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Supplementary Materials for

Commensal bacteria contribute to insulin resistance in aging by activating innate B1a cells

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MATERIALS AND METHODS

Mice, primates and cells. Young (10-12 week) and aged (18-24 month) female C57BL/6 mice were obtained from Charles River Laboratories (Aged Rodent Colony, NIA). Young, middle-aged (12 month) and aged female and male C57BL/6J mice and congenic GFP^+ mice (C57BL/6-Tg(UBC-GFP)30Scha/J), CD45.1 mice (B6.FVB-Ptprca Tg(CAG-luc,-GFP)L2G85Chco Thy1a/J) and J_HT mice with B-cell deficiency (B6.129P2-*Igh-Jtm1Cgn*/J) were originally purchased from The Jackson Laboratory. 4-1BBL deficient mice (4-1BBL KO) were gift from professor Michael Croft (La Jolla Institute for Allergy and Immunology, La Jolla, CA) under a Material Transfer Agreement (MTA) with Amgen (*58*). Germ-free mice were from The National Gnotobiotic Rodent Resource Center, University of North Carolina at Chapel Hill. Mice were housed in a pathogen-free environment at the National Institute on Aging (NIA) Animal Facility, Baltimore, MD; and 19 rhesus monkeys (*Macaca mulatta*) and 9 squirrel monkeys (*Saimiri sciureus*) were housed at the NIH Animal Center in Poolesville, MD, in standard primate caging with controlled temperature and humidity and a 12-hour light cycle. Animal treatment studies were matched with control trials, including animals were randomly chosen and paired based on age and sex. Experiments were performed blinded with respect to treatment.

Flow cytometry. All antibodies (Ab) were from BioLegend except otherwise specified. Monkey cell Fc receptors were blocked with Human TruStain FcX for 5 min prior to staining with anti-human Ab: CD20-PerCP/Cy5.5, -APC, or -PE (clone 2H7), 4-1BBL-PE or -APC (5F4), CD14-PE (clone M5E2), CD8-PE or PE-Cy7 (clone RPA-T8), GrB-FITC or -APC (clone GB11). For murine cell phenotyping, cells were pre-incubated with Tru Stain FcX before immunostaining with different combinations of the following anti-mouse Ab: TNFα-FITC, -APC

or -Pacific Blue (clone MP6-XT22), 4-1BBL-PerCP-eFluor 710 (clone TKS-1), CD5-PE, -FITC or -APC (clone 53-7.3), CD19-APC, -APC-eFluor 780 or -PerCP Cy5.5 (eBio1D3 or 6D5), CD11b-eFluor 506, -BV510, or -FITC (clone M1/70), MHC II-APC, Fitc or -PerCP-eFluor 710 (clone AF6-120.1 or 25-9-17), Ly6C-PerCP Cy5.5, -FITC or - PE Cy7 (clone HK1.4), Ly6G-PE, -PerCP or PE-Cy7 (clone 1A8), CD11c-PE, -FITC, -BV510 or -Pacific Blue (clone N418), F4/80- Pacific Blue, -PerCP Cy5.5, -APC or -APC-eFluor 780 (clone BM8), CD3-PE or -APC eFluor 780 (clone 17A2), NK1.1-PerCP Cy5.5 or -APC eFluor 780 (clone PK136), CD115-APC or -PE (clone AFS98), CCR2-BV421 or -Alexa Fluor 647 (clone 203G11), CX_3CR_1-PerCP Cy5.5, -PE or -PE Cy7 (clone SA011F11), 4-1BB-APC or -PE (clone 17B5), CD40L-PE Cy7 or -APC (clone MR1), GrB-Fitc, -eFluor 660 or -PE (clone NGZB), CD170-eFluor660 (clone 1RNM44N), IFNγ-Pacific Blue, -FITC, - PE or -PE Cy7 (clone XMG1.2), CD4-PerCP Cy5.5 (clone GK1.5), CD8-APC eFluor 780 (clone 53-6.7), CD45.1-PE or –eFluor 450 (clone A20). For intracellular cytokine staining, freshly isolated monkey PBMC or murine cells were activated with Phorbol 12-Myristate 13-Acetate (PMA, 5 ng/ml; R&D Systems) + Ionomycin (500 ng/ml; R&D Systems) in the presence of Monensin (1/1000, eBioscience) for 4-6 h at 37°C. The cells were stained using the Intracellular Fixation and Permeabilization Buffer Kit (eBioscience) following the manufacturer's protocol. Murine omentum was dissociated by incubation at 37°C for 1 h in 1 mg/ml collagenase type IV (Life Technologies) and then were passed through a 70 µM strainer to perform flow-cytometry staining.

In vitro assays. B cells and monocytes from monkey peripheral blood (PB) were isolated by magnetic sort with anti-CD20-PE or anti-CD14-PE Ab and anti-PE microbeads (Miltenyi Biotec), using the autoMACS Pro Separator. B cells from murine spleens were negatively isolated using the EasySep Mouse B-cell Isolation Kit (≥95% purity, StemCell Technologies),

while B1 cells from the peritoneal cavity were isolated with anti-CD19-PE and anti-PE microbeads (Miltenyi Biotec) after depletion of $CD23^+$ B2 cells. Myeloid cells from peritoneal cavity were flowcytometrically sort-purified (MoFlo, Daco Cytomation) either as a Lin^- $CD11b^+$ population, or a Lin^- $CD11b^+$ $F4/80^{hi}$, Lin^- $CD11b^+$ $F4/80^{lo/int}$ $CD11c^+$ and Lin^- $CD11b^+$ $F4/80^{lo/int}$ $CD11c^-$ (Lin includes CD19, CD3, NK1.1 and Ly6G). Both monkey and murine B cells have been labelled with eFluor 450 proliferation dye (5 μM; eBioscience). Labelled B cells were cultured overnight in 96-well plate with myeloid cells at 1:1 ratio. The following day, cells were activated and analyzed for surface and intra-cellular markers by flow-cytometry. To test the ability of the omentum to convert 4BL cells, eFluor 450-labeled B1 cells of young mice were co-cultured overnight with a whole omentum isolated from young or aged mice in 48-well plates at 37^0C. Then, the omentum and free cells were dissociated to analyze the surface or intra-cellular markers in B cells by flow-cytometry.

Immunofluorescence staining. After dissection, whole omentum was fixed in 4% PFA for 3 h at 4°C, then then blocked in PBS with 10% donkey serum and 0.3% Triton X-100 for 1 h at RT with 300 rpm agitation prior to staining with primary Ab in 1.5% donkey serum and 0.3% Triton X-100 in PBS [anti-CD115 (Santa Cruz Biotechnology), anti-B220 Ab (Biolegend), and anti-4-1BBL Ab (R&D Systems)] for 48 h in 4°C with 300 rpm agitation. After three 10 min PBS washes, tissues were incubated with secondary Ab diluted for 1 h at RT with 300 rpm agitation. After three 10 min PBS washes, tissues were mounted onto glass slides and ProLong Diamond with DAPI was applied with a cover slip and imaged using a Zeiss LSM 710 confocal microscope.

Whole Animal Glycemic Function Testing. In mice, standard insulin tolerance tests (ITT) were performed after a 4 h fast. Mice were i.p. administered 1.0 U/kg of Novolin (Novo Nordisk)

and blood glucose was monitored for up to 120 min in distal tail snips. Oral glucose tolerance test (OGTT) was performed after an overnight fast. Mice were orally gavaged with 1.5 g/kg of glucose. Over 120 min, blood glucose levels were measured in tail snips using an Accu-Chek Aviva Plus blood glucose meter (Roche Diabetes Care). Blood was collected into heparinized tubes, centrifuged at 13,200 rpm for 5 min at 4°C, and insulin was quantified was quantified plasma using an Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc.). To perform intravenous glucose tolerance tests (IVGTTs) in macaques, they were fasted overnight and then anesthetized using Telazol (5mg/kg, IM) to measure weight and to collect blood. In addition, each animal received a 0.015 mg/kg IM dose of glycopyrrolate prophylactically to reduce excessive salivation and to stabilize the physiological effects of prolonged anesthesia episodes (e.g. decreased heart rate). For the IVGTT, intravenous serum samples were collected at baseline and at minutes 1, 5, 10, 20, 30, 40, 50, and 60 following 300 mg/kg of 50% dextrose i.v. administered through the saphenous vein. Glucose values were measured using an Ascensia Breeze 2 blood glucose monitoring system (Bayer HealthCare LLC.) and insulin levels were measured using an Insulin ELISA kit (Mercodia).

The in vivo insulin receptor signaling analysis was performed as reported elsewhere (*59*). In brief, mice were fasted for 6 h and then were anesthetized with xylazine and ketamine. Insulin (0.75 IU/kg, Novolin, Novo Nordisk Inc.) or saline was infused into the liver of mice through the portal vein. At 4 min after infusion, the liver was extracted, flash frozen in liquid nitrogen, and stored at -80^{o}C until processing. For western blot analyses and immunoprecipitation, liver was lysed in cell lysis buffer that contained 25 mM Tris (pH 7.4), 2mM Na_3VO_4, 10mM NaF, 10mM $Na_4P_2O_7$, 1mM EGTA, 1mM EDTA, and 1% NP-40. Protease and phosphatase inhibitor cocktails were added to the lysis buffer before each experiment. Protein concentration was

measured by BCA protein assay (Thermoscientific). For immunoprecipitation, proteins were incubated with rabbit anti-insulin receptor antibody (Santa Cruz Biotechnology) overnight at 4°C followed with protein A/G beads for 4h. Then, they mixture was three times washed in 150 mM NaCl washing buffer and subsequently denatured by boiling at 70°C for 10 min in 1x Laemmli buffer. For western blotting, protein lysates were mixed with 5x Laemmli buffer and boiled at 100°C for 5 min. After running in SDS-PAGE, the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in blocking reagent (LI-COR) for 1 h at RT, followed by overnight incubation with primary Ab (anti-phospho-tyrosine Ab [EMD Millipore], anti-AKT and anti-phospho-AKT [Ser473], and anti-beta tubulin Ab [Cell Signaling]) in blocking reagent with 0.1% Tween-20 at 4°C. Then, the membrane was washed three times in 0.1% TBST and incubated with secondary antibody in blocking reagent with 0.1% Tween-20 at RT for 1 h. After 3 washes the immunoblot was developed with a chemiluminescence assay system and visualized using X-ray films. Densitometry quantification was performed using ImageJ software (NIH).

