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Supplemental information

Regulation of splenic monocyte homeostasis and function by gut microbial products

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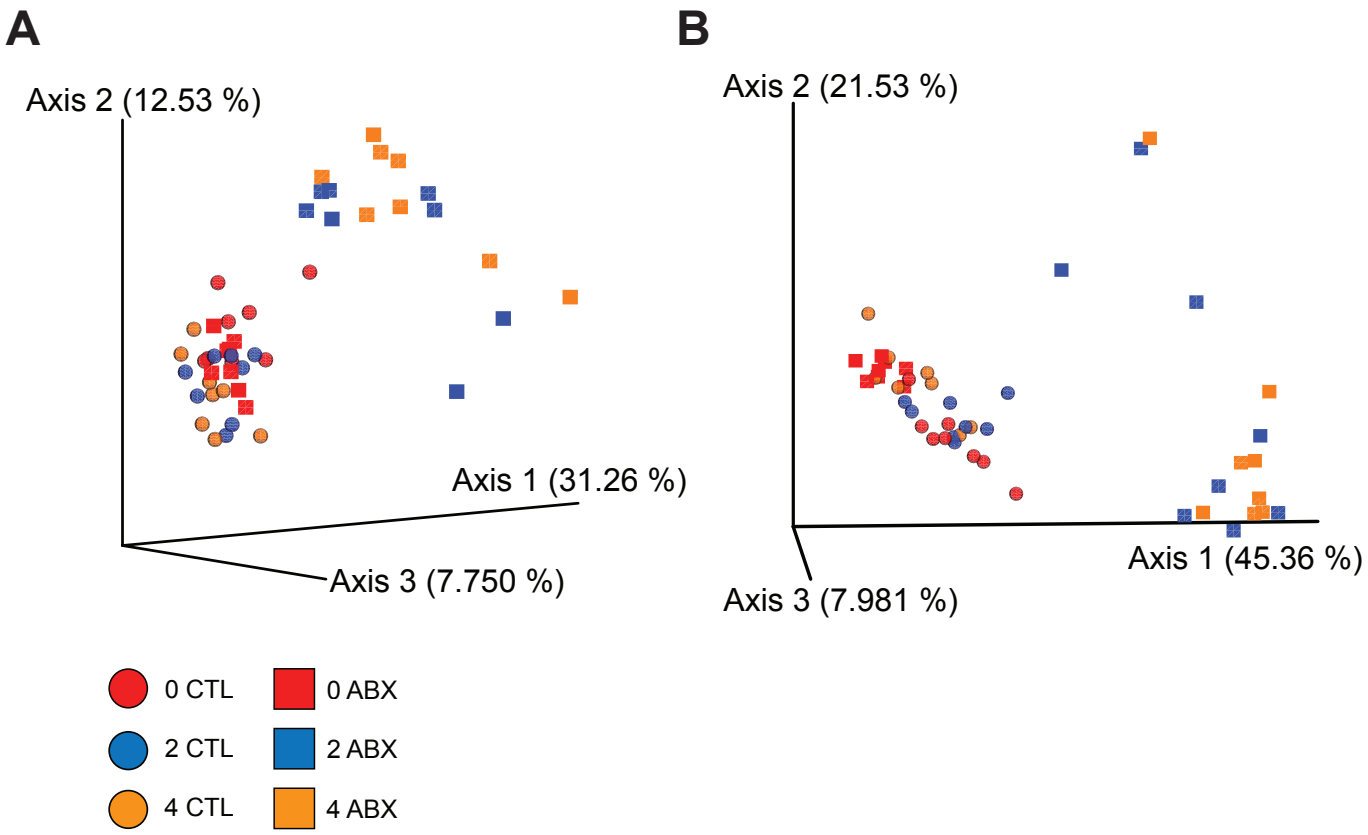

Figure S1. Alterations in the composition of the gut microbiome after a short-term antibiotic treatment. Related to Figure 1.

Principal coordinates analysis (PCoA) of the A) unweighted and B) weighted Unifrac distances of the 16S rRNA sequencing data as visualized by Emperor. The two groups are represented by colored circles (untreated mice) and squares (antibiotic-treated mice). Timepoints are shown in red (day 0), blue (day 2), and orange (day 4) color. Differences between the antibiotic-treated and untreated group were statistical significant as calculated by the permanova paired wise test.

