (n=9 per group) by i.p. injection one day prior to *i.v.* infection with 2 x 10^8 CFU of the pathobiont NI1077. **R,** The survival of NI1077-infected mice was monitored for 7 days. No further deaths were observed beyond 4 days after infection. **S**, The bacterial number in the blood on day 1 post-infection was determined by plating on BHI media (n=5 per group). *, $p < 0.05$; **, $p < 0.01$; ****, ****, $p<0.0001$. Results are representative of three independent experiments.