Akkermansia culture. *Akkermansia muciniphila* (ATCC BAA-835) was stored at -80°C in BD Brain Heart Infusion (BHI) agar supplemented with 0.4% mucin (Sigma) and incubated in liquid BHI medium containing 0.4% mucin under anaerobic condition in anaerobic jar in the GasPack 100 system (BD Biosciences) at 37°C for 36 h. Then, bacteria were suspended in 10 mL of BHI containing 15% glycerol, aliquoted, and stored at -80°C. To determine the colony forming units (CFUs), bacterial colonies were enumerated after 36 h growth on BHI agar.

In vivo manipulations. For adoptive transfer experiments, 5-10x10^6 eFluor 450 or CellVue Maroon-labeled B cells from spleens or B1 cells from peritoneal cavity of WT C57BL/6 or GFP-Tg mice were injected intravenously (i.v.) via tail vein or intraperitoneally (i.p.) in

congenic aged and young mice. For monocyte tracking, bone-marrow monocytes were isolated using the Miltenyi Biotec BM monocyte isolation kit from CD45.1 mice and then labelled with CellVue Jade cell labelling kit (eBioscience). To transiently deplete immune cells, mice were i.p. injected with their respective Ab, such as 20 μg MC21 antibody (depletes $CCR2^+$ monocytes) three times; 250 μg 5D2 antibody (depletes $CD20^+$ B cells/4BL cells, Genentech) twice weekly; 170 μg anti-Ly6G Ab (depletes neutrophils) or 200 μg anti-CD8 Ab (depletes $CD8^+$ T cells, BioXcell) for four times over 20 days. Control mice were injected with rat IgG2b (MC21 control), mouse IgG2b (5D2 control) and rat IgG2a (1A8 and 53-6.7 control). The MC21 and MC67 are gift from Mathias Mack (Universitätsklinikum Regensburg, Regensburg, Germany).

To treat with antibiotic, Enrofloxacin (Baytril) is given for 3-4 months to mice in the drinking water at 170 mg/L and to macaques (at 5 mg/kg once daily in the morning) using palatable 5-gram Baytril tablets in cherry and banana flavors formulated for treatment of nonhuman primates (Bio-Serv). Control animals were similarly treated with control 5-gram Prima-Treat wafer. For fecal suspension transplantation, germ-free mice were orally gavaged with a fecal suspension from young and aged SPF mice (125μg/ml in PBS) weekly for 4 weeks using 20G-1.5" curved 2.25 mm ball needles. To replenish gut *A. muciniphila*, frozen stocks were diluted with PBS. Mice were orally gavaged with *A. muciniphila* ($1x10^8$ CFU/mouse in 200 μl PBS) at days 0, 2, 4, 7, 9, 13, 16 and 20. To test the role of 4BL cells, mice were similarly gavaged with *A. muciniphila* and (on day 13 or 16) were i.p. injected with $3\text{-}5x10^6$ $CD5^+$ $CD19^+$ (B1a) cells isolated from peritoneal cavity of aged WT or congenic GFP^+ or 4-1BBL deficient C57BL/6 mice. Alternatively, B1a cells were similarly i.v. injected into B-cell deficient JHT mice that were fed with high-fat/glucose diet (HFD, Dyets Inc., which contained 14.7% Kcal protein, 36.1% Kcal carbohydrates, 49.2% Kcal fat, 9.4% Kcal sucrose, 11.7% Kcal dextrose)

for 3-4 months. 7 days after transfer of B cells, OGTT, ITT and fasting glucose levels were evaluated as described above. To test butyrate, mice were orally gavaged with sodium butyrate (BA, 1.5 g/Kg, Sigma) daily for 14 days. Some butyrate-treated mice, 2 days after the termination of the treatment, were i.v. transferred with either splenic GFP^+B cells or PeC GFP^+B1a cells (5-10^6 cells/mouse) to quantify 4BL cell conversion in the omentum and PeC after 5-6 days. To evaluate the role of LPS, mice were daily i.p. injected with 5 μg LPS (*Escherichia coli* 0111:B4, Sigma) daily for 5-7 days.

Intestinal permeability assays. Mice were orally gavaged with FITC-dextran beads (Sigma) as described by Gupta et al. (*60*). Then, after 4 h, serum was collected to measure FITC-dextran using Perkin Elmer Victor 3V 1420 multilabel counter. Serum endotoxin was quantified using the LAL endotoxin assay (Pierce), following the manufacturer's instructions. The readings were performed on a Biorad 680 microplate reader. To evaluate activity of serum endotoxin, sera were tested using the HEK-Blue-4 cells stably expressing human *TLR4* and an NF-κB-inducible secreted embryonic alkaline phosphatase reported gene following manufacturer's instructions (InvivoGen). To analyze the gut mucus layer thickness, 1.5 cm-long segments of colon were carefully collected and fixed in a water-free Methanol-Carnoy's fixative (60% dry methanol, 30% chloroform and 10% acetic acid) at 4°C overnight. The tissues were then washed in methanol before embedding in paraffin, and 5 μm sections were placed on glass slides. Slides underwent deparaffinization with xylene, rehydration with 95% and 70% ethanol. The slides were then stained using the standard Periodic acid-Schiff method kit (Sigma) and assessed by light microscope by measuring 4 slices per mouse to calculate the mucus layer thickness in μm. The thickness of the inner stratified mucus layer was measured using a digital camera and AxioVision software (Carl Zeiss).

Gene Expression in resident macrophages, dendritic cells, and monocytes sort-purified from PeC of young and aged C57BL/6 mice was analyzed using NanoString nCounter system (NanoString Technologies) following the manufacturer's instructions. Briefly, RNA was obtained by lysing cells (5 x 10^3 cells/5 μL) in RLT buffer (Qiagen) and then hybridized with the C2566 Mouse Myeloid Panel. Post normalization, genes with mean counts less than two standard deviations above the mean count of the highest negative control were excluded, and differential expression of remaining genes was determined using a nonparametric Welch t-test with correction for multiple testing using the BH false discovery rate (FDR).

Proteome Profiler Mouse Cytokine Array was used to quantify PeC proteins. Mice were i.p. lavaged with 5 ml complete RPMI to characterize proteins with Mouse XL Cytokine Array Kit (R&D Systems) following the manufacturer's instructions. Data were analyzed using ImageJ software (NIH).

Fecal microbiome sequencing was performed at the Johns Hopkins University Deep Sequencing and Microarray Core, JHU, Baltimore, MD. Murine feces were independently collected by placing each mouse in sterile container. DNA was isolated using Qiagen QiaAmp DNA Stool kit. The 16s rRNA gene V3+V4 region was amplified using barcoded primers following Caparose's procedure and cleaned up and size selected using AMPure XP beads (*61*). The resulting amplicon was quantified using Qubit and run on a bioanalyzer to confirm expected size. Amplicons from all samples were then pooled together in equal molar concentration and sequenced on Illumina MiSeq using MiSeq 600 cycle sequencing kit (v3) for paired end 300 bp read length. To analyze 16S rRNA amplicon sequences, raw paired-end reads output by the MiSeq platform were merged into consensus fragments by FLASH (*62*) and subsequently filtered for quality (max error rate 1%) and length (minimum 200 bp) using

Trimmomatic (*63*) and QIIME (*61*). Spurious hits to the PhiX control genome were identified using BLASTN and removed. Passing sequences were trimmed of primers, evaluated for chimeras with UCLUST (de novo mode), and screened for mouse-associated contaminant using Bowtie2 (*64*) followed by a more sensitive BLASTN search against the GreenGenes 16S database (*65*). Chloroplast and mitochondrial contaminants were detected and filtered using the Ribosomal Database Project (RDP) classifier (*66*) with a confidence threshold of 80%. High-quality 16S rRNA sequences were assigned to OTUs with a high-resolution taxonomic lineage using Resphera Insight (www.respherabio.com). Sequences were further analyzed by PICRUSt with default parameters to infer functional content of each sample. To normalize across samples, 16S profiles were subsampled to 2,500 sequences per sample prior to downstream statistical comparisons. Raw PICRUSt counts associated with gene content were also subsampled to an even level of coverage. Differentially abundant taxa, alpha diversity measures and PICRUSt functional categories were detected using the nonparametric difference test with p-value correction using the False Discovery Rate (FDR) with supplemental testing including Mann-Whitney and negative binomial test. Alpha and beta-diversity calculations as well as principal coordinate analyses were performed using QIIME (*61*).

In addition, the 16S rRNA raw sequences were also processed using *dada2* (*67*) and *phyloseq* (*68*), as follows. First and second reads were trimmed to 280 bp and 175bp for first batch and 290 bp and 165 bp for second batch, error-corrected and then merged using overlapping ends (with parameters maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE). After that, sequences shorter than 402 or longer 429 bp have been removed. Finally, chimeric sequences were removed using consensus method separately for each batch. The resulting sequences were assigned to taxonomy using naive RDP classifier (*66*) with 50% confidence

threshold. RDP v16 (*69*) and SILVA rRNA database v128 (*70*) were separately used as reference database. Non-bacterial sequences have been removed from downstream analysis. Differential analysis was performed separately for each strain/batch with DESeq2 (*71*) using Wald significance test and False Discovery Rate to control for multiple testing (*72*).

To confirm bacterial colonization, DNA was isolated from frozen fecal pellets using the stool DNA isolation kit (Norgen Biotek Corp.) following the manufacturer's instructions. Samples were homogenized using bead-beater with the beads provided in an Precellys 24 homogenizer. DNA was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Scientific). A total of 10 ng of DNA was used in quantitative PCR (qPCR) reactions with primers, such as for *A. muciniphila* (F: 5′-CAGCACGTGAAGGTGGGGAC-3′, R: 5′-CCTTGCGGTTGGCTTCAGAT), Roseburia spp (F: 5'- AAGCGACGATCAGTAGCCGA- 3', R: 5'- TTCTTCTTCCCTGCTGATAGAG -3'), total bacteria (F: 5′-ACTCCTACGGGAGGCAGCAG-3', R: 5′- ATTACCGCGGCTGCTGG-3') (*48*), Mouse Intestinal Bacteroides (MIB) (F: 5'- CCAGCAGCCGCGGTAATA-3' and R: 5'-CGCATTCCGCATACTTCTC-3') and Segmented filamentous bacteria (SFB) (F: 5'-GACGCTGAGGCATGAGAGCA-3' and R: 5'-GACGGCACGGATTGTTATTC-3') (*73*). qPCR was performed using PowerUp SYBR Green Master Mix and Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems).