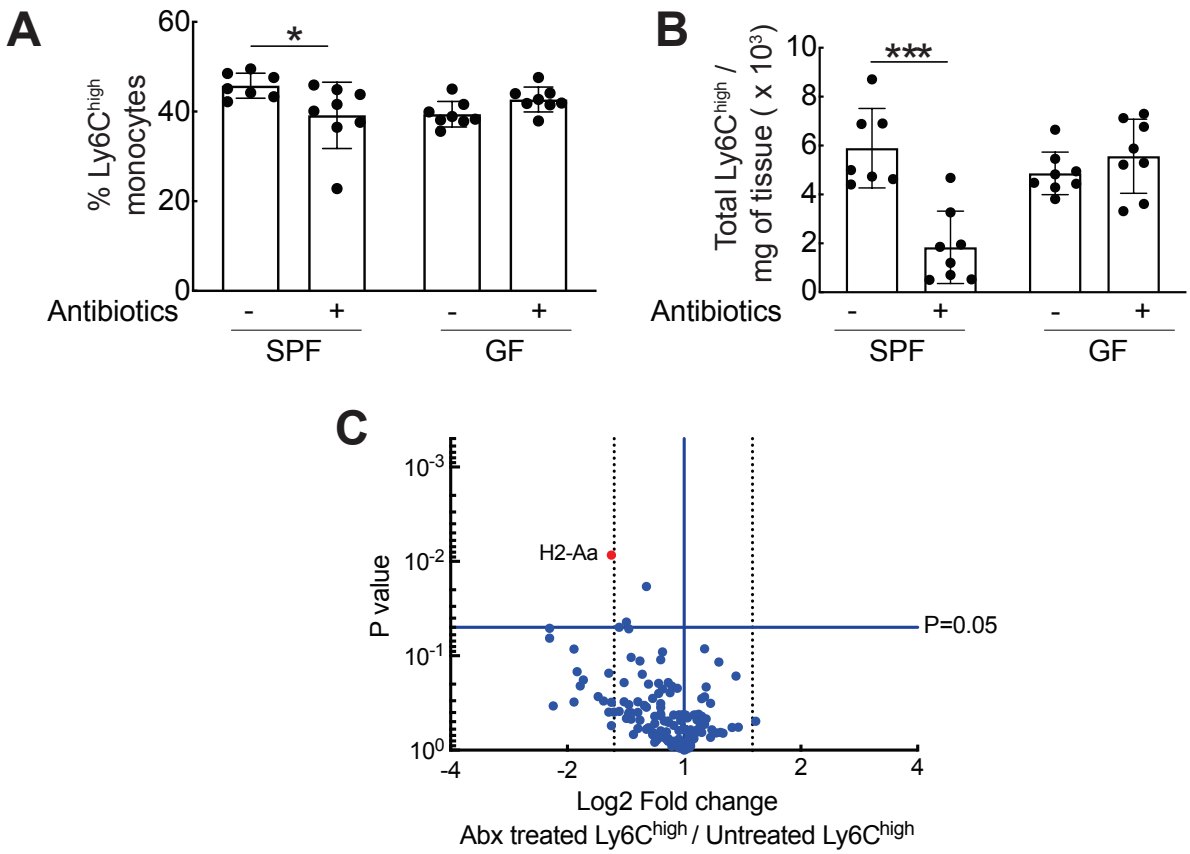

Figure S2. Perturbation of splenic Ly6C^{high} monocyte homeostasis in antibiotic-treated mice is microbiome-mediated. Related to Figure 2 and 3.

