Supplementary on-line material

Comprises:

Materials and Methods

Mice

Myd88$^{-/-}$ *Ticam1*$^{-/-}$ mice on a C57BL/6 background were a kindly provided by Prof B A Beutler, The Scripps Research Institute (la Jolla, CA, USA) (*1*). F1 controls were generated by crossing these mice to C57BL/6 mice originally purchased from Taconic and maintained at McMaster University. Animals were bred and maintained at three different hygiene statuses. SPF mice were housed in individually ventilated cages (IVC racks) with unlimited access to food and water and were continuously monitored for pathogens. Germ-free and "ultra-clean" SPF mouse colonies were obtained by 2-cell embryo transfer, as described below. *CybbNOS2* mice on a C57BL/6 background and corresponding C57BL/6 control animals were co-housed in clean SPF conditions at the Swiss Federal Institute of Technology (ETH) Zurich, Switzerland. MyD88$^{-/-}$J$_H^{-/-}$ mice were generated as F2 litters from interbreeding of *MyD88*$^{-/-}$ mice and J$_H^{-/-}$ mice (*2*) in "ultra-clean" IVC housing. All experiments were carried out in accordance with the McMaster university animal utilization protocols and the Canadian Council on Animal Care (CCAC) guidelines.

Germ-free rederivations, recolonizations and monocolonizations

Briefly, 4-6 week old *Myd88*$^{-/-}$ *Ticam1*$^{-/-}$ females were superovulated by intra-peritoneal (i.p.) injection of 5 IU pregnant mare serum gonadotropin (PMS, VWR) on d 0, and 5 IU of human chorionic gonadotropin (hCG) on d 2. Females were paired with stud *Myd88*$^{-/-}$ *Ticam1*$^{-/-}$ males on d 2. Two days later, plugged females were euthanized, oviducts collected, and the embryos were flushed out of the oviducts. The fertilised 2-cell embryos were collected and extensively washed in PenStrep (Invitrogen) containing M2 medium (Sigma). Subsequently these embryos were transferred into pseudopregnant germ-free NMRI or Swiss Webster recipient females under aseptic conditions. Resulting litters were maintained in flexible film isolators with unlimited access to autoclaved food and water, and were regularly checked for germ-free status by aerobic and anaerobic culture and sytox-green and gram staining of cecal content to detect unculturable contamination. In addition, serological testing for known viruses and pathogens was performed periodically (Charles River Laboratories). Some animals were exported to IVCs with irradiated food and autoclaved water and colonized with an "ultra-clean" SPF flora, including the altered Schaedler flora bacteria with limited diversification ("ultra-clean" colony). For monocolonizations, 12-week old knockout and control germ-free mice as well as

outbred germ-free mice were exported to a small flexile-film isolator. *Enterococcus faecalis* or *E. coli* K-12 overnight cultures were prepared and aseptically imported into the isolator. The equivalent of 100µl of overnight culture was gavaged into the stomach of each outbred mouse. Knock-out and control animals were co-housed with outbred "colonizers" for indicated time-points. Monocolonization was confirmed by quantitative bacteriology on the feces during the experiment and on cecal content at termination. DSS treatment was carried out in IVCs with unlimited access to food. Drinking water was supplemented with 5% DSS, sterile-filtered and provided to the mice 48h before analysis.

Bacterial strains and plasmids

E. coli K-12 strain JM83 was a gift from Roy Curtis 3rd. The non-virulent *Salmonella typhimurium* mutant M557 (Δ*invG sseD::aphT*) has been described previously (*3*). The other commensal bacterial strains used in this study were isolated from mice housed in the McMaster University Central Animal Facility, or Swiss Federal Institute of Technology (ETH) Zurich, Switzerland. Bacterial clones were isolated from cecal or fecal content by streaking onto blood agar, followed by aerobic culture. Individual colonies were then picked, re-streaked and stocked in 10% glycerol for long-term storage. The isolated species were identified based on 16S rDNA gene sequencing. The RFP expression plasmid pDsRed has been described previously (*4*).

CD4 T cell depletions

Sterile filtered anti-CD4 (clone) was sterile-filtered and imported into surgical isolators. Mice were injected with 200µg I.P. at d-3 and d-1 before monocolonization with *E. faecalis*, and every 3rd day during the recolonization. A second group of mice were monocolonized with *E. faecalis*, then injected with 200µg of anti-CD4 I.P on d26 and d28. All animals were then challenged with 10^7 *E. faecalis* and 10^8 *E. coli* K-12 I.V. and spleens recovered for CFU determination 3hrs later.

CD20 B cell depletions

Anti-mouse CD20 (MB20-11) and Anti-human CD20 (B1-B220) were sterile filtered and imported into surgical isolators. 200µg was injected IP into each mouse at d0, and 100µg injected at d7. At d14, blood counts of B220+CD19+ cells were determined by flow cytometry and fecal albumin levels were determined by ELISA.

Bacterial FACS

3ml LB cultures were inoculated from single colonies of plated cloned bacteria and cultured overnight at 37ºC without shaking. 1ml of culture was gently pelleted for 2mins at 8000rpm in an Eppendorf minifuge and washed 3x with sterile-filtered PBS/1%BSA/Azide before resuspending at approximately 10^7 bacteria per ml. Mouse serum was diluted 1:10 in PBS/1%BSA/Azide and heat-inactivated at 60ºC for 30mins. The serum solution was then spun at 13000rpm in an Eppendorf minifuge for 10mins to remove any bacteria-sized contaminants and the supernatant was used to perform serial dilutions. 25µl serum solution and 25µl bacterial suspension were then mixed and incubated at 4ºC for 1hr. Bacteria were washed twice before resuspending in monoclonal FITC-anti-mouse IgG2a, IgG2b or IgA, PE-anti-mouse IgG1 and APC-anti-mouse IgM (all BD Pharmingen). After a further hour of incubation the bacteria were washed once with PBS/1% BSA/Azide and then resuspended in 2% PFA/PBS for acquisition of a FACSCalibur or FACSArray using FSc and SSc parameters in logarithmic mode. Data were analysed using FlowJo (Treestar, USA). Analysis of specific IgA in intestinal washes was achieved using an identical protocol, substituting cleared intestinal lavage for serum. Intestinal lavages were collected as described previously (*5*).