Quantitation of short-chain fatty acids. Chemicals used: 3-nitrophenylhydrazine (3NPH), pyridine, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), regular and deuterated fatty acid standards (d4-acetic acid, d5-propionic acid, d7-butyric acid, d9-pentanoic acid, d11-hexanoic acid, d23-lauric acid, d27-myristic acid, d31-palmitic acid, d35-stearic acid, d32-linoleic acid, and their native forms) were purchased from Sigma. Ethanol (Omnipur, 200 proof)

and Methyl-t-Butyl Ether (MTBE) were purchased from EMD Millipore. High purity water and acetonitrile (ACN), both with and without 0.1% formic acid pre-blended, LC-MS grade, were purchased from Honeywell Burdick & Jackson. The protocol of 3NPH derivatization of fatty acids and subsequent LC-MS analysis was adapted from (*74*) with the expansion to cover mid-chain fatty acids in this work. Mouse cecum sample preparation: The above 10 deuterated fatty acids (d-mix10) were made in 70% ethanol at 500 μM each. A derivatization solution was freshly made consisting of: 3NPH 15.3 mg/ml, EDC 14.2 mg/ml, pyridine 10.5 μl/ml, (75% ACN in water) 960 μl/ml. Sections of mouse cecum (31 samples, average weight 142 mg/each) were stored in 400 μl ethanol at -80 °C until used. Per the exact weights of individual samples, a 4-solvent mix (1:1:1:1 v/v/v/v MeOH, ACN, MTBE, H2O) was added to make the total-solvent to tissue ratio (μl/mg) of 6.5. Samples were transferred to homogenization tubes (Omni International) and homogenized on an Omni Bead Ruptor 24 at speed 5, period 1 min, 4 cycles. 150 μl of samples were transferred to 1.5 ml Eppendorf tubes (=20 mg tissue) and another 150 μl of the above mix4 solvent was added and samples were vortexed and left at -20°C overnight. Samples were then centrifuged at 13,000 rpm for 20 min on an Eppendorf 5424 centrifuge and 280 μl supernatants were carefully pipetted into 2 ml glass HPLC vials. 10 μl of the 500 μM d-mix10 (=5 nmol) were added to samples and equilibrated at RT for 30 min. 50 μl of the above derivatization solution was added and the reaction lasted for 4 h at RT. Samples were then dried on a Genevac evaporator (model EZ-2, HPLC mode, 30 °C, 40 min). Samples were not completely dried (~10 μl left) under these settings for easy resuspension. Ethanol was added to make final volume of 100 μl for all samples and 2 μl was injected into LCMS for analysis.

Mouse feces sample preparation. The above 10 deuterated fatty acids (d-mix10) were made in 70% ethanol at 500 μM each. A derivatization solution was freshly made consisting of: 3NPH

15.3 mg/ml, EDC 14.2 mg/ml, pyridine 105 μl/ml, (75% ACN in water) 865 μl/ml. Mouse feces were stored at -80 °C until used. Appropriate volume of 70% ethanol was added to feces (weighing 21 mg or more) to end at 1 mg/30 μl. Samples were vortexed and sonicated for 10 min and centrifuged at 13,000 rpm for 20 min. 280 μl of each supernatant was transferred to 2 ml glass HPLC vial and 10 μl of the 500 μM d-mix10 (5 nmol) were added. After 30 min equilibration at RT, 50 μl of the above derivatization solution was added and the reaction lasted for 2 hr at RT. Samples were then dried on a Genevac evaporator (model EZ-2, HPLC mode, 30 °C, 40 min). Samples were not completely dried (~10 μl left) under these settings for easy resuspension. Ethanol was added to make final volume of 100 μl for all samples and 2 μl was injected into LCMS for analysis.

Mouse peritoneal cavity lavage (PCL) sample preparation. The above 10 deuterated fatty acids (d-mix10) were made in 70% ethanol at 100 μM each. A derivatization solution for PCL was freshly made consisting of: 3NPH 30.6 mg/ml, EDC 28.4 mg/ml, pyridine 6 μl/ml, (75% ACN in water) 935 μl/ml. 41 mouse PCL samples were stored at -80 °C until used. To 200 μl samples in 2 ml Omni tubes, 1 ml pure ethanol was added and homogenized at speed 4, 1 min, 2 cycles. The homogenates were transferred to 1.5 ml Eppendorf tubes and centrifuged at 13,000 rpm for 15 min. All the supernatants were transferred into 2 ml glass HPLC vials and dried in Genevac (35 °C, HPLC mode, 1 h), ending 90-140 μl. Proper volumes of water were added to make them all ending with 140 μl. 7 μl of the 100 μM d-mix10 (=700 pmol) was added to samples and equilibrated at RT for 30 min. 48 μl of the derivatization solution was added and the reaction was carried out at RT for 30 min. 1 μl was injected into LCMS for analysis.

Monkey feces sample preparation. The above 10 deuterated fatty acids (d-mix10) were made in 70% ethanol at 100 μM each. A derivatization solution was freshly made consisting of: 3NPH

61.4 mg/ml, EDC 56.9 mg/ml, pyridine 60 μl/ml, (75% ACN in water) 820 μl/ml. Monkey feces were stored at -80 °C until used. Appropriate volume of 70% ethanol was added to feces (weighing 43 mg or more) to end at 91 mg/ml. Samples were vortexed and sonicated for 10 min and centrifuged at 13,000 rpm for 15 min. 10 μl of each supernatant was transferred to 2 ml glass HPLC vial and diluted with 90 μl of 70% ethanol. 10 μl of the 100 μM d-mix10 (1 nmol) were added to each sample and equilibrated at RT for 30 min. 30 μl of the above derivatization solution was added and the reaction lasted for 30 min at RT. One μl was injected into LCMS for analysis.

Standard curves sample preparation. A 9-level calibration experiment was carried out with d-fatty acid concentrations stayed at 50 μM while regular fatty acid concentrations varied from 0.5 μM to 5 mM corresponding to h- to d- fatty acid concentration ratios of 0.01, 0.05, 0.25, 1, 5, 10, 20, 50, 100, respectively. A mixture of the 10 deuterated fatty acids (d-mix10) was made in 70% ethanol at 500 μM per acid and 10 μl of it is used per tube. A mixture of the 10 regular fatty acids (h-mix10) was also made in 70% ethanol at appropriate concentrations and 10 μl of it is used per tube. After mixing the d- and h- acids, 280 μl ethanol was added to the tubes. A derivatization solution was freshly made before use which consists of: 3NPH 15.3 mg/ml, EDC 14.2 mg/ml, pyridine 10.5 μl/ml, (75% ACN in water) 960 μl/ml. 50 μl of the derivatization solution was added and reacted overnight at RT. Samples were then dried on Genevac (HPLC mode, 30 °C, 40 min). Samples were not completely dried (~10 μl left) under these settings for easy resuspension. Ethanol was added to make final volume of 100 μl for all samples. Samples with regular acids at or above 50 μM (levels 4-9) were further diluted 20x with ethanol to avoid mass detector saturation. Two μl was injected into LCMS for analysis. To generate the standard

curves for each acid, the actual concentration ratios (h-acids / d-acids) were plotted against the observed extracted ion chromatogram (EIC) peak area ratios (h-acids / d-acids), and the curves were fitted into either a linear or a quadratic equation. For some acids (e.g., acetic acid), different curves were drawn across different concentration ranges to get the most accurate fits.

Liquid chromatography - Mass spectrometry. Fatty acids were separated on an Agilent 1290 HPLC (Agilent Technologies) with Kinetex C18, 2.6u, 100A, 150 x 2.1 mm column (Phenomenex) at 40 °C, flow rate 0.4 ml/min, mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid). The LC gradient program was: 0-0.5 min 15% B; 8.5 min 40% B; 10 min 70% B; 14 min 99% B; 16 min stay at 99% B; 16.5 min 15% B; 20 min 15% B. The gradient was linear between adjacent steps. The mass spectrometric detection was carried out on an Agilent 6550 qTOF system with internal mass referenced m/z errors < 2 ppm. Data acquisition was done under MassHunter (version B.05.00) with MS-only in electrospray positive ion mode with the following parameters: Gas temp 250 °C; Drying gas 15 L/min; Nebulizer 30 psi; Sheath gas temperature 250 °C; Sheath gas flow12 L/min; VCap 4000 V; Nozzle voltage 250 V; 100-1500 m/z range; 2 spectra/sec. Mass chromatogram was integrated within ±5 ppm in MassHunter qTOF Quantitative Analysis (version B.05.01) after Gaussian smoothing (width parameters 8, 4). Relative quantification was obtained by calculating the peak area ratios between each fatty acid and its deuterated internal standard, and the ratios were converted to fatty acid concentrations by using external calibration curves assuming 1 mg cecum has the volume of 1 μl.

