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Supplemental Information

T Follicular Helper Cells Promote a Beneficial Gut Ecosystem for Host Metabolic Homeostasis by Sensing Microbiota-Derived Extracellular ATP

Lisa Perruzza, Giorgio Gargari, Michele Proietti, Bruno Fosso, Anna Maria D'Erchia, Caterina Elisa Faliti, Tanja Rezzonico-Jost, Daniela Scribano, Laura Mauri, Diego Colombo, Giovanni Pellegrini, Annalisa Moregola, Catherine Mooser, Graziano Pesole, Mauro Nicoletti, Giuseppe Danilo Norata, Markus B. Geuking, Kathy D. McCoy, Simone Guglielmetti, and Fabio Grassi

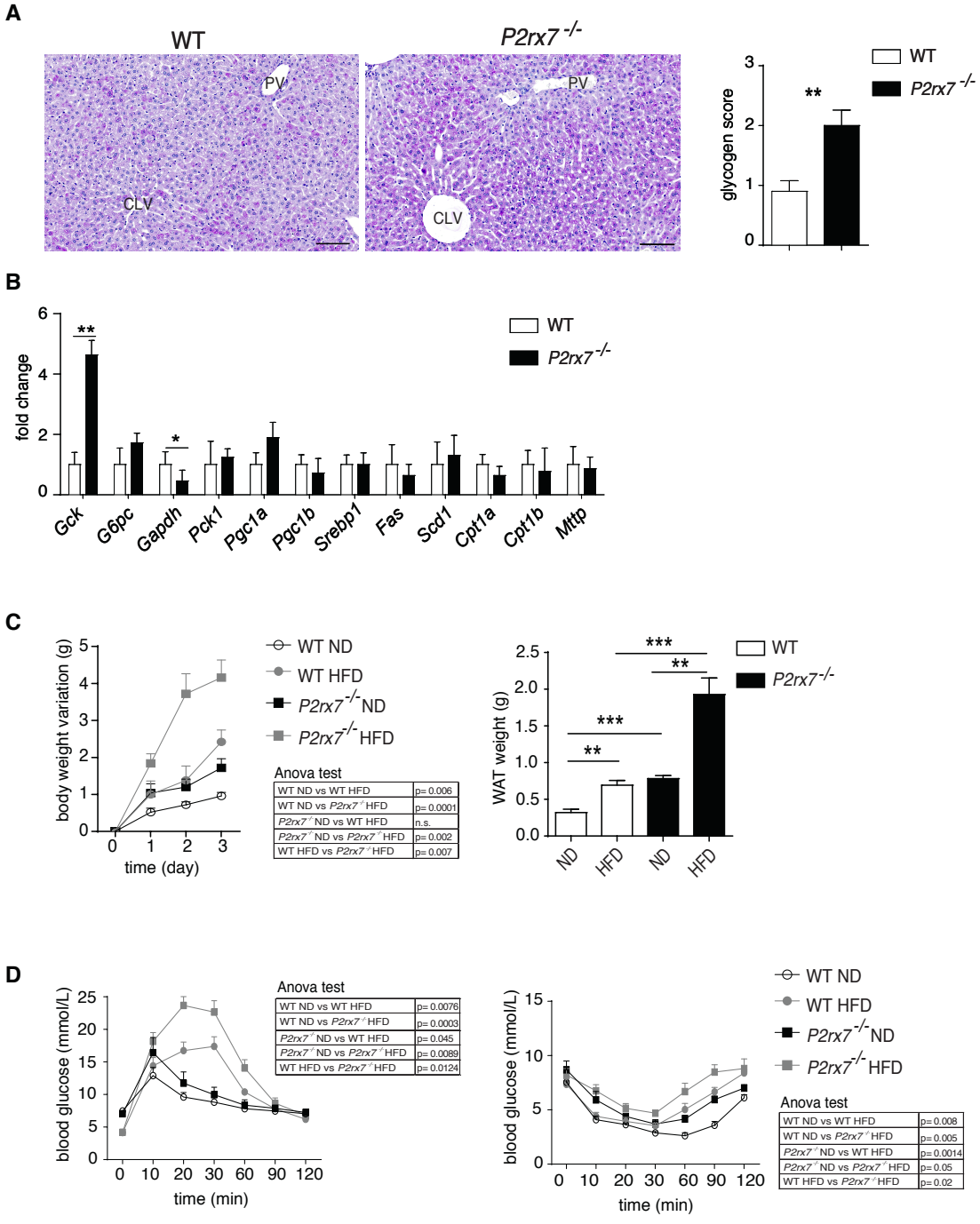

Figure S1. Glycogen accumulation in the liver and enhanced deterioration of glucose metabolism by high-fat diet in *P2rx7*$^{-/-}$ mice, Related to Figure 1 (A) PAS-stained liver sections from *P2rx7*$^{-/-}$ and WT littermates and statistical analysis of glycogen score (see Experimental Procedures). Relative glycogen accumulation is indicated by presence of magenta staining within the hepatocyte cytoplasm (CLV: centrilobular veins, PV: portal veins, scale bar: 100 μm). (B) Hepatic mRNA levels of genes involved in glycolysis (GK, GAPDH), fatty acid catabolism (CPT1a, CTP1b, PGC1α, PCGC1β), fatty acids and lipoprotein synthesis (SREBP1, FAS, SCD1, MTTP). (C) Increase in body weight during 3 weeks of high fat diet (HFD) in WT (grey dot) and *P2rx7*$^{-/-}$ (grey square) mice (n=5); control mice with normal diet (ND) are also shown (left) and WAT weights at 3 wks (right). (D) GTT and ITT after 3 weeks of HFD. Mean ± SEM are shown, Mann-Whitney (A, B and C) and Two-way ANOVA (C, D) tests were used. *p < 0.05, **p < 0.01, ***p < 0.001.