SPF and GF mice were treated with or without the antibiotic cocktail for 4 days. On day 4, the frequency and absolute numbers of Ly6C^{high} monocytes were estimated by flow cytometry. A) Frequency and B) absolute number per mg of tissue of splenic Ly6C^{high} monocytes from antibiotic-treated and untreated SPF and GF mice. Data show the mean ± s.dev. (n=7-8 mice per group). Statistical significance was assessed by two-tailed student's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; C) Volcano plot showing fold changes in gene expression in splenic Ly6C^{high} monocytes from antibiotic-treated vs untreated GF mice using the nCounter Nanostring Immunology panel. Each dot presents one gene while the color of the dot shows the significance in the change of gene expression. A fold change ≥ 1.5 (upregulation) and ≤ -1.5 (downregulation) was considered significant whereas p values were calculated by the nSolver analysis software. Blue dots represent genes with no statistically significant change in their expression whereas red dots show genes which expression levels were significantly affected by the antibiotic treatment.

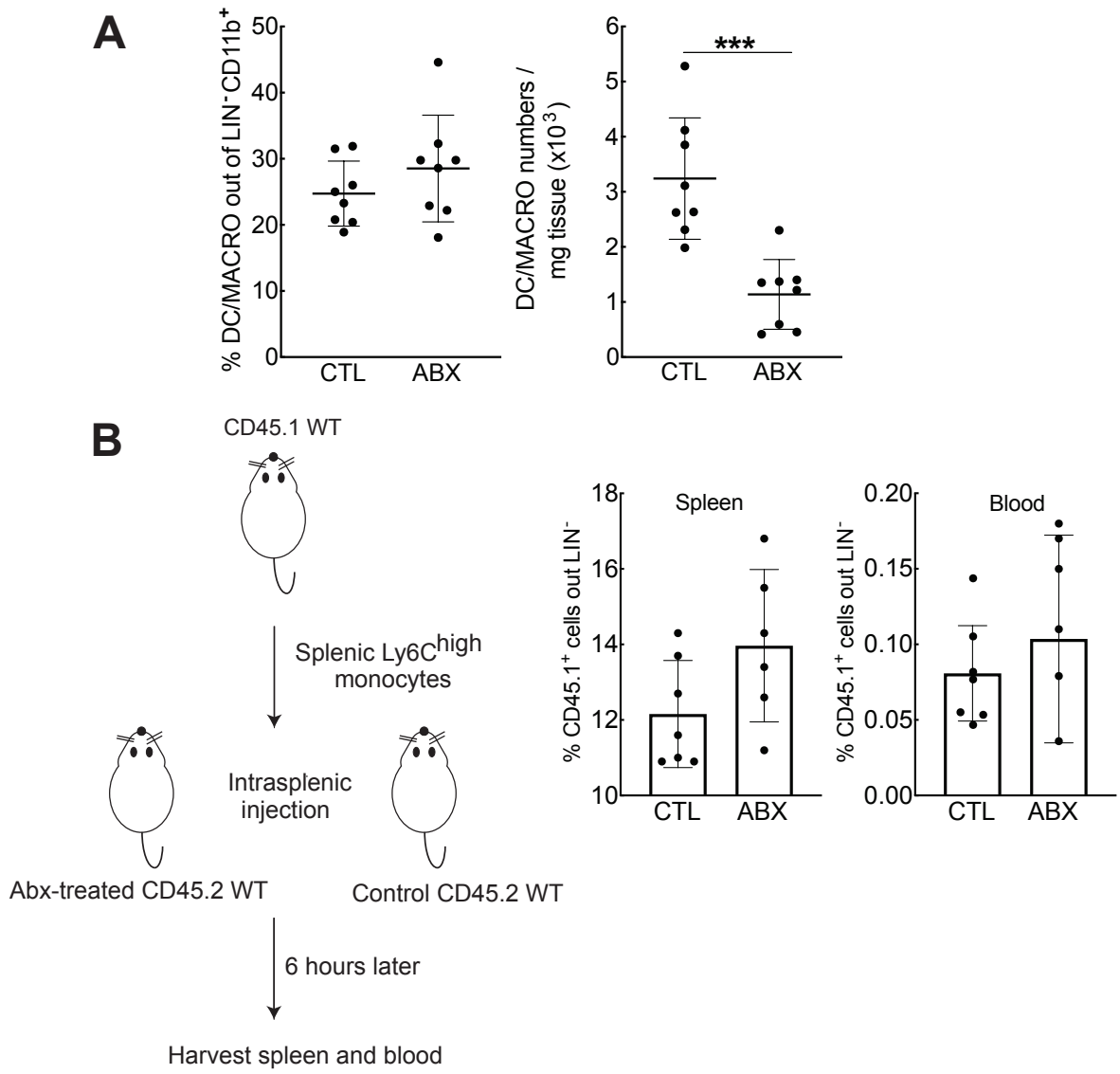

Figure S3. Perturbation of the splenic Ly6C^{high} monocyte homeostasis after gut dysbiosis is not related to increased differentiation to DC/macrophages or increased exit from the tissue. Related to Figure 2.