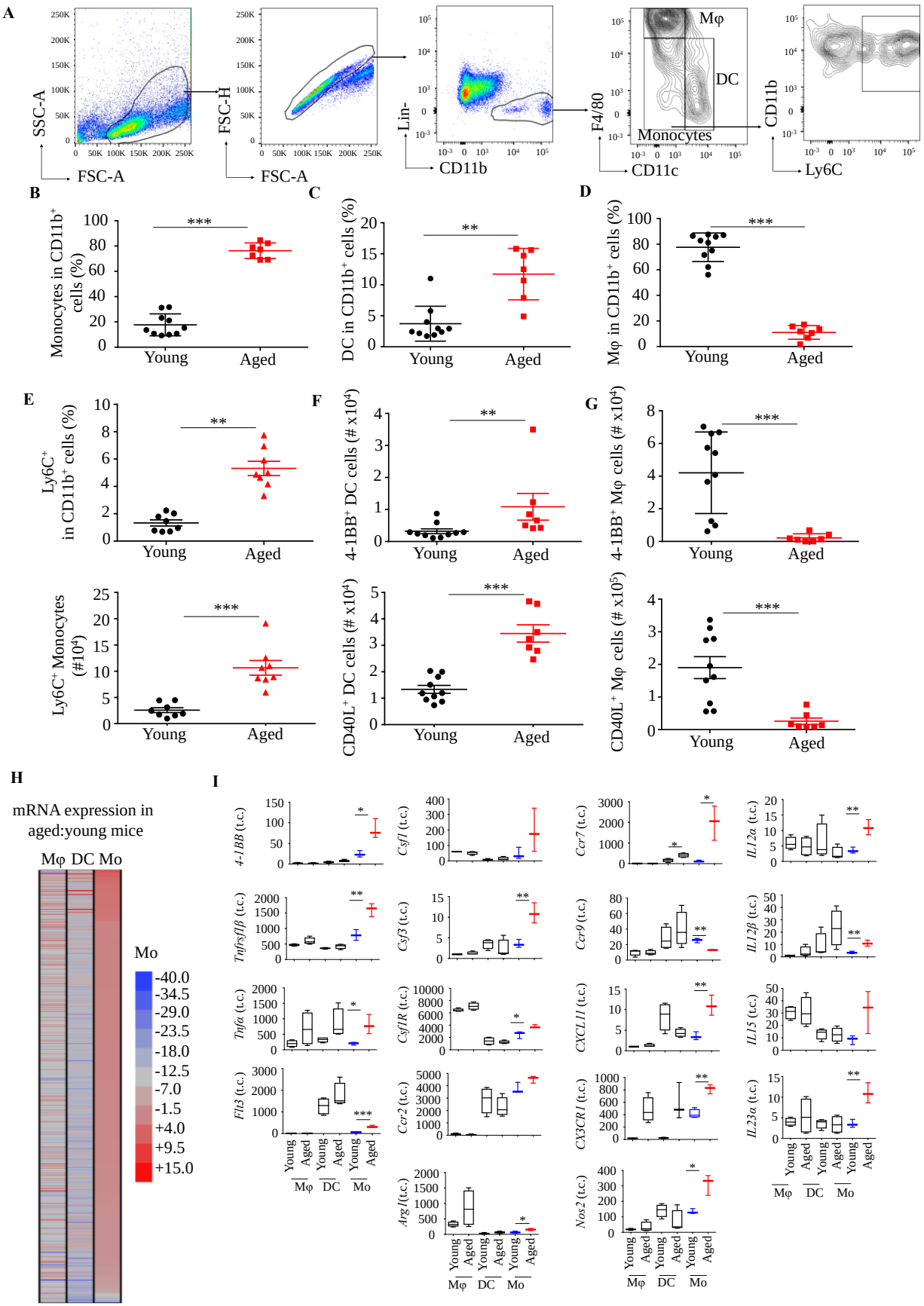


Figure S1. Aging induces monocyte accumulation and activation in the PeC. **(A)** Flow cytometric analysis of Lin^{-} CD11b^{+} myeloid cells (Lin includes CD19, CD3, NK1.1 and Ly6G cells) in PeC of young and aged mice used in the study. Shown is the gating strategy used to

characterize monocytes (MO), dendritic cells (DC) and macrophages (Mϕ). (**B**-**D**) PeC myeloid populations change with age as shown by frequency (%) of indicated cells (y-axis). Plots show frequency (%, top) and absolute numbers (bottom panel) of $Ly6C^+$ MO (in **E**) and 4-1BB- and CD40L-expressing DC and Mϕ (upper and bottom panels, respectively in **F** and **G**). Each symbol in **B**-**G** is for an individual mouse (n=7-10). (**H** and **I**) Z-score heatmap of the aged:young expression ratio of Mϕ, DC, and MO in mRNA expression analysis (n=4, also see Fig.1E). (**I**) Expression (transcript counts, T.C.) of selected genes significantly changed in aged mouse MO, DC and Mϕ. From here on, statistical significance is indicated as $*P < 0.05$, $**P < 0.001$ and $***P < 0.0001$ (Kruskal-Wallis test with Dunn's test for multiple corrections or Mann-Whitney test).

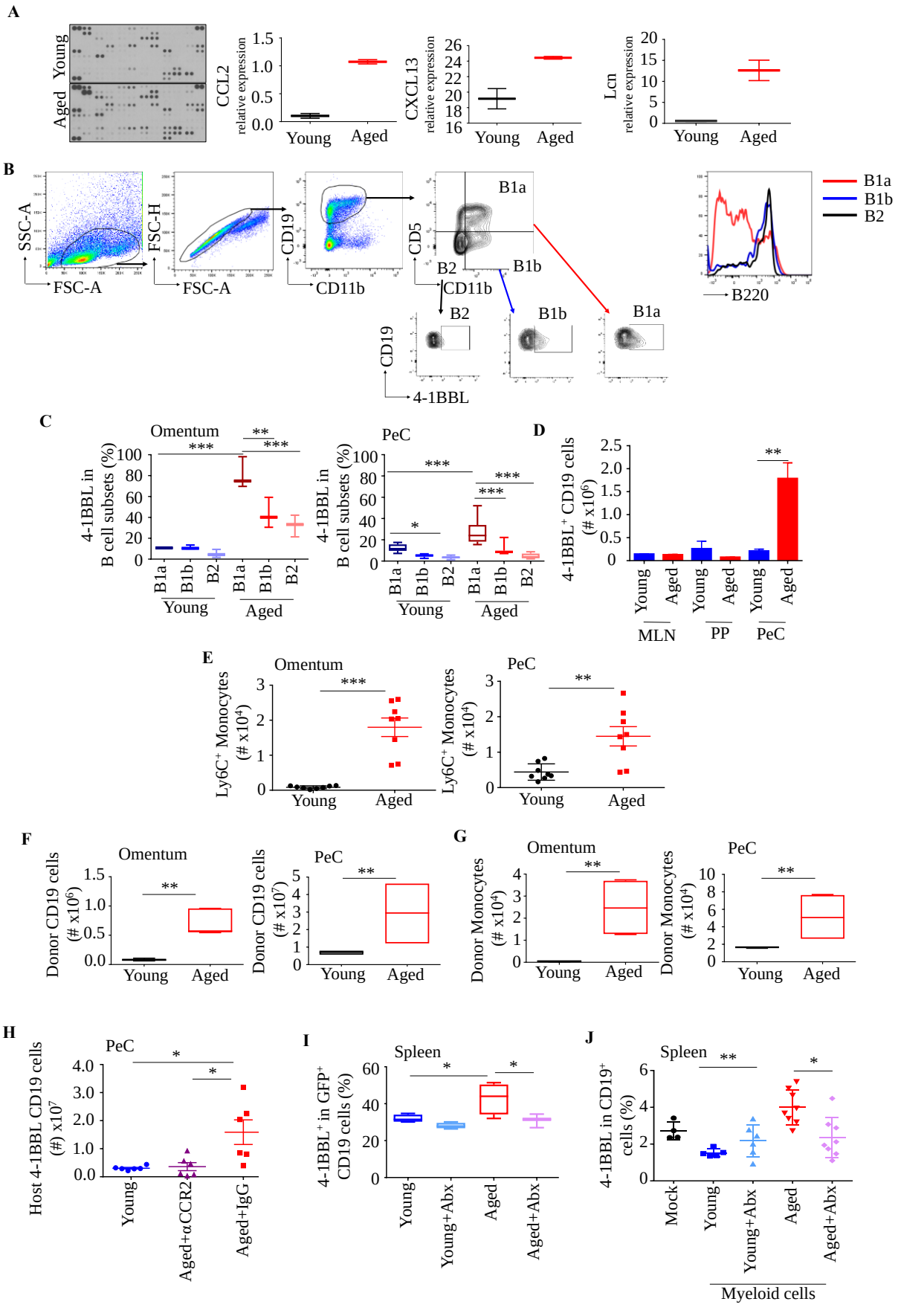


Figure S2. The PeC and ometum of aged mice are inflamed. (**A**) Compared to young mice,

factors associated with inflammation, including chemokines CCL2 and CXCL13, and lipocalin 2 (Lcn2) increased in PeC lavage of aged mice (representative blots and data of proteome profiler arrays; shown as normalized pixel density to controls (lavage media) determined by densitometry analysis, n=3). (**B**) Shown is the flow cytometric gating strategy used to characterize 4-1BBL$^+$ B cell subpopulations in the omentum. Arrows indicate amounts of 4-1BBL (contour plot) and B220 (histogram, right panel) in B1a, B1b and B2 B cells. (**C** and **D**) 4-1BBL expression was highest in B1a cells as compared to B1b and B2 cells. Shown are the frequency (%) of 4-1BBL$^+$ B cell subsets in the omentum (n=3) and PeC (n=8) and absolute numbers in PeC, MLN and Peyer's patches (PP, n=4) of young and aged mice. (**E**) Aging also markedly increased Ly6C$^+$ monocytes in the omentum (left) as in PeC (right panel, n=8, each symbol represents an independent mouse, reproduced twice). (**F** and **G**) Aging omentum preferentially recruited B1 cells and monocytes. CD45.2$^+$ young and aged mice were i.v. transferred with a mixture of PeC GFP$^+$ B1 cells and bone marrow CD45.1$^+$ monocytes from young mice and euthanized after 2 days to quantify donor and host B cells and monocytes in the omentum (left) and PeC (right panels, n=5 mice per group, the experiments is reproduced twice). (**H**) The 4BL cell conversion was induced by CCR2$^+$ monocytes. After 12 days of treatment with IgG or CCR2$^+$ monocyte-depleting Ab (αCCR2), host PeC B cells were analyzed for 4-1BBL expression (n=6 mice per group, experiment was reproduced three times). (**I)** Enrofloxacin (Abx) impaired 4BL cell conversion in aged mice. The Abx-treated mice were switched to water without Abx and after 2 days, GFP$^+$ B1 cells from young mice were transferred i.p. into young and aged mice. After 6 days, mice were euthanized to quantify 4BL cell conversion within GFP$^+$ B1 cells (n=5-8 mice per group, the experiment was reproduced three times). (**J**) Abx abolished the ability of PeC Lin$^-$ CD11b$^+$ myeloid cells to convert 4BL cells from eFluor 450-labelled Young splenic B1 cells in vitro. Cells were overnight cocultured at 1:1 ratio. Shown is 4-1BBL expression on CD19$^+$ cells (n=3-4 mice per group, reproduced twice, each symbol is for individual mouse).

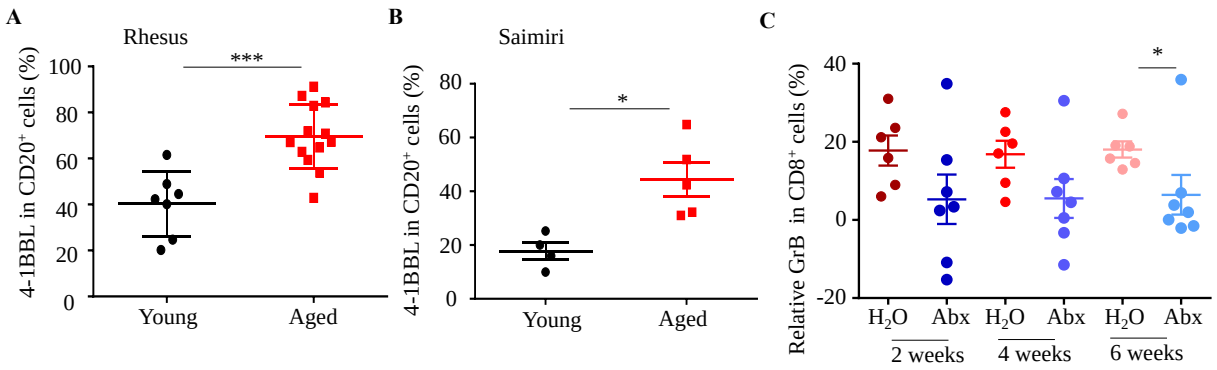


Figure S3. Aging induced 4BL cells in primates. 4BL cells accumulated in aged *M. mulatta* (**A**, peripheral blood, n=7-13 per group) and *S. sciureus* monkeys (**B**, PeC, n=5). (**C**) Enrofloxacin treatment decreased GrB^+CD8^+ T cells in peripheral blood of aged macaques (Abx, n=6-7 per group and each symbol is for an individual primate).