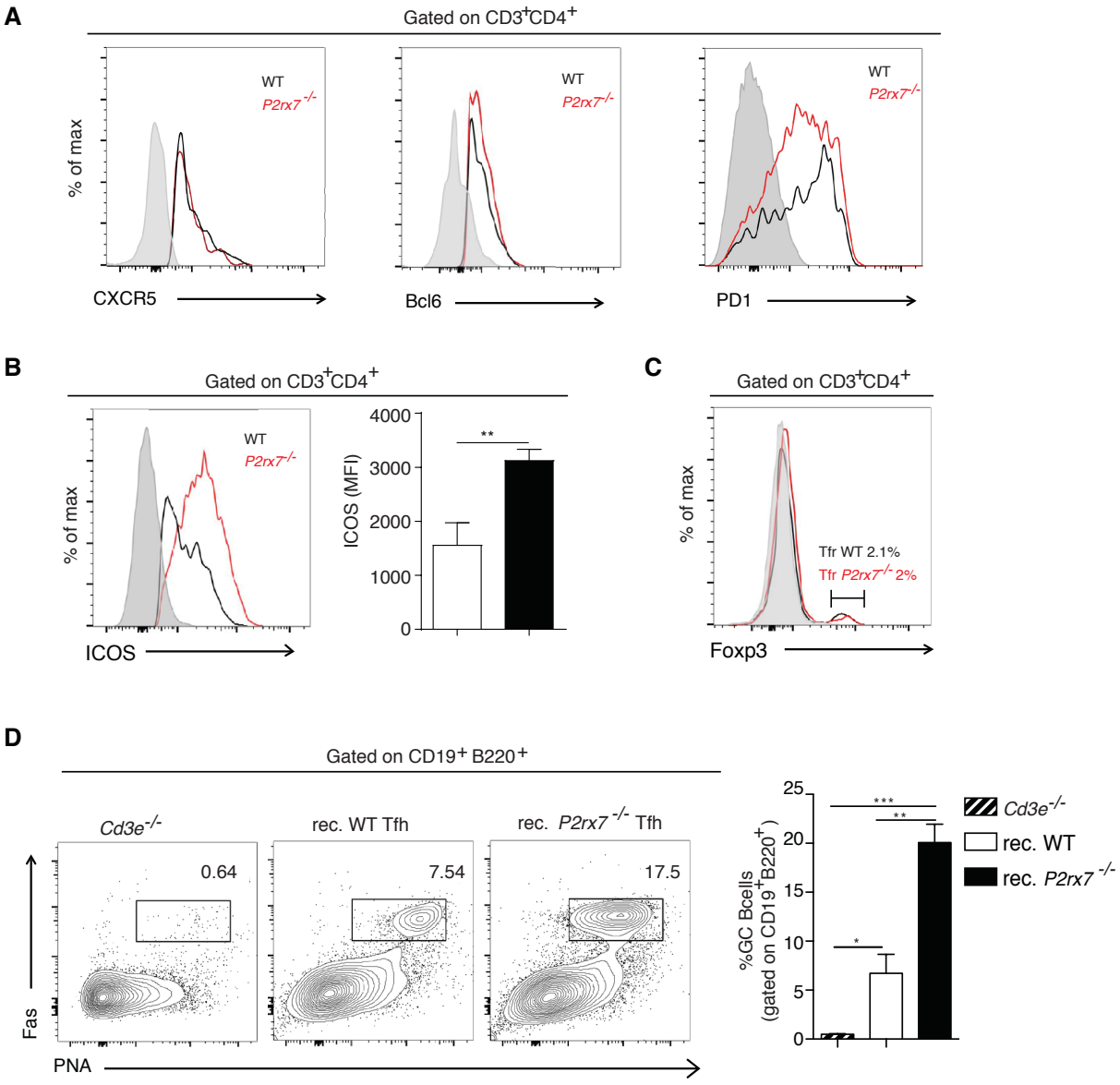

Figure S2. Phenotype of Tfh cells and GC B cells at one month after transfer into *Cd3e*$^{-/-}$ mice, Related to Figure 2. (A) Quantification of Tfh cells in PPs of *Cd3e*$^{-/-}$ mice reconstituted with purified WT or *P2rx7*$^{-/-}$ Tfh cells. (B-D) FACS histograms overlays of gated WT or *P2rx7*$^{-/-}$ CD4 cells isolated from PPs of reconstituted *Cd3e*$^{-/-}$ mice for: (B) CXCR5, Bcl6 and PD1; (C) ICOS with statistical analysis of MFI; (D) Foxp3. (E) Representative contour plots and statistical analysis of Fas^{+}PNA^{+} GC B cells in *Cd3e*$^{-/-}$ mice either non-reconstituted or reconstituted with WT or *P2rx7*$^{-/-}$ Tfh cells. Means ± SEM are shown, Mann-Whitney tests was used. **p < 0.01, ***p < 0.001.