ELISAs

Total concentrations of antibody isotypes in mouse serum were determined by sandwich ELISA. Coating antibodies were goat-anti-mouse IgG1, 2a, 2b, A and M (Serotech) and detection antibodies were HRP-conjugated anti-mouse IgG, IgM or IgA (Sigma). Standards were myeloma-derived purified IgG1, IgG2a, IgG2b, IgA and IgM from Hycult. Fecal albumin levels were determined using the “Mouse albumin ELISA kit” (Bethyl Labs) as per the manufacturer’s instructions. Fresh feces were collected and suspended at 10mg/ml in PBS for analysis of serial dilutions.

Bacterial containment after gavage

10^{10} CFU of washed *E. coli* K-12 pDsRed (DsRED and Chloramphenicol resistance) in 250µl PBS were gavaged into the stomach of “ultra-clean” knockout and control mice. After 18h, animals were euthanized and spleen and mesenteric lymph nodes were removed aseptically. Finally, the cecum was opened and an aliquot of cecal content was taken. Organs were homogenised in 0.5% Tergitol/PBS using a Tissuelyser (Qiagen) and sterile stainless-steel ball bearings. Cecal content, and organ suspensions were then plated on LB containing chloramphenicol for overnight culture at 37ºC and CFU counting. Control animals not treated with *E. coli* K12

harbouring pDsRed did not contain any chloramphenicol-resistant bacteria in cecum or any organs tested.

Vaccination with peracetic-acid killed bacteria

An overnight culture of nalidixic-acid resistant *Enterococcus faecalis* was washed twice with sterile PBS then incubated with 1% peracetic acid/water for 1hr. The suspension was then washed 4x with PBS and neutral pH values were confirmed. The equivalent of 10^6 or 10^7 CFU of *E. faecalis* was injected i.p. into knockout and control mice on d0 and d7. Mice were bled weekly to measure total and *Enterococcus*-specific Ig levels. Complete bacterial killing and bacterial density was confirmed by plating a mock-treated culture and the calculated equivalent of 10^9 CFU from the peracetic acid killed culture on LB agar and culturing overnight at 37ºC.

Systemic bacterial killing assays

The indicated dose of *E. coli* K12 pDsRed or nalidixic acid resistant *Enterococcus faecalis* were injected i.v. into knockout and control mice. Animals were bled and sacrificed at the indicated time-points and organs were collected aseptically. Organs were homogenised in 0.5% Tergitol/PBS using a tissuelyser and sterile stainless-steel ball bearings. Cecal content, and organ suspensions were then plated on LB containing the appropriate antibiotics and CFU determined. Blood detection limit per ml was defined as 1 CFU per minimum blood volume taken. Whole spleen was analysed therefore the detection limit is 1 CFU/spleen.

Small intestine FACS

1 cm sections of jejunum were dissected and flushed with PBS. Each section was then dissected to open the gut tube and cut into 6 slices. These were incubated in PBS containing 1% BSA (Sigma) and Liberase C1 (Roche) for 25mins at 37ºC with shaking. Digested tissues were mashed through a 20µm cell strainer and the resulting cell suspension stained with PerCP-anti-CD45, APC-anti-B220 (all BD Pharmingen). The cells were then fixed and permeabilized using BD Fix/Perm as per the manufacturer's instructions and stained with FITC-anti-IgA and PerCP-anti-CD45 (BD Pharmingen).

Histology and Immunofluorescence

Intestinal tissue samples were embedded in OCT, snap-frozen in liquid nitrogen, and stored at –80°C. Cryosections (5 µm) were mounted on glass slides, air dried for 2 h at room temperature, and either stained with hematoxylin and eosin (H&E) or fixed in

2% fresh PBS-buffered para-formaldehyde (PFA) before staining with FITC-anti-Ki-67 or FITC-anti-IgA (BD Pharmingen) and DAPI and mounting in Vectashield (Vector labs). To analyse RFP-expressing bacteria spleens were fixed immediately in 4% PFA overnight, followed by overnight treatment in 20% sucrose. Tissues were then embedded in OCT and snap frozen. 5µm sections were mounted on glass slides, stained with DAPI and imaged using an Olympus fluorescence microscope.

Permeability measurements: Ussing Chamber Studies

Two sections of jejunum and colon from each mouse were used for Ussing chamber studies. 5 cm of jejunum and colon samples were collected and divided into 2 segments. Each segment was opened along the mesenteric border, flattened and mounted in an Ussing chamber with an opening of 0.6 cm^2. Tissues were bathed in oxygenated Krebs buffer containing 10 mM glucose (serosal side) or 10 mM mannitol (luminal side) at 37°C, and net active transport across the epithelium was measured via a short circuit current (Isc: µA) injected through the tissue under voltage clamp conditions. After a 20-minute equilibration period, baseline Isc (µA/cm2) and conductance (G: mS/cm2) were recorded. The permeability of the tissue to the inert probe ^{51}CR-EDTA was measured by adding 6µCi/ml ^{51}CR-EDTA (hot) to the mucosal buffer and taking samples every 30 minutes from the serosal side of the Ussing chamber. ^{51}CR-EDTA was measured in a beta counter and expressed as % recovery of the hot sample.

Intestinal permeability in ex-vivo loop

Intestinal permeability ex-vivo was investigated using an isolated arterially perfused jejunal loop as described previously (*6*) . Briefly, after ketamine/xylazine anaesthesia, 4 cm segment of the distal jejunum was selected and a terminal branch of the superior mesenteric artery was cannulated and perfused with Hemoglobin Vesicles (Waseda University, Japan). Luminal ends of the jejunal segment were cannulated using polyethylene cannulas. After transferring the loop into a custom-built organ chamber, saline was perfused intraluminally at 5ml/h using a syringe infusion pump (Harvard, Massachusetts). Venous outflow was sampled continuously for the entire duration of the experiment (36 min) in 3-minute fractions. Intestinal segment was perfused luminally with iso-osmotic mixture of ^{51}CR-EDTA (0.6 mCi/ml) and ^{14}C-mannitol (0.1 mCi/ml (Perkin Elmer, Boston, MA) solution for a period of 9 minutes at 5ml/h. Concentration of the radiolabeled macromolecules in the venous outflow was detected using a liquid scintillation Beta counter (LS 5801, Beckman Coulter, Mississauga, ON). The recovery of radioactivity in each venous outflow fraction is

expressed as a proportion of that found in an identical volume of the luminal perfusate.