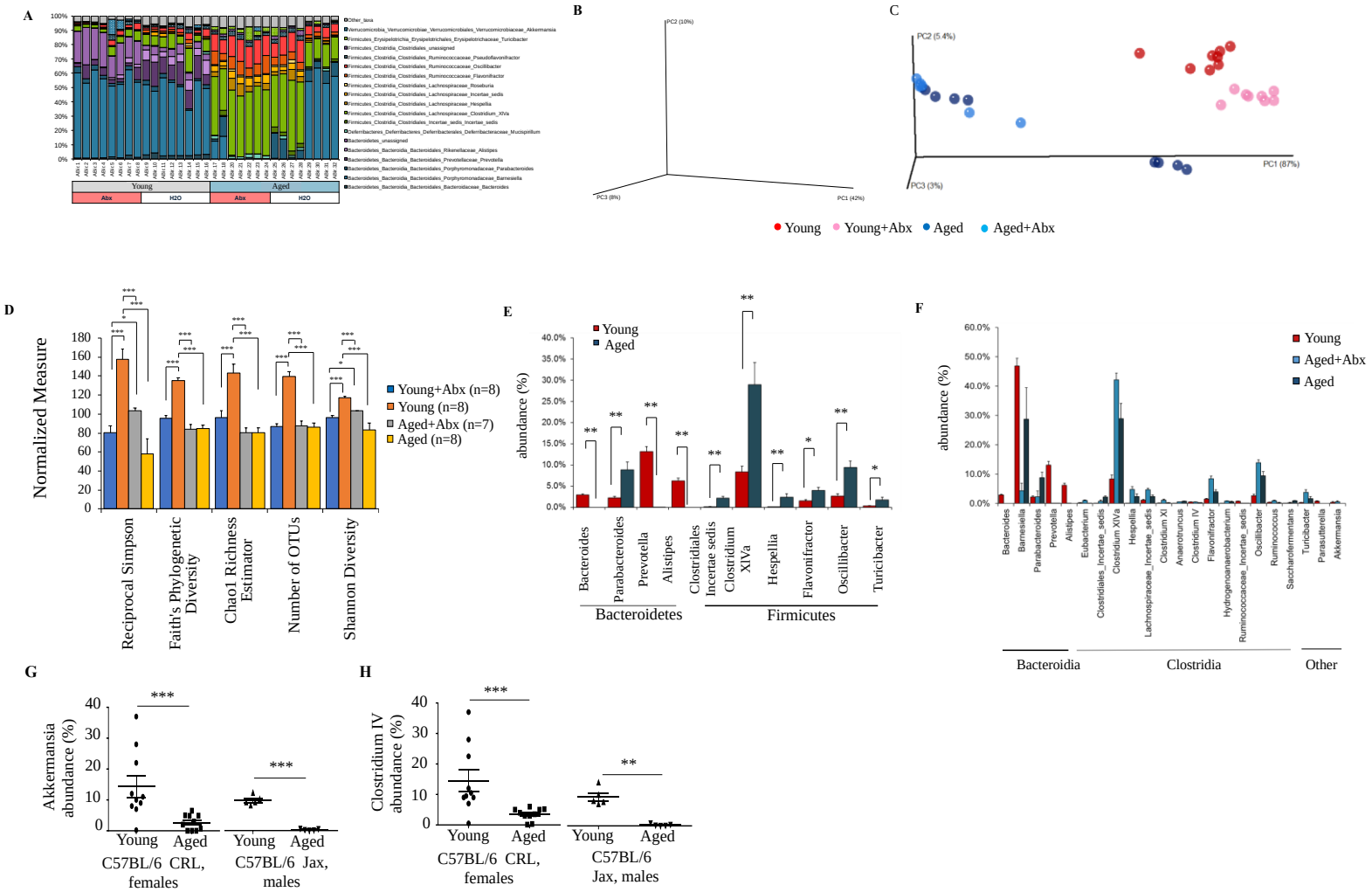


Figure S4. Aging significantly changed gut microbiome. (**A**) Total 16S rRNA gene sequencing shows changes in fecal microbiome profiles (genus level) of young and aged mice. The y-axis displays microbial abundance (%) per sample (n=7-8 mice per group). Principal component analysis based on unweighted (**B**) and weighted (**C**) UniFrac distances confirmed that microbiota from young and aged mice significantly differ (P<1e-3 for all comparisons, Mann-Whitney U). Each symbol is for an individual mouse. **(D**) Alpha diversity of microbiota is decreased in aged or after Abx treatment. Measures are re-scaled to an overall mean of 100. Aging differentially affected abundance of members of *Bacteroides* and *Firmicute*s phyla (**E**), including *Bacteroidia* and *Clostridia* (**F**). Shown are the means of relative microbial abundances in young and aged mice with or without antibiotic treatment. (**G** and **H)** Because in (**A**-**F**) we used female C57BL/6 mice received from a single vendor (Frederick, Charles River), to rule out the "cage effect" we also sequenced fecal microbiota of mice from North Carolina, Charles River (CRL, female, n=10 mice per group), and Jackson laboratory (Jax, males, n=5 mice per group). Shown is abundance (%) of *A. muciniphila* (**G**) and *Chlostridium* cluster *IV* (**H**) in individual mice (each symbol).

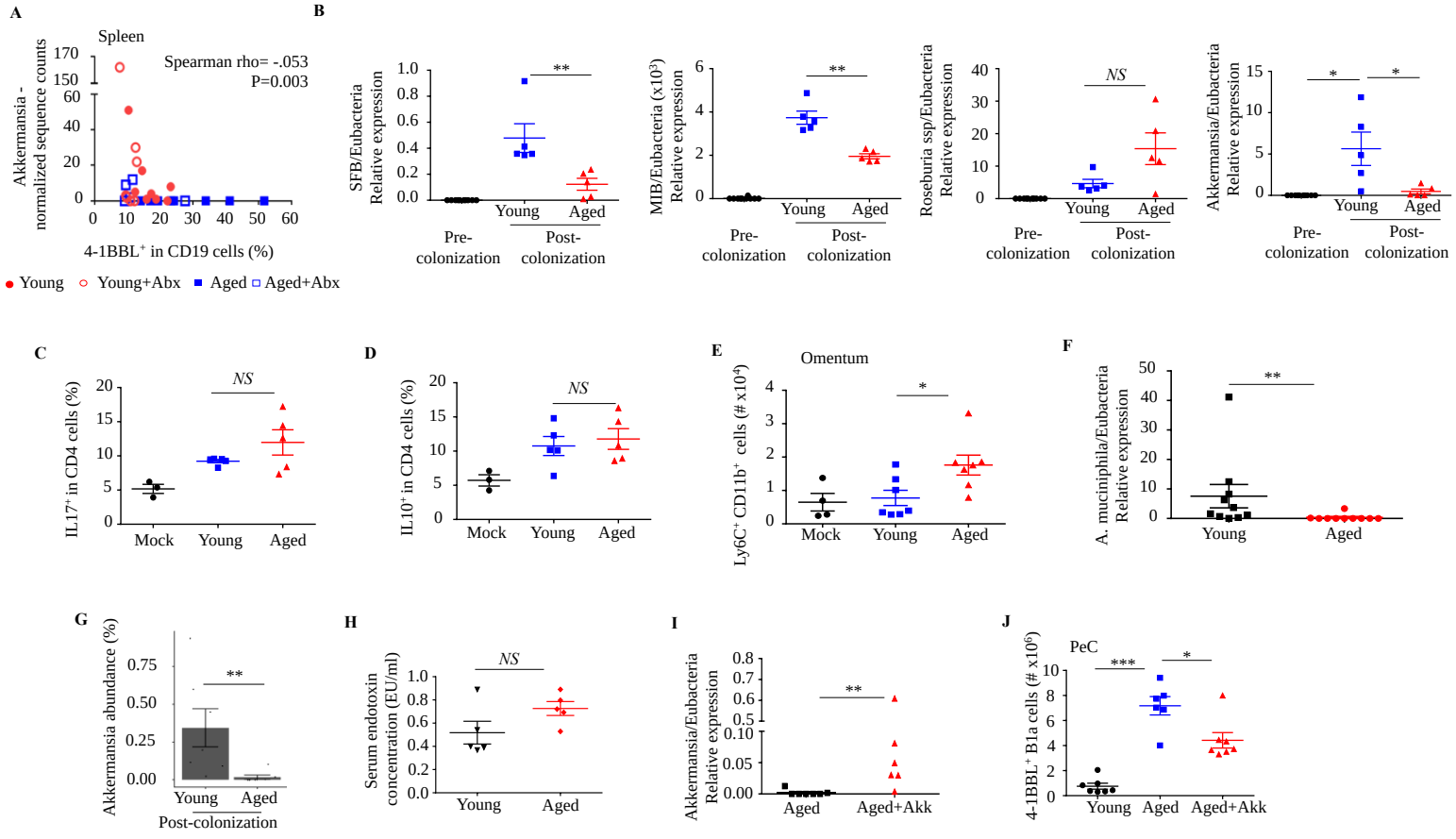
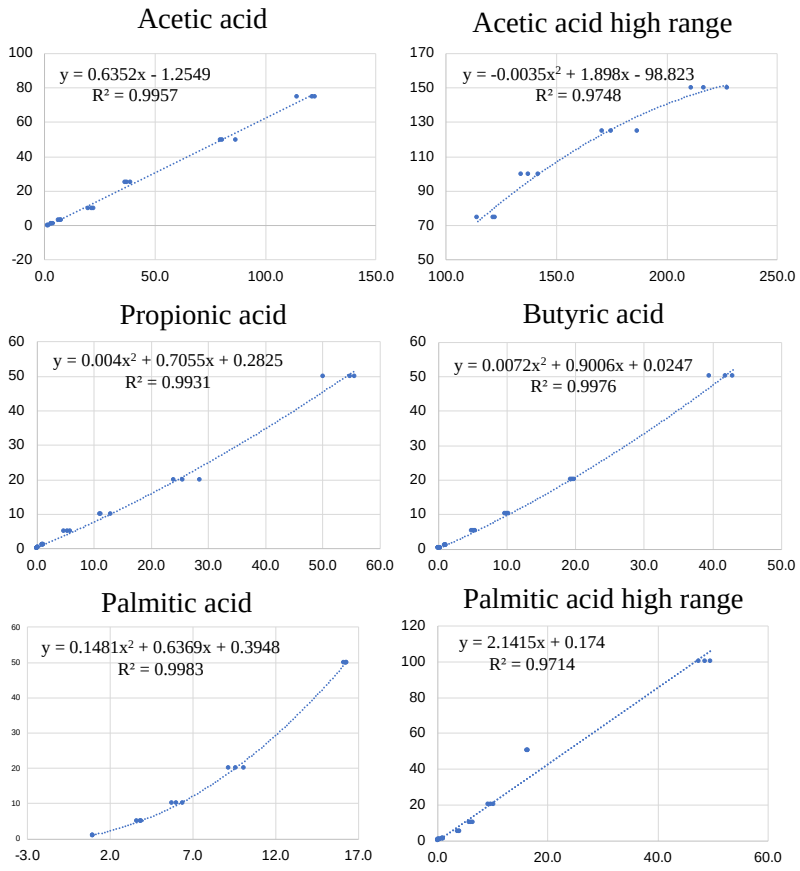