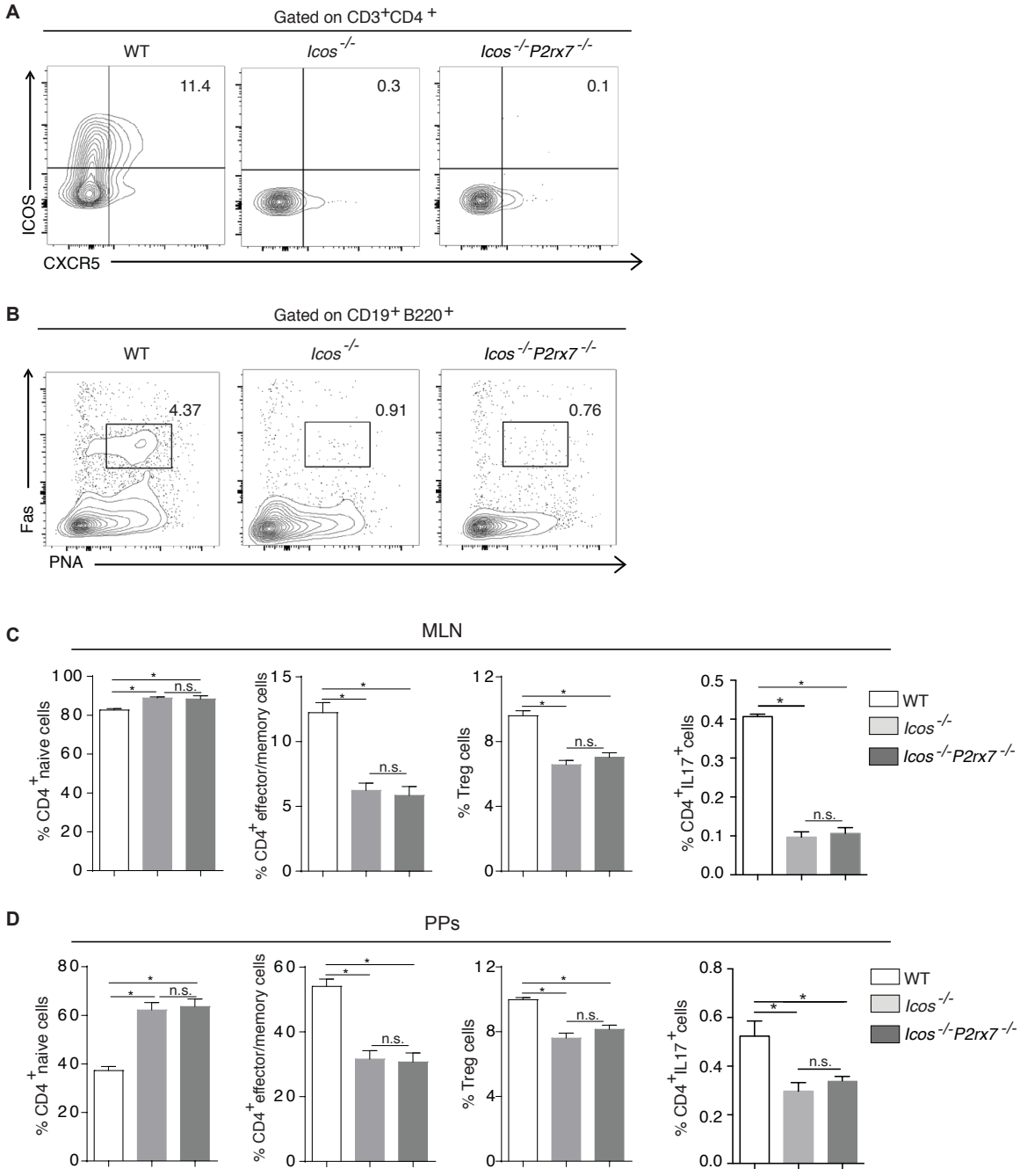

Figure S3. Phenotype of CD4 cells in *Icos*$^{-/-}$ and *Icos*$^{-/-}$*P2rx7*$^{-/-}$ mice, Related to Figure 3. (A) Contour plots of $CD3^+CD4^+$ cells from PPs stained for CXCR5 and ICOS. (B) Contour plots of $CD19^+B220^+$ B cells stained for Fas and PNA. (C, D) Statistical analysis of naïve, effector/memory, Treg and IL-17 secreting CD4 cells in MLN (C) and PPs (D). *$p < 0.05$; n.s., non-significant.