A) Frequency and absolute number of DC/macrophages in splenic cell suspensions of mice treated with or without antibiotics for 3 days. Data show mean ± s.dev. and they are representative of two experiments (n=6 per group). Statistical significance was assessed by Student's t-test. P values are shown as *p<0.05, **p<0.01, ***p<0.001; B) Experimental design of intrasplenic transfer of CD45.1^{+} splenic Ly6C^{high} monocytes to CD45.2^{+} C57BL/6 mice, pretreated with or without antibiotics for 3 days. Data show frequency of CD45.1^{+} cells out of LIN- in splenic cell suspensions and blood six hours after cell transfer. Data show mean ± s.dev. and they are representative of two experiments (n=6 per group).

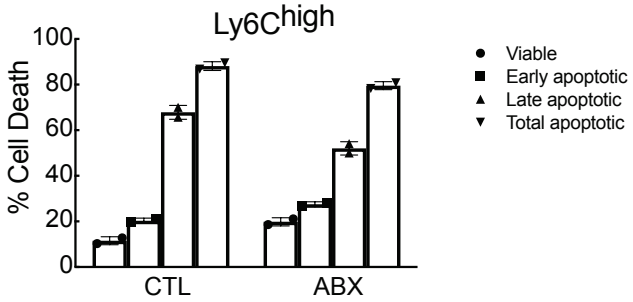

Figure S4. Short antibiotic treatment does not impair splenic Ly6C^{high} monocyte survival *ex vivo.* Related to Figure 3.

FACS-sorted splenic Ly6C^{high} monocytes from mice treated with or without antibiotics for 3 days were stimulated ex vivo with 100 ng/ml LPS. Twenty-four hours later, cells were stained with Annexin V and viability dye eFluor780 to enumerate the frequency of cell death by flow cytometry. Data show the frequency of viable (black bars), early apoptotic (white bars), late apoptotic (blue bars) and total apoptotic cells (red bars) in LPS-stimulated splenic Ly6C^{high} from antibiotic-treated and control animals. Data represent the mean ± s.dev. of technical duplicate wells.

Supplemental Table 1. Relative abundance of taxa significantly reduced during antibiotic treatment. Related to Figure 1

Phylum	Class	Order	Family	Genus	D0	D2	D4
Bacteroidetes	Bacteroidia	Bacteroidales	S24_7	unclassified	40.7%[a]	1.01%	0.49%
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	unclassified	20.6%	2.58%	1.14%
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	unclassified	6.5%	8.34%	3.09%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified	3.1%	1.26%	0.33%
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	1.8%	1.58%	0.62%
Tenericutes	Mollicutes	RF39	unclassified	unclassified	1.5%	0.44%	0.29%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	0.9%	0.21%	0.03%
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.5%	0.16%	0.03%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	0.4%	0.00%	0.00%
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified	0.3%	0.07%	0.03%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium piliforme	0.2%	0.00%	0.00%
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus	0.1%	0.03%	0.00%
Firmicutes	Clostridia	Clostridiales	Mogibacteriaceae	unclassified	0.1%	0.03%	0.01%
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Allobaculum	0.1%	0.04%	0.01%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea	0.04%	0.01%	0.02%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerostipes	0.04%	0.00%	0.00%
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	0.36%	0.07%	0.22%

[a] Data show mean relative abundance of taxa averaged from eight mice.

Supplemental Table 2. Relative abundance of taxa significantly increased during antibiotic treatment. Related to Figure 1.