Statistics

Differences were analysed for statistical significance using Prism 4 for Macintosh (GraphPad software Inc). The details of the test carried out are indicated in figure legends. Where data was approximately normally distributed, values were compared using either a student t-test for single variable, or 2-way ANOVA for two variables. Approximate P values were computed for 2-way ANOVA. Where data were not normally distributed, (e.g. including bacterial CFU counts close to or equal to zero) non-parametric 2-tailed Mann-Whitney U tests were applied. In all cases, $p < 0.05$ was considered significant.

Author contributions

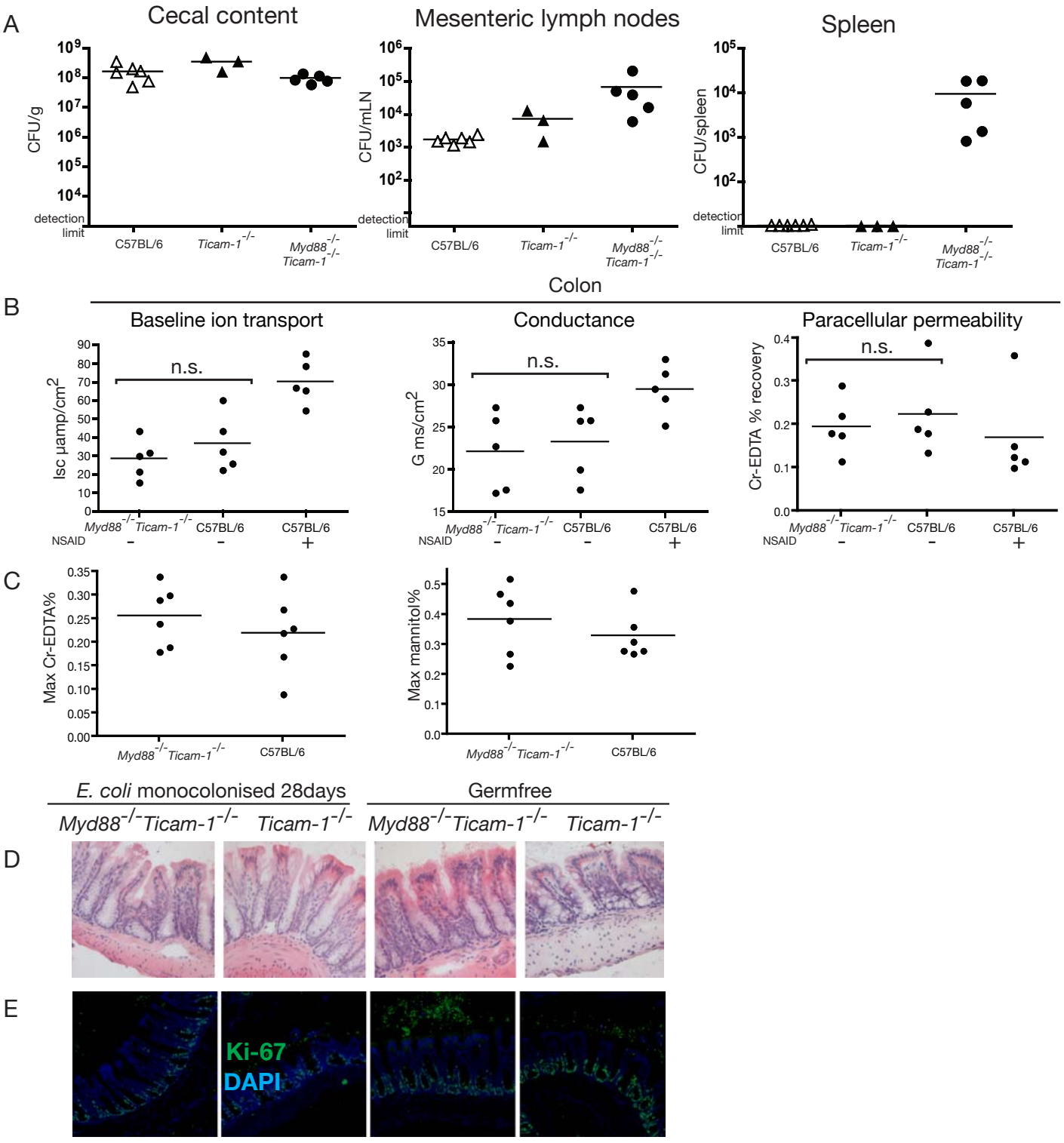
A.J.M and K. D. M oversaw and obtained funding for the research and contributed to experiment planning and editing of the manuscript. E.F.V and P. B. planned and carried out all intestinal permeability measurements. W-D. H. and B. S. planned and provided samples for the analysis of *CybbNOS2*-deficient and appropriate control mice. B.B. provided the original *MyD88-/-Ticam-1-/-* aniamls. T.F.T. provided the reagents for B cell depletion *in vivo.* M.G. contributed to experimental results shown in fig. 4 and fig S6, 7 and 8. Y.V. carried out some experimental work show in Fig. S5. M.S. and M. L. contributed to data shown in fig S7 and S8. S.H. contributed to the planning of experiments, initiation of the project and carried out quantitative bacteriology. E. S. planned and carried out all other experimental work shown, analysed the data and wrote the paper.