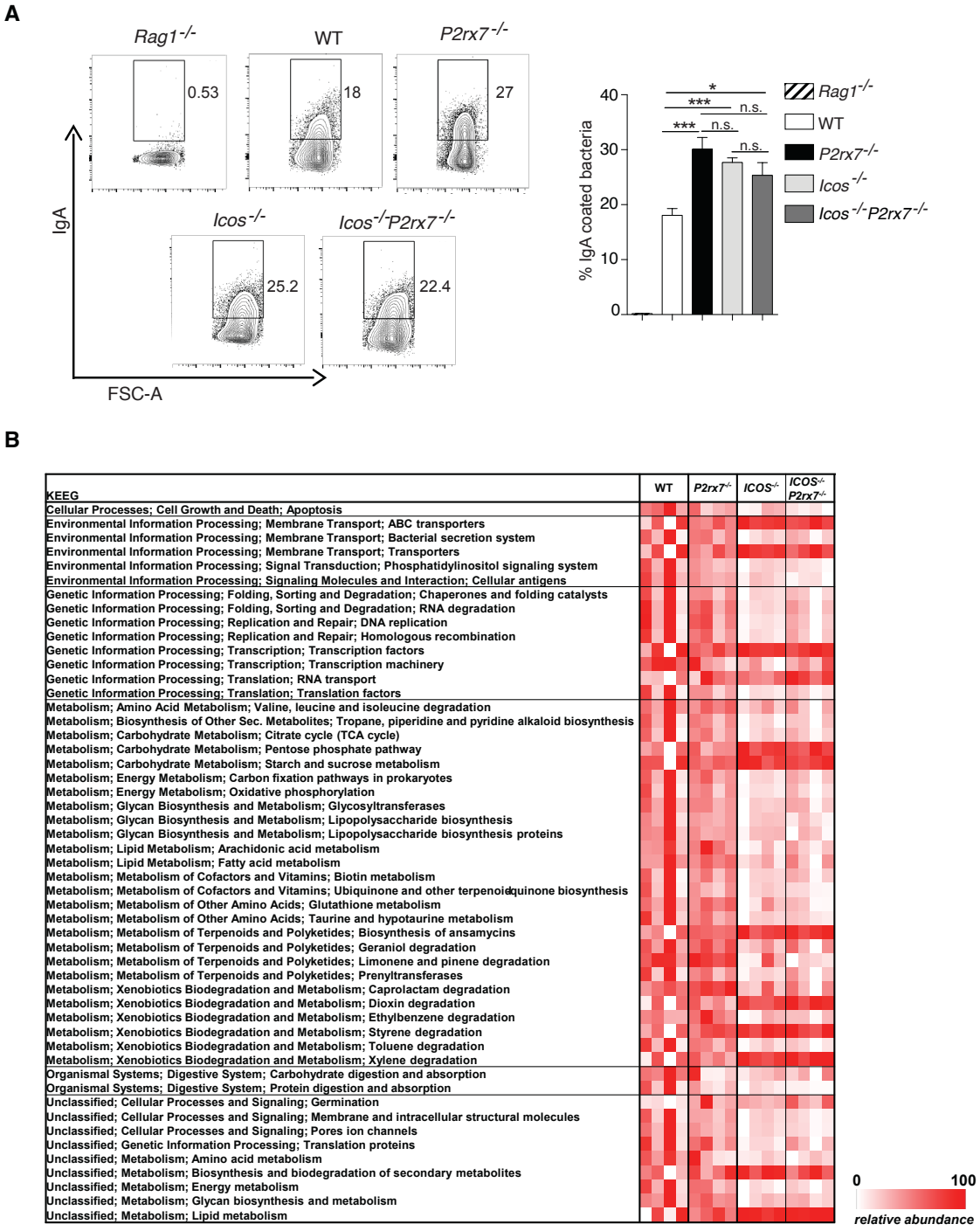

Figure S4. IgA coating in fecal bacteria and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), Related to Figure 3. (A) Representative contour plots of IgA coating and forward scatter at FACS of fecal bacteria isolated from the indicated mice with statistical analysis (Mann-Whitney test, n=10). Percentages of positive bacteria in the indicated quadrant are shown. Mean values ± SEM. ***$p < 0.001$, *$p < 0.05$, n.s., non-significant. (B) Heat map of the predicted metabolic potential of caecal microbiota samples discriminating WT, *P2rx7*$^{-/-}$, *Icos*$^{-/-}$ and *Icos*$^{-/-}$*P2rx7*$^{-/-}$ mice. The relative abundances of KEEG categories have been determined through the PICRUSt software (Langille et al., 2013). Color scale: increasing relative abundance from white to red. Each column represents an individual mouse.