Phylum	Class	Order	Family	Genus	D0	D2	D4
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	0.83%[a]	23.3%	36.7%
Firmicutes	Bacilli	Bacillales	Bacillaceae	Staphylococcus	0	15.4%	17.2%
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia municiphila	0.35%	5.57%	5.59%
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	0	0.26%	2.42%
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	0	0.8%	1.72%
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus gnavus	0.66%	0.85%	1.64%
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	unclassified	0	0.04%	0.8%
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	0	0.05%	0.54%
Firmicutes	Bacilli	Lactobacillales	unclassified	unclassified	0	0	0.48%
Firmicutes	Bacilli	Bacillales	Bacillaceae	Geobacillus vulcani	0	0.03%	0.4%
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Coprobacillus	0.03%	0.1%	0.39%
Firmicutes	Clostridia	Clostridiales	Tissierellaceae	Gallicola	0	0	0.26%
Firmicutes	Clostridia	Clostridiales	Tissierellaceae	Anaerococcus	0	0.03%	0.24%
Actinobacteria	Actinobacteria	Actinomycetales	Sanguibacteraceae	Sanguibacter	0	0	0.13%
Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	0	0	0.09%
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	0	0	0.09%
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium	0	0	0.09%
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0.1%	0.11%	0.06%
Firmicutes	Bacilli	Bacilllales	unclassified	unclassified	0	0	0.04%
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia	0	0	0.01%

[a] Data show mean relative abundance of taxa averaged from eight mice.

TRANSPARENT METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD3-APC	Biolegend	100236; RRID: AB_2561456
Anti-mouse CD3-PerCP-Cy5.5	Biolegend	100218; RRID: AB_1595492
Anti-mouse B220-APC	Biolegend	103212; RRID: AB_312997
Anti-mouse NK1.1-APC	Biolegend	108710; RRID: AB_313397
Anti-mouse CD49b-APC	Biolegend	108910; RRID: AB_313417
Anti-mouse LY6G-APC	Biolegend	127614; RRID: AB_2227348
Anti-mouse Siglec-F-APC	Miltenyi Biotec	130-102-241; RRID:AB_2653452
Anti-mouse CD11c-BV421	Biolegend	117330; RRID: AB_11219593
Anti-mouse CD11c-BV605	Biolegend	117334; RRID: AB_2562415
Anti-mouse F4/80-BV421	Biolegend	123132; RRID: AB_11203717
Anti-mouse I-A^b-BV421	BD Biosciences	562928; RRID: AB_2737897
Anti-mouse I-A^b-APC/Fire™ 750	Biolegend	116424; RRID: AB_2721487
Anti-mouse CD11b-PeCy7	Biolegend	101216; RRID: AB_312799
Anti-mouse Ly6C-Alexa488	Biolegend	128022; RRID: AB_10639728
Anti-mouse Ly6C-Brilliant Violent 785	Biolegend	128041; RRID: AB_2565852
Anti-mouse Ly6C-PE	Biolegend	128008; RRID: AB_1186132
Anti-mouse CD115-PE	Biolegend	135506; RRID: AB_1937253
Anti-mouse CD45.1-BV711	Biolegend	110739; RRID: AB_1937253
Anti-mouse CD45-BUV395	BD Biosciences	564279; RRID: AB_2651134
Fixable Viability Dye eFluor™ 506	ThermoFisher Scientific	65-0866-18
Fixable Viability Dye eFluor™ 660	ThermoFisher Scientific	65-0864-18
Fixable Viability Dye eFluor™ 780	ThermoFisher Scientific	65-0865-18
Bacterial Strains		
Citrobacter rodentium	ATCC	51459
Escherichia coli, strain K12	The Coli Genetic Stock Center at Yale	CGSC#7296
Cell lines		
HEK-Blue mTLR2	Invivogen	hkb-mtlr2
HEK-Blue mTLR3	Invivogen	hkb-mtlr3
HEK-Blue mTLR4	Invivogen	hkb-mtlr4
HEK-Blue mTLR5	Invivogen	hkb-mtlr5
HEK-Blue mTLR7	Invivogen	hkb-mtlr7
HEK-Blue mTLR9	Invivogen	hkb-mtlr9
HEK-Blue mNOD1	Invivogen	hkb-mnod1
HEK-Blue mNOD2	Invivogen	hkb-mnod2
HEK-Blue Null1	Invivogen	hkb-null1
HEK-Blue Null1-k	Invivogen	hkb-null1k
HEK-Blue Null1-v	Invivogen	hkb-null1v