Supplementary References

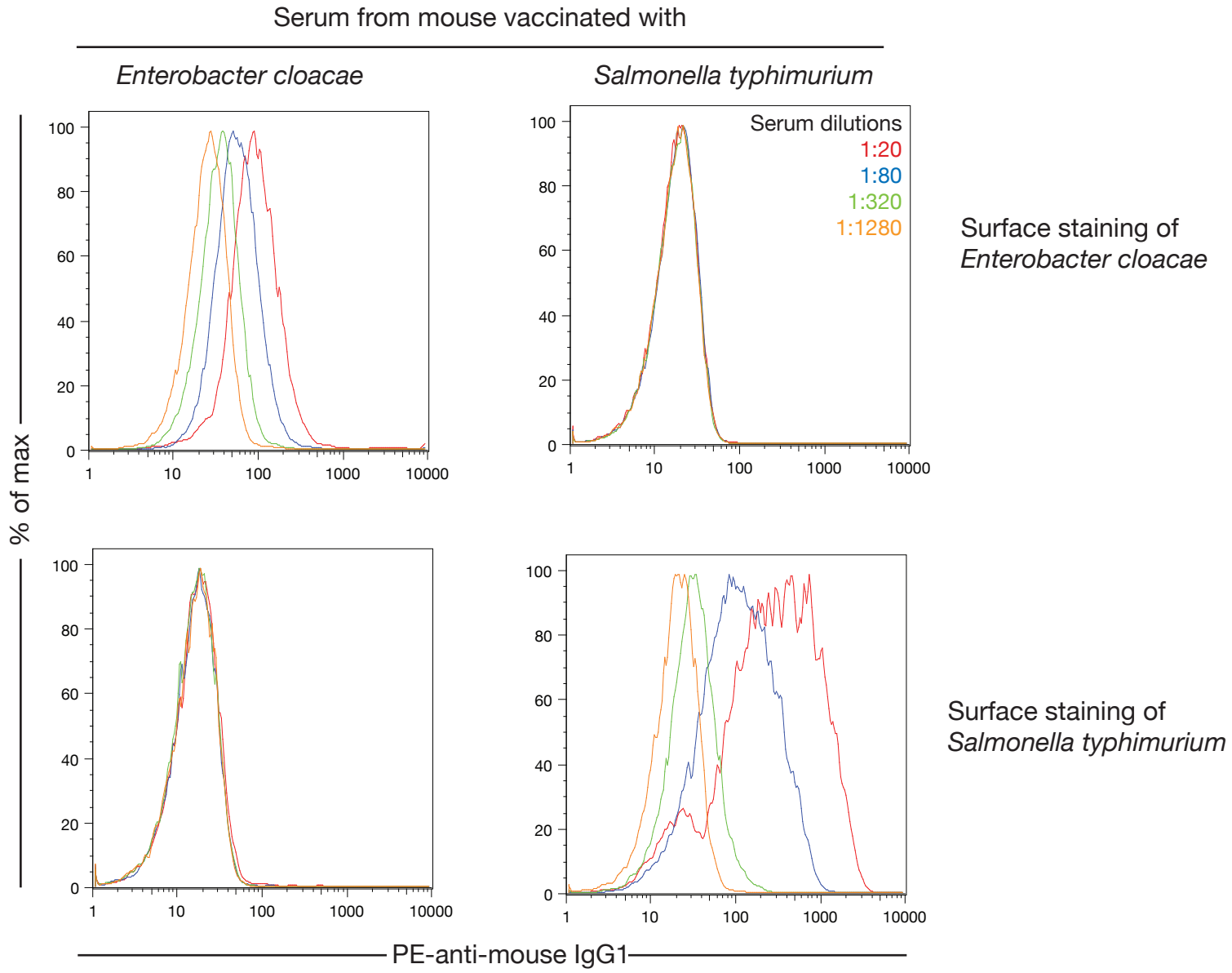
1. K. Hoebe *et al.*, *Nature* **424**, 743 (2003).
2. J. Chen *et al.*, *International Immunology* **5**, 647 (1993).
3. S. Hapfelmeier *et al.*, *Infect Immun* **72**, 795 (2004).
4. S. Hapfelmeier *et al.*, *J Exp Med* **205**, 437 (2008).
5. A. J. Macpherson, T. Uhr, *Science* **303**, 1662 (2004).
6. A. K. Varghese *et al.*, *Gastroenterology* **130**, 1743 (2006).

Supplementary table 1: Titres of anti-commensal IgG1 antibodies obtained from knockout and wild-type mice housed in different animal facilities and hygiene statuses. Upper values show range and mean values of Median fluorescence intensity obtained at a serum dilution of 1:20 in representative experiments. Lower values show the number of mice analysed for each experiment shown.

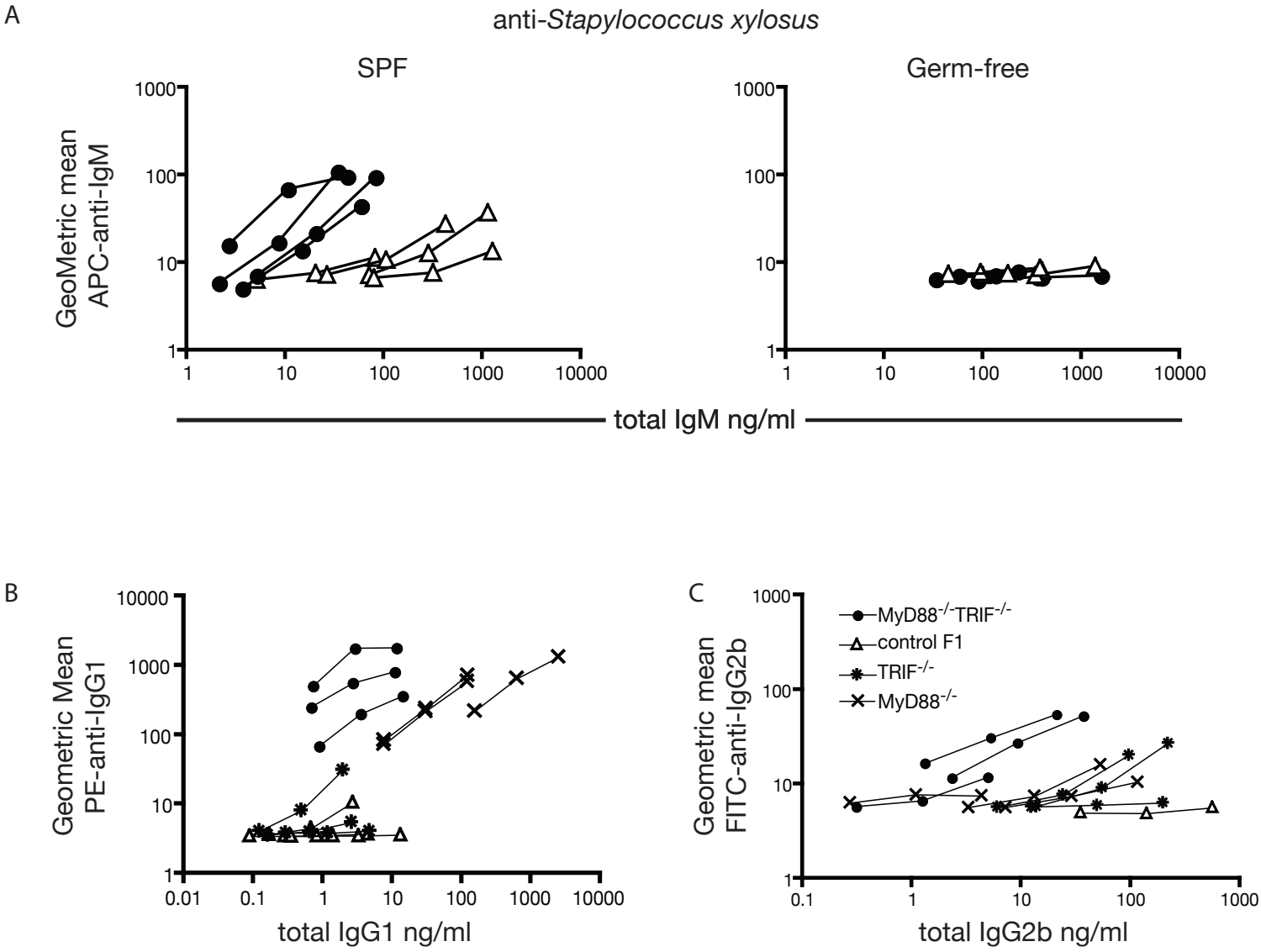
Bacterial species checked	WT Median Fluorescence intensity at 1:20	*Myd88*$^{-/-}$ *Ticam1*$^{-/-}$ Median Fluorescence intensity at 1:20
Room 1U13 (SPF) McMaster University, Canada		
Enterococcus faecalis	4.6 < 4.7 <4.8 n=5	74.6 <2 49.5 < 473 n=5
Staphylococcus xylosus	8.9 < 16.9 < 21.2 n=5	126 < 194.7 < 382 n=5
Room 1U69 (SPF) McMaster University, Canada		
Mixed aerobic fecal cultures	3.7 < 4.1 < 4.78 n=3	11.8 < 20.93 < 27.6 n=3
Room 1U19 (Ultraclean) McMaster University, Canada		
Mixed aerobic fecal cultures	6.6 < 15.8 < 27.7 n=4	47 < 96.78 < 169 n=4
(SPF) Institute of Microbiology, ETH Zürich		
	Litter mate controls	*MyD88*$^{-/-}$
Mixed aerobic culture of whole feces	8.9 < 11.2 < 15.6 n=4	24.0 < 35.37 < 57.8 n=4