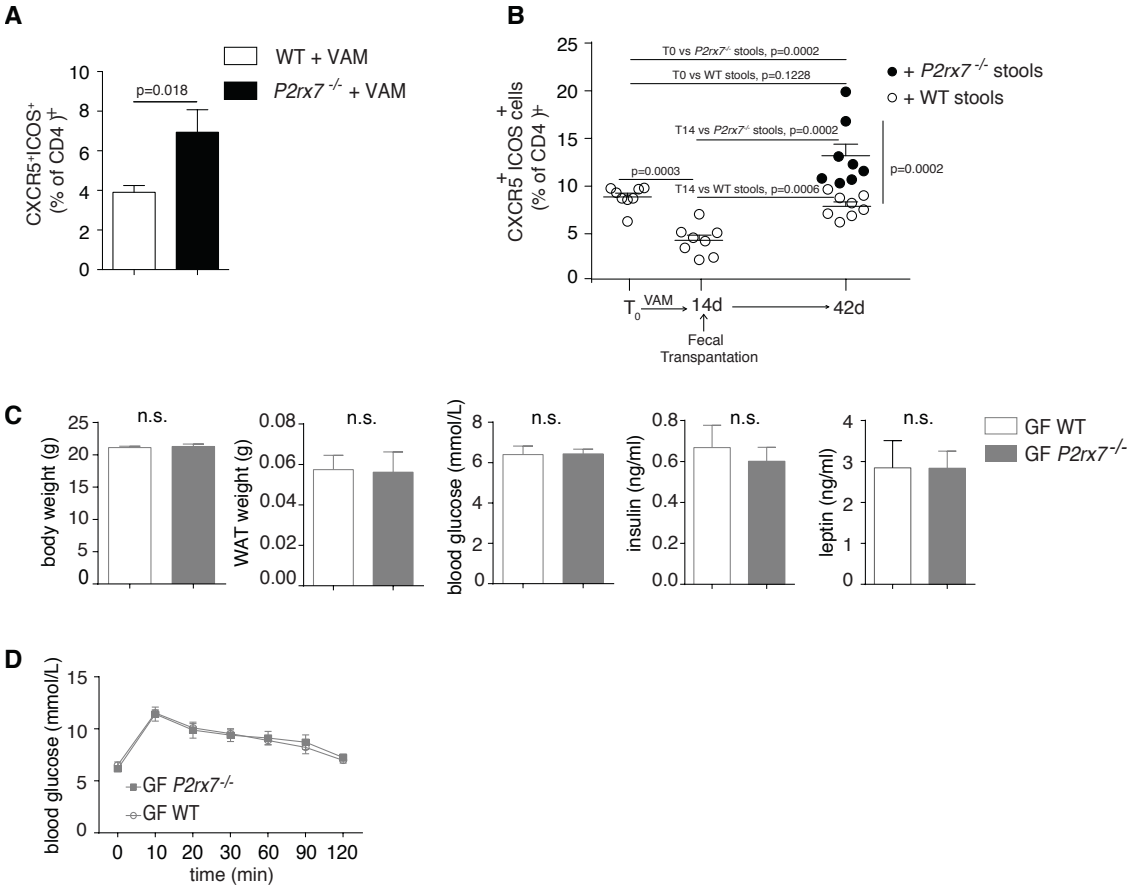

Figure S5. Tfh cells frequency after administration of antibiotics and fecal transplantation, Related to Figure 4. (A) Statistical analysis of Tfh cells in PPs from VAM treated WT or *P2rx7*$^{-/-}$ mice. (B) Statistical analysis of Tfh cells from PPs of WT mice 14 d after administration of VAM and 42 d after transplantation of stools isolated from WT or *P2rx7*$^{-/-}$ mice. (C) Body and WAT weights, blood glucose, serum insulin, leptin levels and (D) GTT in germ-free WT and *P2rx7*$^{-/-}$ mice at 9 wk (n=7). Means ± SEM and p values obtained with Mann-Whitney test are shown. Two-way ANOVA for GTT was not significant.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice and *in vivo* experiments

C57BL/6J, *P2rx7*$^{-/-}$, *Icos*$^{-/-}$ (Jackson Laboratory), and *Cd3e*$^{-/-}$ (Malissen et al., 1995) mice were bred in specific pathogen-free (spf) facility at the Institute for Research in Biomedicine, Bellinzona, Switzerland. The colony of C57BL/6J, *P2rx7*$^{-/-}$ was maintained onsite with heterozygous breeders and littermates kept in the same cages until weaning at 4 week of age. C57BL/6J and *P2rx7*$^{-/-}$ germ free mice were maintained in flexible film isolators at the Clean Animal Facility, University of Bern, Switzerland. To deplete gut flora, 8 week old C57BL/6J and *P2rx7*$^{-/-}$ mice were treated daily with an antibiotic association containing Metronidazole (2,5 mg), Ampicillin (2,5 mg) and Vancomycin (1,25 mg) (VAM) in 200µl per mouse by oral gavage for 2 weeks. For adoptive transfer of Tfh cells, CD4^{+}CD8^{-}CXCR5^{+}ICOS^{+} cells were sorted at FACSAria from pooled PPs of C57BL/6J or *P2rx7*$^{-/-}$ mice. Eight week old *Cd3e*$^{-/-}$ mice were injected with 1x10^{5} Tfh cells. Recipient mice were sacrificed 4 weeks after reconstitution. All animal experiments were performed in accordance with the Swiss Federal Veterinary Office guidelines and authorized by the Cantonal Veterinary. For body and WAT weights, and blood glucose variations, we show the difference between the initial value and the value obtained at the indicated time points.