Figure S5. ***A. muciniphila*** **loss in aging induced gut leakiness and 4BL conversion**. **(A)** The abundance of *A. muciniphila* inversely correlates with splenic 4-1BBL^{+}CD19^{+} B cells of SPF mice. Spearman correlations and associated p-values are shown (n=7-8 mice per group). **(B-E** and **G)** Shown are results of fecal microbiota transplantation in germ free (GF) mice. **(B)** Fecal microbiota of GF mice was quantified for segmented filamentous bacteria (SFB), mouse intestinal bacteroides (MIB), Roseburia ssp, and *A. muciniphila* using qPCR. Results were normalized to eubacteria in samples before (pre-colonization) and after oral gavage with SPF fecal suspension (30 days post colonization). Microbiota from both aged and young SPF mice activated IL17- (**C**) and IL10-expressing CD4^{+} T cells (**D**). Gavage with SPF fecal suspension of aged, but not young mice, increased monocytes in the omentum of GF mice (**E**). Consistent with the presence of *A. muciniphila* in feces of SPF mice (**F**, shown by qPCR analysis), *A. muciniphila* was increased in GF mice only after transfer with microbiota from young, but not

aged, SPF mice (**G**, n=6 mice per group, shown by 16S rRNA gene sequencing). (**H**) Shown is serum endotoxin levels in GF mice after gavage with fecal suspension from young and aged SPF mice (n=5-6 mice per group, *NS*, not statistically significant). Gavage with *A. muciniphila* increased *A. muciniphila* (**I**) and reversed the 4BL cell accumulation (**J**) in aged SPF mice. Shown are fecal qPCR (**I**) and PeC flowcytometry analysis (**J**) after a 20-day gavage with *A. muciniphila* (n=5-7 mice per group, each symbol in A-J is for individual mice).

A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Acetic acid high range
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R² = 0.9748
Propionic acid
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R² = 0.9931
Butyric acid
y = 0.0072x² + 0.9006x + 0.0247
R² = 0.9976
Palmitic acid
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Palmitic acid high range
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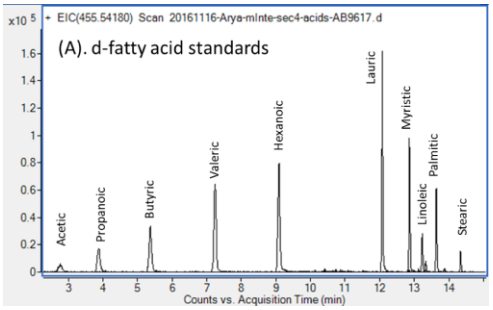
B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Propanoic
Butyric
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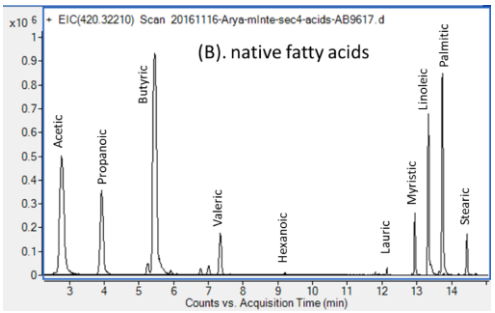
Stearic
Counts vs. Acquisition Time (min)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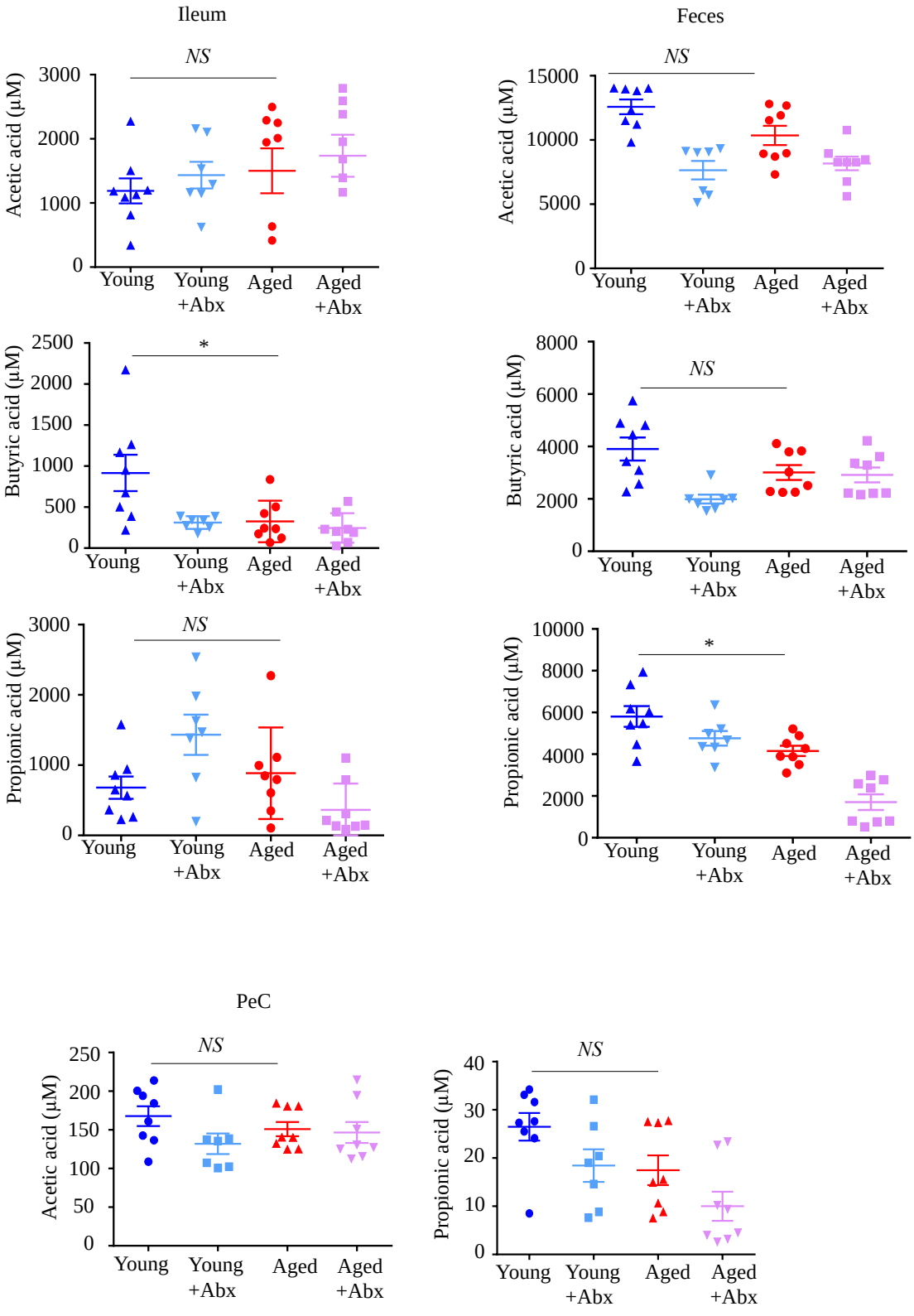
(B). native fatty acids
Acetic
Propanoic
Butyric
Valeric
Hexanoic
Lauric
Myristic
Linoleic
Palmitic
Stearic
Counts vs. Acquisition Time (min)