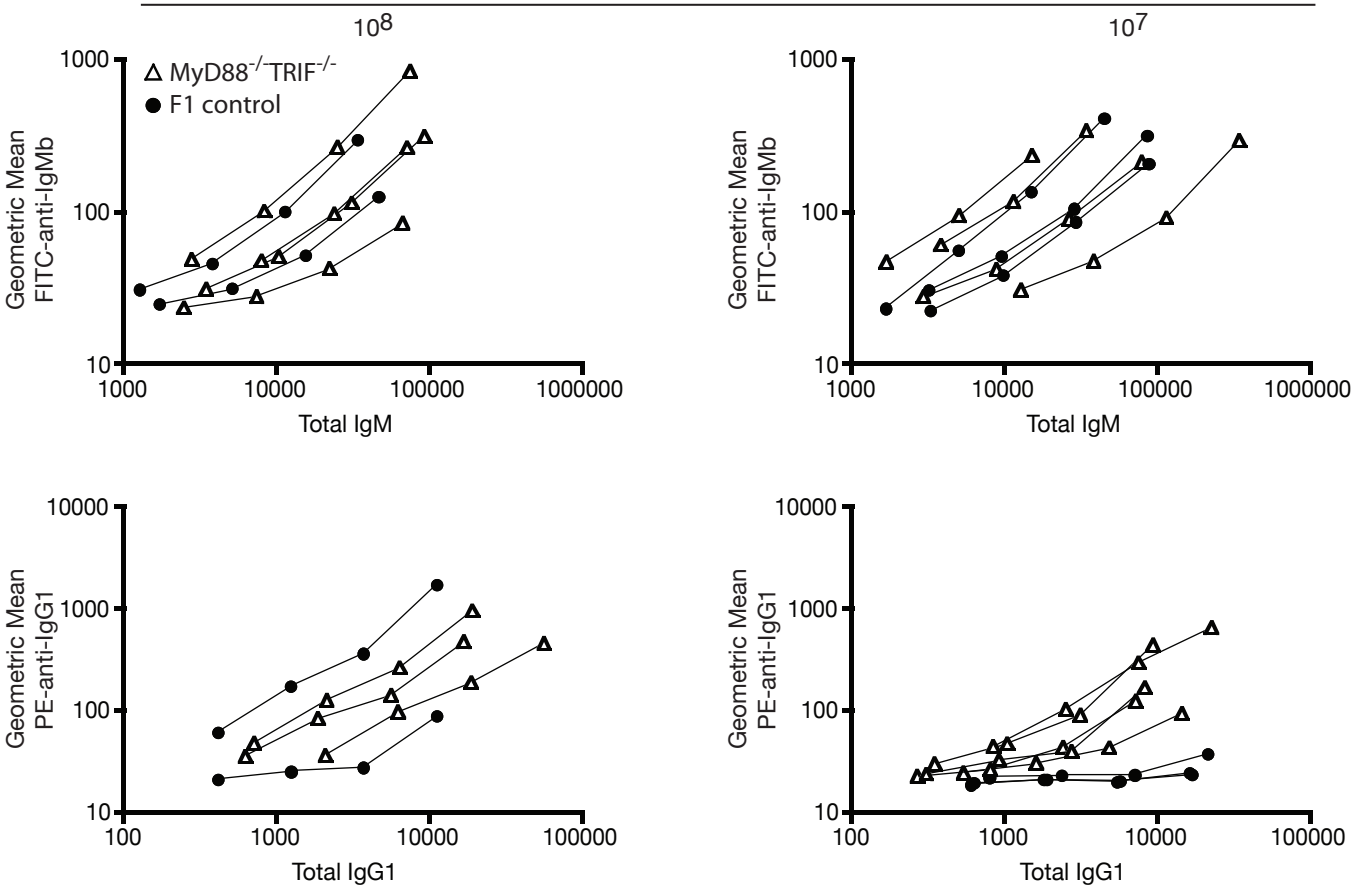
Supplementary figure 1: Co-housing with SPF animals, *ex-vivo* intestinal loop measurements and histology confirm Ussing chamber studies. A. *Myd88*$^{-/-}$*Ticam-1*$^{-/-}$ mice were cohoused for 48h with SPF C57BL/6 mice and CFU of aerobic bacteria determined by plating on blood agar from the indicated organs. B. Ussing chamber measurements of baseline ion transport, conductance and paracellular permeability, as assessed by serosal ^{51}Cr-EDTA recovery, of colon from *Myd88*$^{-/-}$*Ticam-1*$^{-/-}$ mice, co-housed C57BL/6 control mice (B6), and positive control (C57BL/6) mice treated with 7.5 mg/kg Indomethacin (NSAID). C. Total ^{51}Cr-EDTA and mannitol flux from intestinal lumen to blood/lymph in artificially perfused jejunum loops from *Myd88*$^{-/-}$*Ticam-1*$^{-/-}$ and control C57BL/6 (B6) mice. D. HE stained frozen sections of colon from germ-free and monocolonized *Myd88*$^{-/-}$*Ticam-1*$^{-/-}$ and *Ticam-1*$^{-/-}$ control mice. E. Ki-67 stained frozen sections of colon from germ-free and monocolonized *Myd88*$^{-/-}$*Ticam-1*$^{-/-}$ and *Ticam-1*$^{-/-}$ mice. Ki-67 staining and HE staining is representative of 5 mice per group.