Tissue collection and histology

All animals were fasted overnight for 12 h prior to sacrifice. Livers were collected shortly after euthanasia, weighed and fixed in 4% buffered paraformaldehyde (PFA). Paraffin-embedded sections (3-5 µm thick) were routinely stained with hematoxylin and eosin

(H&E) and Periodic Acid Schiff (PAS) reagent for histological evaluation and visualization of glycogen content, respectively. The amount of glycogen was assessed in the PAS-stained sections according to a 0-3 scale modified from Villano et al. (Villano et al., 2013): Grade 0: negligible glycogen levels; Grade 1: patchy glycogen accumulation with a predominant midzonal distribution, affecting less than 30% hepatocytes; Grade 2: uniform glycogen accumulation with a predominant midzonal to centrilobular distribution (31-50%); Grade 3: abundant glycogen accumulation with a panlobular distribution (> 50%).

Liver gene expression

Quantitative PCR analysis of transcripts for gene involved in metabolic pathways in the liver was performed as described (Braccini et al., 2015).

GTT, ITT, serum insulin and leptin quantification.

Animals were fasted for 12 (GTT) or 6 (ITT) h and then received an i.p. injection of glucose (2 g/kg of body weight) or insulin (0.6 U/kg). Blood glucose was monitored for 120 min using a glucometer (Healthpro-x1, Axapharm) on samples collected from the tip of the tail vein. Insulin in serum was quantified using an ELISA kit (High sensitive mouse insulin ELISA kit, Biorbyt Ltd). Leptin was quantified using an ELISA kit (Mouse Leptin Quantikine ELISA Kit, Bio-Techne AG).