C


Ileum
Feces
PeC
Acetic acid (μM)
Butyric acid (μM)
Propionic acid (μM)
NS
*
Young
Young +Abx
Aged
Aged +Abx
0
1000
2000
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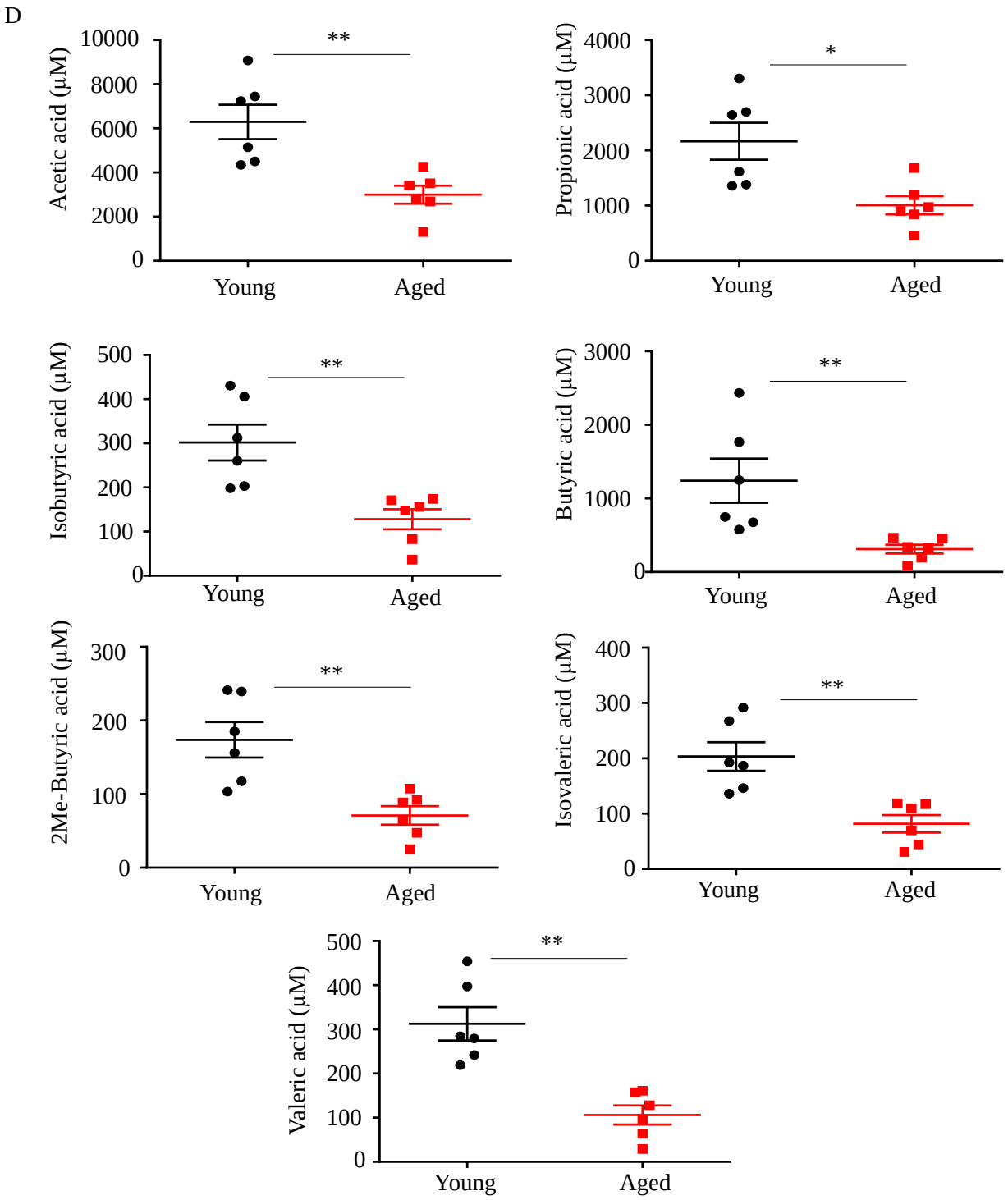

Figure S6. Aging changed fatty acids in the intestine and feces. Shown are examples of standard curves (**A**) and (**B**) LCMS chromatograms in fatty acid analysis. For each acid, the actual concentration ratios (h-acids / d-acids) are plotted against the observed extracted ion chromatogram peak area ratios (h-acids / d-acids), and the curves were fitted into either a linear or a quadratic equation. (**A**) depicts extracted ion chromatograms from a mouse cecum sample

for d-fatty acid standards and (**B**) regular fatty acids. (**C**) Shown are concentrations (μM) of acetic acid, butyric acid, propionic acid and palmitic acid in ileum, feces and PeC of individual young and old mice (each symbol is for individual mice with n=7-9) after 16 weeks of Abx or mock treatment. (**D**) Feces of elderly macaques were reduced in SCFAs (μM) as compared to young primates (n=6). Each symbol is for an individual primate.

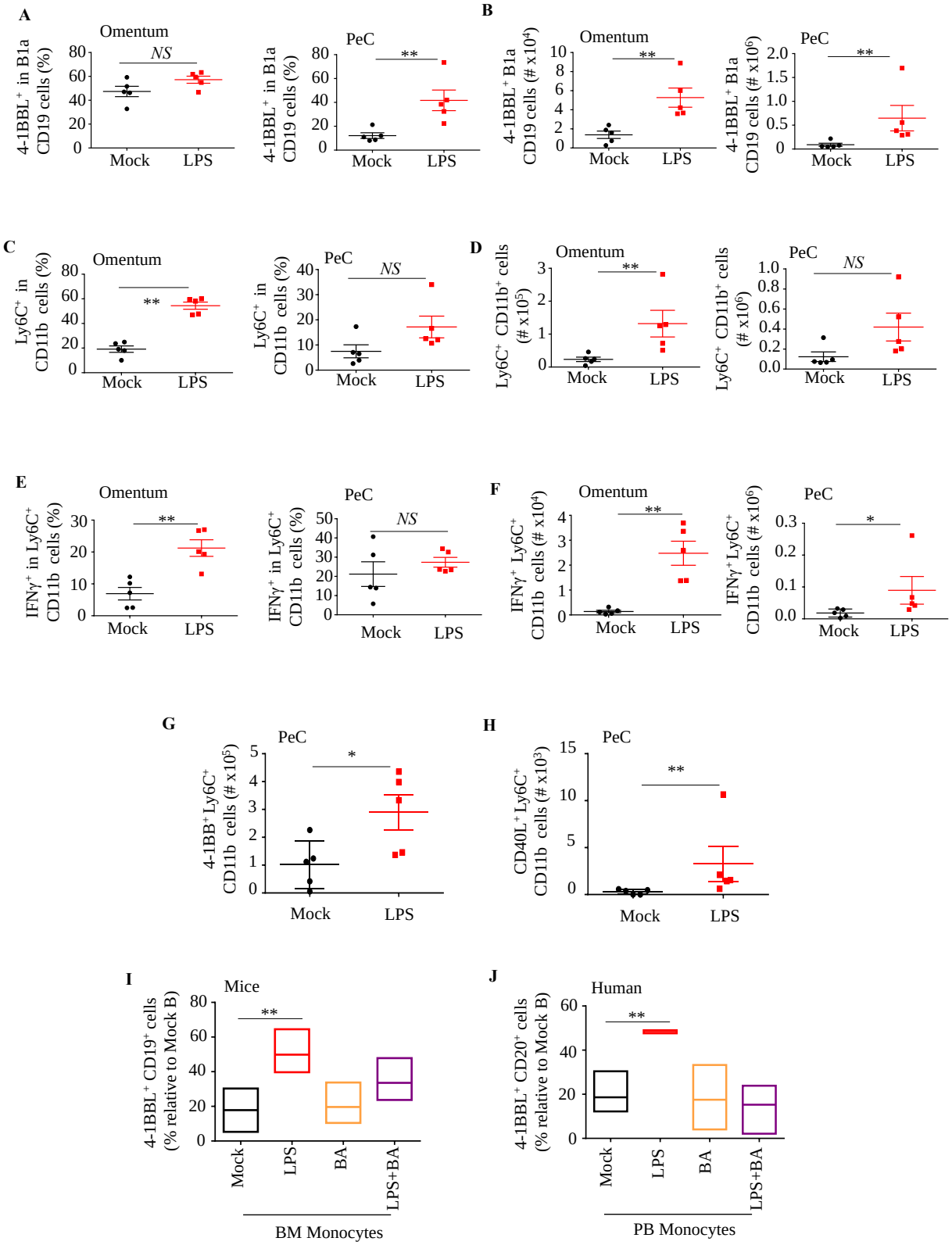

Figure S7. Chronic LPS injection imitated aging-induced 4BL cell accumulation. (**A-H**) LPS injection (i.p., 5 µg LPS daily for 5 days) in young mice increased frequency (%, in **A**, **C**, **E**) and absolute cell count (**B**, **D**, **F**, **G**, **H**) of 4-1BBL$^+$ B1a cells (**A**, **B**) and Ly6C$^+$ CD11b$^+$ monocytes (**C**, **D**) that express IFN-γ (**E**, **F**) in the omentum (left) and PeC (right panel, n=5 mice per group; the experiment was reproduced twice). LPS also upregulates 4-1BB (**G**) and

CD40L (**H**) in PeC Ly6C^{+} CD11b^{+} monocytes. Young mouse bone marrow-derived (BM, in **I**, n=5 mice per group) and human peripheral blood monocytes (**J**, n=9 human donors per group) acquired the ability to convert 4BL cells if treated with LPS in vitro. Monocytes were treated with 100 ng/ml LPS alone or together with 10 µM butyrate (BA). 24 h later young B cells isolated from mouse PeC (**I**) or human peripheral blood (**J**) were added and incubated for additional 48 h.