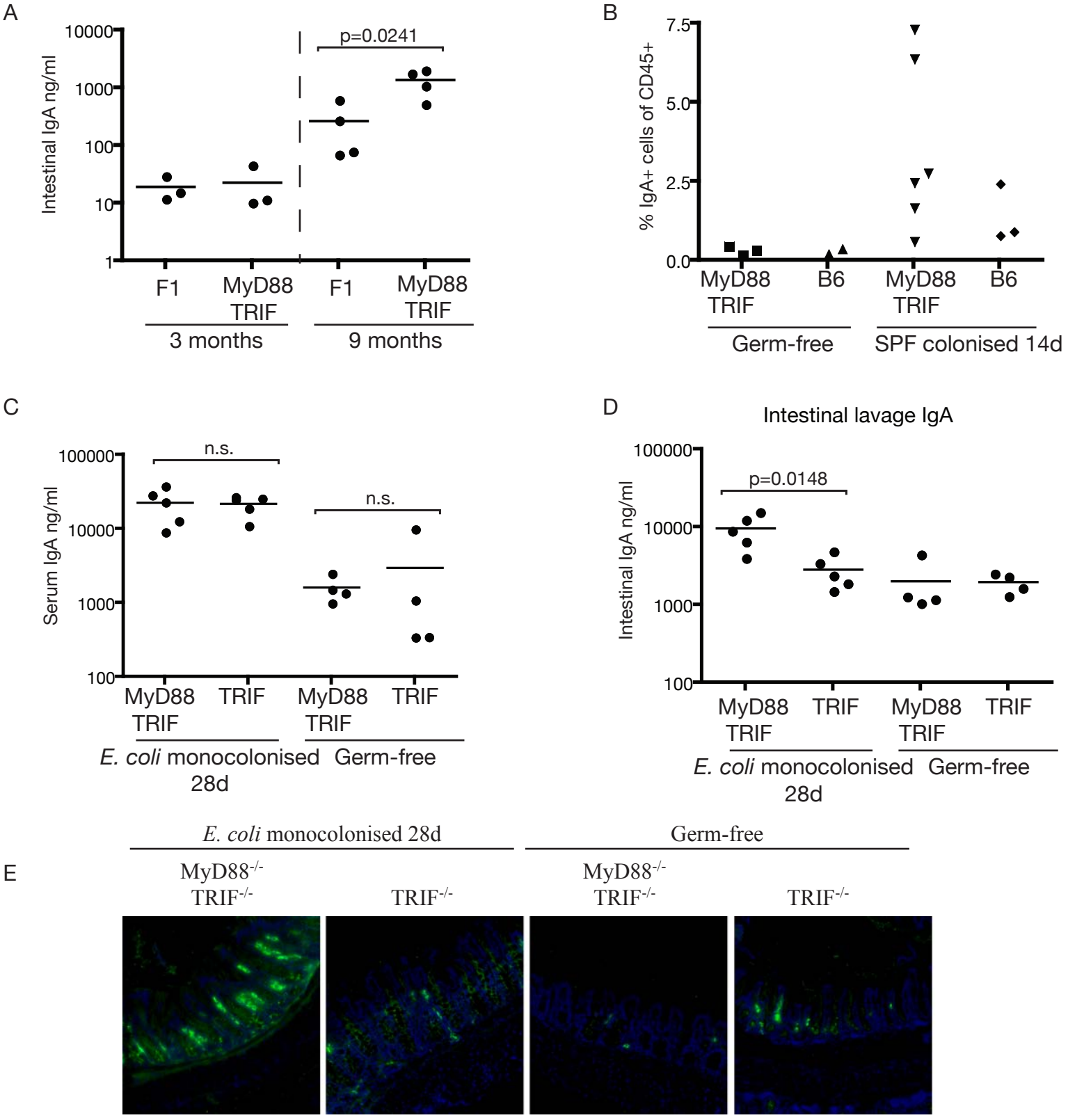
Supplementary figure 2: FACS staining of live bacterial surfaces with whole serum accurately distinguishes between anti-*Salmonella* and anti-*Enterobacter* anti-sera.
Serum from mice vaccinated with live *Enterobacter cloacae* or live attenuated *Salmonella typhimurium* M556 was used to stain the surface of live *Enterobacter cloacae* and *Salmonella typhimurium*. PE-anti-IgG1 was used as a secondary reagent. Representative histograms are shown with serial dilutions of serum. Red 1:20, Blue 1:60, Green 1:180, Orange 1:540. One representative plot is shown of n = 20 *E. cloacae* vaccinated mice and one of n = 8 *Salmonella* vaccinated animals.