SCFA Analysis

An aliquot (100-200 mg) of caecal content was suspended while frozen in 100 μL PBS containing 10 μL stock solution of internal standards (each of the following components at 20 mM: [$^2H_3$]acetate, [$^2H_5$]propionate, and [$^2H_7$]butyrate). After acidification with 10 μl of 37% HCl, SCFAs were extracted twice with 2 ml diethyl ether (Samuel and Gordon, 2006). A 60 μL aliquot of the extracted sample was mixed with 20 μL of *N*-tert-

butyldimethylsilyl-*N*-methyltrifluoracetamide (MTBSTFA, Sigma) at room temperature. An aliquot (2 μL) of the resulting derivatized material was injected into a gas chromatograph coupled with e mass spectrometer. GC-MS analyses were performed by using a Trace GC-Ultra 60 chromatograph coupled with a Trace DSQ mass spectrometer (Thermo Scientific), equipped with a capillary HP-5MS column (Agilent Technologies), and recorded in positive-ion full scan mode with electronic impact for ionization (70 eV) and source temperature 200 °C. Sample solutions (1 μL) were introduced in a splitless mode at 250 °C for the injector and 280 °C for the MS transfer line. Helium at a constant flow-rate of 1 mL/min was used as a carrier gas. The initial oven temperature was held at 80 °C for 5 min, increased to 200 °C at 15°/min and maintained at this value for further 5 min. Data were processed with the aid of the Finnigan Xcalibur™ software system.

Microbiota transplantation

Microbiota transplantation was performed in 8 week old C57BL/6J mice previously treated with VAM for 2 weeks. Fresh caecal content was collected from 5 donor mice (C57BL/6J or *P2rx7*$^{-/-}$*),* resuspended in PBS (0.01 g/ml) and delivered by gavaging in 200 μl for 3 days to recipient animals. Mice were analyzed after 4 weeks.

Antibodies

The following mAbs were purchased from BD Biosciences: biotin conjugated anti-CXCR5 (clone: 2G8, Cat.#: 551960), PE conjugated anti-ICOS (clone: 7E.17G9, Cat.#: 552146). The following mAbs were purchased from Biolegend: PE-Cy7 conjugated anti-CD4 (Clone:GK1.5, Cat.# 100422). Efluo405 conjugated streptavidin was from eBioscience (Cat.#: 48-4317-82).

Cell isolation and adoptive transfer

Single-cell suspensions were prepared from pooled PPs harvested from C57BL/6J or *P2rx7*$^{-/-}$ mice. For adoptive transfer of Tfh cells, CD4^{+}CD8^{-}CXCR5^{+}ICOS^{+}cells were sorted at FACSAria from pooled PPs of C57BL/6J or *P2rx7*$^{-/-}$ mice. Eight week old *Cd3e*$^{-/-}$ mice were injected with 1x10^{5} Tfh cells. Recipient mice were sacrificed 4 weeks after reconstitution.

Fecal IgA Flow Cytometry

For analysis of IgA coated bacteria in flow cytometry fecal samples were collected and homogenized in PBS (0.01 g/ml). The homogenized samples were centrifuged at 400 x g for 5 min to remove larger particles from bacteria. Supernatants were centrifuged at 8'000 x g for 10 min to remove unbound Igs. Bacterial pellets were resuspended in PBS 5% goat serum (Jackson Immunoresearch), incubated 15 minutes on ice, centrifuged and resuspended in PBS 1% BSA for staining with APC conjugated rabbit anti-mouse IgA antibodies (http://www.brookwoodbiomedical.com/ Cat.#: SAB1186, working dilution 1:200). After 30 min incubation, bacteria were washed twice and resuspended in 2% paraformaldehyde in PBS for acquisition at LSRFortessa (BD Biosciences). FSC and SSC parameters in logarithmic mode were used. SYBR Green was added to identify bacteria-sized particles containing nucleic acids.

Taxonomic analysis of microbiota

For the evaluation of intestinal microbiota, the bacterial microbiota of caecal samples from WT, *P2rx7*$^{-/}$, *Icos*$^{-/-}$ *and Icos*$^{-/-}$*P2rx7*$^{-/-}$ has been investigated by sequencing the V5-V6 hypervariable regions of 16S rDNA gene by using the Illumina MiSeq platform as described in Manzari et al. (Manzari et al., 2015). The prokaryotic composition of the

tested samples has been assessed by the BioMaS (Bioinformatic analysis of Metagenomic AmpliconS) pipeline (Fosso et al., 2015) on the paired-end (PE) reads generated by Illumina MiSeq sequencing. The overlapping 2x250 bp PE reads were merged into consensus sequences by using Flash (Magoc and Salzberg, 2011) and sequences shorter than 50nt were removed. Non-overlapping PE reads were further cleaned by removing low-quality regions (quality-score threshold equal to 25) and discarding PE reads containing sequences shorter then 50nt by using Trim-Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). In order to minimize the background noise due to host DNA contamination both consensus and non-overlapping denoised PE reads were mapped against a collection of *Mus musculus* genome by using Bowtie2 (Langmead and Salzberg, 2012). Sequences with an identity percentage ≥ 97% were discarded. The retained consensus and unmerged PE reads were compared to the 11.2 release of the RDP II database (Cole et al., 2009) by using Bowtie2. In order to obtain the taxonomic classification, mapping data were filtered according to two parameters: identity percentage (≥ 90%) and query coverage (≥ 70%). The taxonomic data at family level were normalized by converting the raw count in RPM (reads per million) by using the following formula: RPM = assigned reads / (total assigned reads at the rank level/1.000.000). The alpha-diversity index (Shannon Index) was measured by applying the R package phyloseq (McMurdie and Holmes, 2013) on the taxonomic data at family level (Segata et al., 2011). In experiments with adoptive transfer of Tfh cells into *Cd3e*$^{-/-}$ mice, microbial 16S rRNA gene segment spanning the variable V5 and V6 regions was amplified using the barcoded forward primer 5'-*CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG* BARCODE ATT AGA TAC CCY GGT AGT CC-3' in