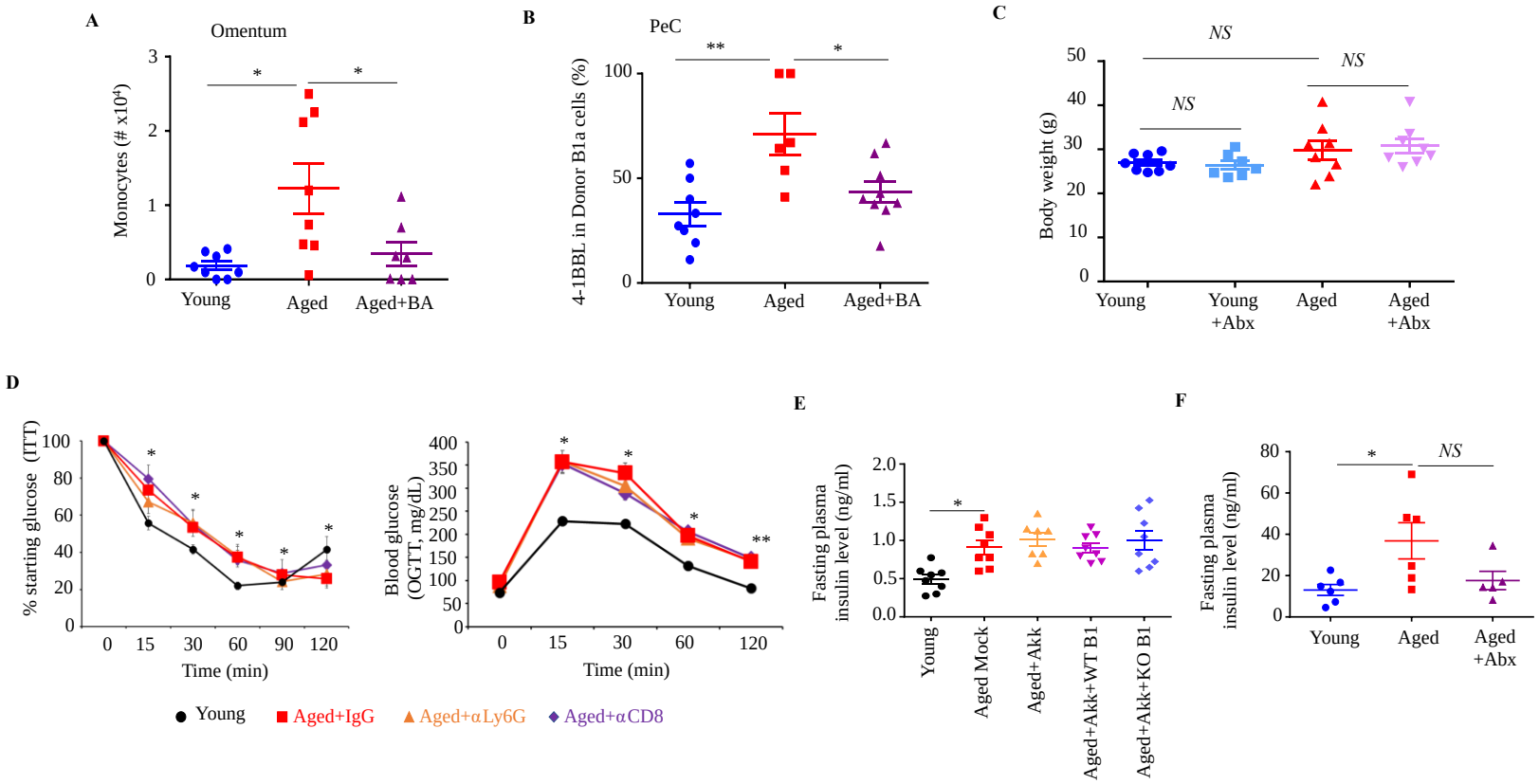

Figure S8. 4BL cells increased IR in aging. (**A** and **B**) Aged mice were orally gavaged with butyrate (BA, 2.5 g/kg body weight) every second day for 4 weeks. Then, 2 days after the termination of BA gavage, the mice were i.v. transferred with PeC GFP$^+$ B1 cells from young mice to evaluate their conversion into 4BL cells after 5 days. Shown is host monocytes and converted donor 4BL cells (n=6-9 mice per group, the experiment was reproduced twice). (**C**) Enrofloxacin (Abx) did not affect body weight of mice after a 16-weeks of treatment. (**D**) Depletion of CD8$^+$ T cells or granulocytes/neutrophils did not affect IR in mice. Aged mice were treated for 16 days with 200µg/mouse αCD8 Ab (depletes CD8$^+$ T cells) or 170 µg/mouse αLy6G Ab (depletes granulocytes) every 4 days (n=8-10 mice per group). ITT and OGTT were performed on day 20 and 22, respectively. (**E**) The IR benefit of *A. muciniphila* (Akk) in aged mice was abolished by transfer of 4BL cells. Aged mice were orally gavaged for 14 days with Akk and then were i.v. transferred with PBS (Aged+Akk) or with 4BL cells (B1 cells) from PeC of aged WT or 4-1BBL deficient mice (Aged+Akk+WT B1 and Aged+Akk+KO B1, respectively). Fasting plasma insulin was increased in aged mice (**E**) and elderly macaques (**F**). Shown are fasting plasma insulin levels of individual mice. In **E**, each mouse was tested at day 20 (n=8); the experiment was reproduced three times). In **F**, each symbol is for an individual animal.

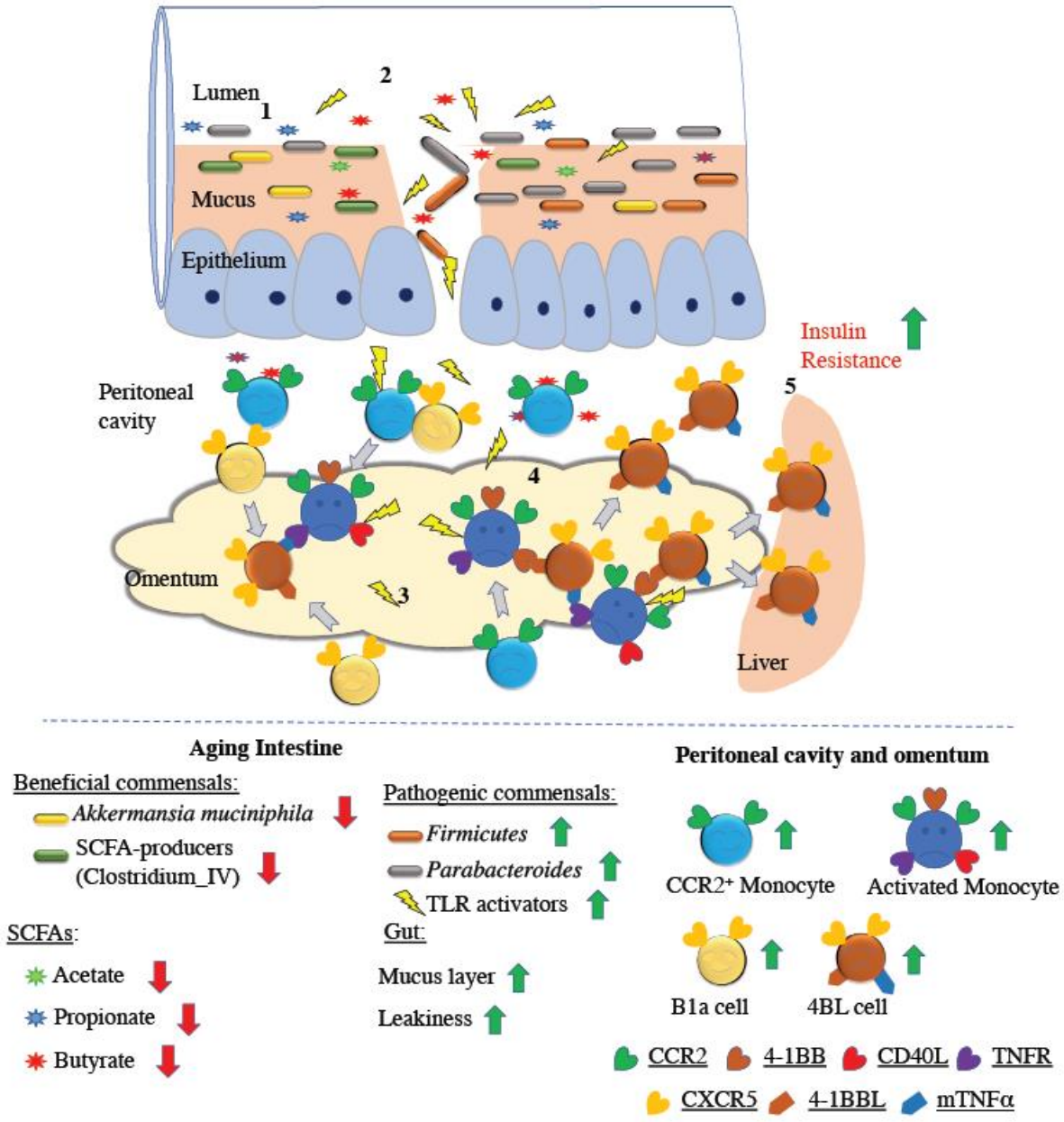

Figure S9. Schema summarizing our findings. 1, Gut dysbiosis was increased in aging. We found that the abundance of *A. muciniphila* and other beneficial commensal bacteria and their immunoregulatory SCFA were reduced in the intestine of aged mice. 2, The loss of *A. muciniphila* led to reduction of the protective inner mucus layer and leakiness of the gut, thereby inflaming the peritoneal cavity and omentum. Because of importance of *A. muciniphila* for other commensals and production of SCFA, its reduction also decreased intestinal SCFA. 3, Inflamed PeC and omentum recruited $CCR2^{+}$ monocytes and B1a cells by upregulating production of

CCL2 and CXCL13 chemokines, respectively. 4, When butyrate was decreased, bacterial stimuli (such as LPS) activated $CCR2^{+}$ monocytes to convert B1a cells into 4BL cells via 4-1BB/4-1BBL, TNFR2/mTNFα and CD40/CD40L axes (*22, 23*). 5, 4BL cells inhibited liver insulin receptor signaling and promoted insulin resistance requiring 4-1BBL.

Table S1. Operational taxonomic units (OTUs) for several butyrate inducers were markedly reduced in aging of C57BL/6 mice. Shown is mean % ±SEM abundance values in 16S rRNA gene sequencing of fecal microbiota of individual 8 young and 8 aged mice. ***** is for a species-level *ambiguous assignment* not reported in table S1.

	Species/OTUs			
Mouse ID #	***Intestinimonas butyriciproducens***	***Faecalibacterium prausnitzii / Gemmiger formicilis***	***Anaerostipes butyraticus / Anaerostipes caccae***	***Roseburia faecis***
Young # 1	0.80%	0.00%	0.12%	0.16%
Young # 2	0.24%	0.00%	0.04%	0.00%
Young # 3	0.16%	0.04%	0.04%	0.28%
Young # 4	0.36%	0.12%	0.08%	0.12%
Young # 5	0.36%	0.00%	0.00%	0.04%
Young # 6	0.48%	0.40%	0.00%	0.00%
Young # 7	0.08%	0.00%	0.00%	0.00%
Young # 8	0.08%	0.00%	0.40%	0.04%
Young (n=8) Mean % Abundance (s.e.m.)	**0.32% (0.09%)**	**0.07% (0.05%)**	**0.09% (0.05%)**	**0.08% (0.04%)**
Aged # 1	0.00%	0.00%	0.00%	0.00%
Aged # 2	0.00%	0.00%	0.00%	0.00%
Aged # 3	0.00%	0.00%	0.00%	0.00%
Aged # 4	0.00%	0.00%	0.00%	0.00%
Aged # 5	0.00%	0.00%	0.00%	0.00%
Aged # 6	0.00%	0.00%	0.00%	0.00%
Aged # 7	0.00%	0.00%	0.00%	0.00%
Aged # 8	0.00%	0.00%	0.00%	0.00%
Aged (n=8) Mean % Abundance (s.e.m.)	**0% (0%)**	**0% (0%)**	**0% (0%)**	**0% (0%)**
Negative binomial test P-value	**3.59E-16**	**0.00059**	**0.00010**	**0.00013**
FDR Adjusted P-value	**2.11E-15**	**0.00158**	**0.00031**	**0.00036**

Table S2. Compared to middle-aged mice, aged C57BL/6 mice increase insulin resistance.

N=8-10 per group	Age: 12 months		Age: 24 months		P value	
	Glucose (mg/dL)	Insulin (pmol/L)	Glucose (mg/dL)	Insulin (pmol/L)	Glucose (mg/dL)	Insulin (pmol/L)
Female C57BL/6	117±15	44±13	147±16	237±53	<0.001	<0.001
Male C57BL/6	103±13	76±15	157±14	425±134	<0.001	<0.001