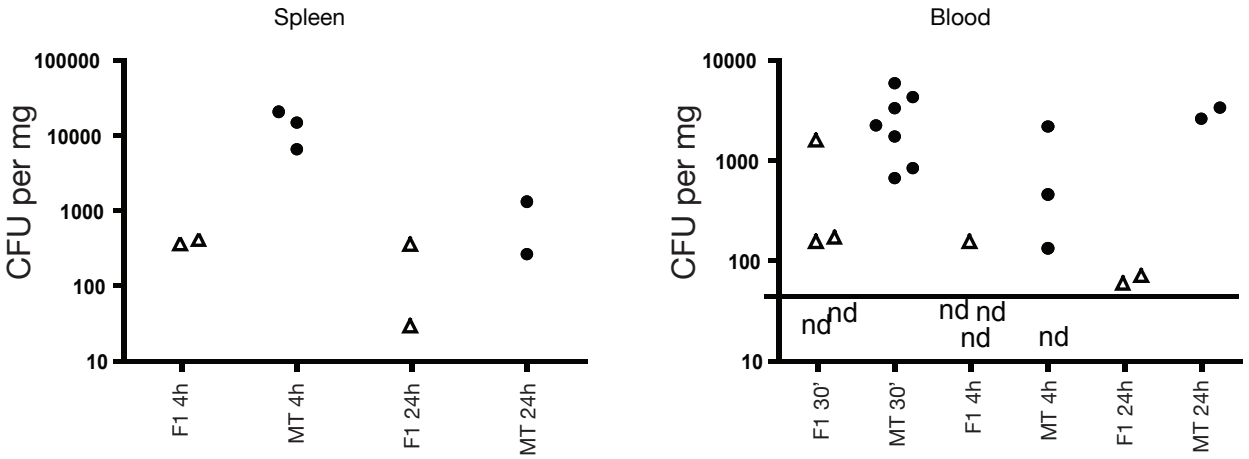
Supplementary figure 3: IgM, IgG2b and IgG1 titres against *Staphylococcus xylosus* in MyD88$^{-/-}$ TRIF$^{-/-}$, MyD88 single-knockout and TRIF single-knockout mice. A. IgM staining of the commensal bacterium Staphylococcus xylosus from the serum of "clean SPF" or germ-free mice. B and C. Titrations of anti-Staphylococcus xylosus surface-specific IgG1 (B) and IgG2a (C) reactivity in 24wk old MyD88-/- and TRIF-/- mice, compared to double-knock out and F1 controls.



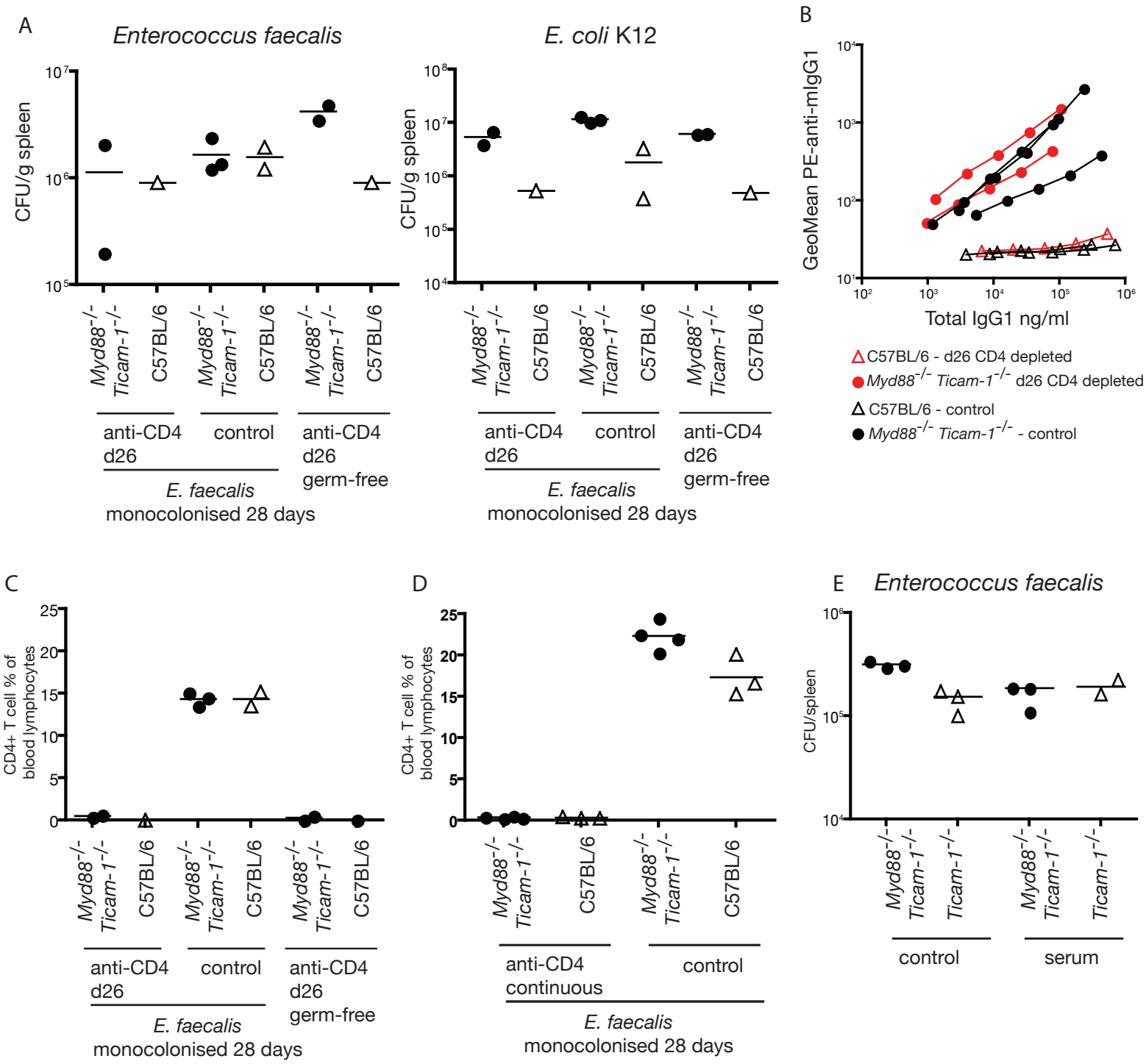
Supplementary figure 4: MyD88$^{-/-}$TRIF$^{-/-}$ mice have a higher threshold for induction of anti-*Enterococcus* antibodies when vaccinated with killed bacteria. MyD88$^{-/-}$TRIF$^{-/-}$ mice and F1 controls were vaccinated with 10^7 or 10^8 peracetic-acid killed *Enterococcus faecalis* intraperitoneally on d 0 and d 7. Blood was collected at d 21 after the first injection and total and specific IgM and IgG1 was measured by sandwich ELISA and bacteria FACS. No Enterococcus-specific antibodies were detected in any mice at d 0.