combination with the reverse primer 5'-*CCT CTC TAT GGG CAG TCG GTG AT* ACG AGC TGA CGA CAR CCA TG-3'. The sequences in italic are Ion Torrent PGM-specific adaptor sequences required for high throughput sequencing. 16S PCR amplicons were gel purified and sequenced on the Ion Torrent PGM system using a 316v2 chip according to the manufacturer's instructions (Life Technologies). At least 10'000 reads were obtained per samples. The microbial profiles were analyzed using QIIME V1.8.0 (Caporaso et al., 2010). Operational taxonomic units (OTU) were generated using uclust and a 97% identity threshold. Taxonomy assignment was performed by blasting representative OTU sequences against the latest Greengenes database.

Colonization of germ-free mice with *E. coli*

E. coli K-12 transformed with pBAD28 or pHND10 were previously described (Scribano et al., 2014). Bacterial suspensions (10^{10} CFUs in 300 µl) were gavaged into the stomach. After 28 days the small intestine was flushed with 10 ml of intestinal wash buffer (PBS, 0.5M EDTA, Soybean trypsin inhibitor, PMSF), spun at 14'000 rpm in a sterile tube and filtered (0.22 µm) to remove any bacteria-sized contaminants. For FACS analysis of anti-*E. coli* IgA, 3ml of LB broth were inoculated with single colonies and cultured overnight at 37°C. Cultures were subsequently centrifuged (3 min at 7'000 rpm), washed 3 times with sterile-filtered PBS, 2% BSA, 0.005% NaN_3 and resuspended at a density of approximately 10^7 bacteria per ml. Intestinal wash and bacteria were then mixed and incubated at 4°C for 1h. Bacteria were washed twice, before being stained with monoclonal FITC-anti-mouse IgA (Southern Biotech, Cat.#: 1040-02). After 1 h incubation, bacteria were washed twice and resuspended in 2% paraformaldehyde in PBS for acquisition on a FACSCanto using FSC and SSC parameters in logarithmic

mode. For each animal analyzed, ELISA was used to determine the total IgA concentration in an undiluted aliquot of the same intestinal wash sample used for surface staining of *E. coli*. This value was used to calculate the total IgA concentration at each dilution of intestinal wash used for FACS analysis of *E. coli* and was plotted against the geometric mean fluorescence obtained in flow cytometry. ATP concentration was evaluated by bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin according to the manufacturer's protocol (Molecular Probes, Cat.# A22066).

Statistical analysis

The displayed data are representative of at least three independent experiments. Results were analyzed using the nonparametric Mann Whitney test, Student's unpaired t test and two-way ANOVA with Bonferroni post-test analysis. Results are presented as mean ± SEM. Values of $p < 0.05$ were considered statistically significant.

For statistical analyses of microbiota R statistic software (version 3.1.2) was used. Differences between the effects on microbiota composition were evaluated by analyzing the data with non-parametric Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction, using paired data when possible, with which we could decide whether the population distributions were identical without assuming them to follow the normal distribution. The choice for a non-parametric test derived from the Shapiro-Francia test performed for the composite hypothesis of normality. The p-value was computed from the formula given by Royston (Royston, 1993). Two-tailed unpaired t-test with a cut-off p-value of 0.1 was performed to select bacterial taxa that could evidence differences between study groups in a heatmap. To describe the variation between genotypes, a non-metric multidimensional scaling (NMDS) was performed using "vegan" library in R on

UNIFRAC dissimilarity matrix, supported by ANOSIM analysis of similarities test. Statistical significance was set at $p \leq 0.05$, and the mean differences with $0.05 < p \leq 0.10$ were accepted as trends. Estimation of metabolic potential from 16S rRNA gene sequencing data in caecal and fecal samples was computationally predicted using PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) (Langille et al., 2013). PICRUSt profiles were expressed as Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ferrario et al., 2014).

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