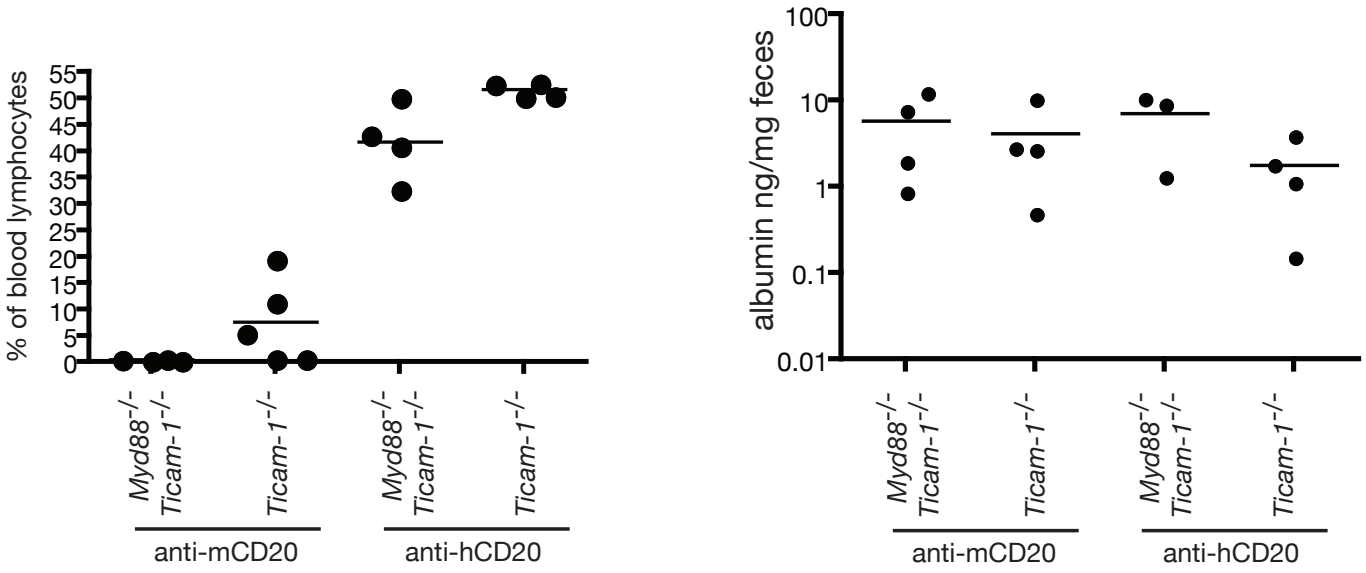
Supplementary figure 5: Normal or elevated IgA production in MyD88$^{-/-}$TRIF$^{-/-}$mice. A. Total IgA measured in the intestinal wash of SPF MyD88$^{-/-}$TRIF$^{-/-}$ and F1 control mice at 3 and 9 months of age. IgA levels appear to be elevated in older knockout mice. B. Jejunum lamina propria of germ-free and 14 day recolonized MyD88$^{-/-}$TRIF$^{-/-}$ (MyD88TRIF) and F1 mice was analysed by flow cytometry for CD45 and intracellular IgA. % of CD45+ cells with intracellular IgA is quantified. One representative experiment of 3 is shown. C. Induction of total serum IgA after monocolonization of germ-free MyD88$^{-/-}$TRIF$^{-/-}$ and TRIF$^{-/-}$ mice for 28 days with *E. coli* K-12. D. Total IgA in intestinal lavage from the same mice as (C). *p=0.0148 by 2-tailed t-test. E. Frozen sections of colon from germ-free and *E. coli* K12 monocolonized mice were stained with FITC-anti-IgA and DAPI. Images are representative of 5 mice per group.



Supplementary figure 6: MyD88$^{-/-}$TRIF$^{-/-}$ mice are inefficient at killing bacteria injected IV. 10^8 *E. coli* K12 expressing RFP and a chloramphenicol resistance cassette were injected into the tail vein of MyD88$^{-/-}$TRIF$^{-/-}$ (black circles) and F1 control (white triangles) mice. Animals were bled and sacrificed at the indicated time-points and *E. coli* density quantified in the spleen and blood by selective plating.



Supplementary Figure 7: Serum antibody and not CD4+ T cells are required during the effector phase to protect *MyD88-/-Ticam-1-/-* mice from intravenously injected *Enterococcus faecalis*. A-C: *MyD88-/-Ticam-1-/-* and C57BL/6 germ-free mice were monocolonized with *Enterococcus faecalis* or left germ-free as indicated for 28 days. On day 26 and day 28 mice were injected with 200µg anti-CD4 I.P. to acutely deplete CD4+ T cells. On day 29, all mice were injected intravenously with 10^8 *E. coli* K-12 and 10^7 *Enterococcus faecalis*. A. Three hours post-injection spleens were homogenised and plated on selective agar to determine bacteria CFU. Each point represents a single mouse. B. titres of anti-*Enterococcus faecalis* IgG1 in serum of mice shown in (A). C) T cell percentage in blood of animals shown in (A). D. Confirmation of T cell depletion in mice shown in fig 4A-C. E. Serum-antibody-coated *E. faecalis* are efficiently cleared in *MyD88-/-Ticam-1-/-* mice. Germ-free *MyD88-/-Ticam-1-/-* and *Ticam-1-/-* controls were injected intravenously with 10^7 *E. faecalis* that had been mock-treated or coated with serum-antibodies from *E. faecalis*-monocolonized *MyD88-/-Ticam-1-/-* mice. CFU in spleen were determined 3h after injection.



Supplementary figure 8: Germ-free *MyD88-/-Ticam-1-/-* mice depleted of B cells with anti-CD20 do not display protein-losing enteropathy. Germ-free *MyD88*$^{-/-}$*Ticam*$^{-/-}$ and control *MyD88*$^{+/+}$*Ticam*$^{-/-}$ mice were injected on d0 with 200µg and boosted on d7 with 100µg of anti-mouse CD20 clone MB20-11 (anti-mCD20) or anti-human CD20 clone B1 hCD20 (anti-hCD20) as a control. A. B cell percentages in blood 14 days after injection. B. Protein-losing enteropathy as quantified by concentration of albumin in feces.