HEK-Blue Null2	Invivogen	hkb-null2
HEK-Blue Null2-k	Invivogen	hkb-null2k
Chemicals, peptides and enzymes		
Histopaque-1077	Sigma	10771
Ampicillin trihydrate	Sigma	A6140
Buprenorphine SR	Wildlife Pharmaceuticals	https://wildpharm.com/medications/labanimals/item/3-buprenorphine-sr-1ml.html
Meloxicam SR	Wildlife Pharmaceuticals	https://wildpharm.com/meloxicamsr5mllab.html
Metronidazole	Sigma	M1547
Neomycin trisulfate salt hydrate	Sigma	N1876
Vancomycin HCL	Research Products International	V06500-5.0
Gentamicin reagent solution	ThermoFisher Scientific	15750-060
Commercial assays		
eBioscience™ Annexin V Apoptosis Detection Kit APC	ThermoFisher Scientific	88-8007-74
FAM-FLIVO® In vivo Polycaspase Assay	ImmunoChemistry Technologies, LLC	981
Legendplex Mouse Inflammation Panel	Biolegend	740446
Mouse TNF-α Duoset ELISA kit	R&D Systems	DY410-05
Mouse IL-6 Duoset ELISA kit	R&D Systems	DY406-05
Mouse IL-10 Duoset ELISA kit	R&D Systems	DY417-05
Mouse IL-1α/IL-1F1 Duoset ELISA kit	R&D Systems	DY400-05
Mouse IL-1β/IL-1F2 Duoset ELISA kit	R&D Systems	DY401-05
PRR ligands		
Pam2CSK4	Invivogen	tlrl-pm2s-1
Poly (I:C) HMW	Invivogen	tlrl-pic
LPS, *E.coli* O55:B5	Sigma	L4524
FLA-ST	Invivogen	tlrl-stfla
CL264	Invivogen	tlrl-c264e-5
ODN1826	Invivogen	tlrl-1826-1
iE-DAP	Invivogen	tlrl-dap
MDP	Invivogen	tlrl-mdp
Software		
FlowJo v.10.5.3.	FlowJo, LLC	https://www.flowjo.com
BioRender		Biorender.com
GraphPad Prism v.8.2.0.	Prism-graphpad.com	https://www.graphpad.com/scientific-software/prism/
Metacore	Clarivate Analytics	https://portal.genego.com/
LefSE		https://huttenhower.sph.harvard.edu/galaxy/

QIIME		https://view.qiime2.org/
Taqman Probes		
Mouse *IL-1α*	Applied Biosystems	Mm00439620_m1
Mouse *IL-1β*	Applied Biosystems	Mm00434228_m1
Mouse *IL-6*	Applied Biosystems	Mm00446190_m1
Mouse *IL-10*	Applied Biosystems	Mm01288386_m1
Mouse *TNF-α*	Applied Biosystems	Mm00443258_m1
Mouse *Trem1*	Applied Biosystems	Mm01278455_m1
Mouse *S100a8*	Applied Biosystems	Mm00496696_g1
Mouse *S100a9*	Applied Biosystems	Mm00656925_m1
Mouse *CD36*	Applied Biosystems	Mm00432403_m1
Mouse *Camp*	Applied Biosystems	Mm00438285_m1
Mouse *Ciita*	Applied Biosystems	Mm00482914_m1
Mouse *Gapdh*	Applied Biosystems	Mm99999915_g1
Others		
Fluoresbrite® Yellow Green Microspheres, 0.5 micrometer	Polysciences, Inc	17152-10
Deposited data	Mendeley data	http://dx.doi.org/10.17632/tjdwjnzskr.1

Animals

Female C57BL/6 (stock number 000664) and B6 CD45.1 (stock number 002014) mice were purchased from The Jackson Laboratory and were used at 6-8 weeks of age. All animals were kept in a pathogen-free facility at Brigham and Women's Hospital (BWH) in accordance with the animal protocol guidelines prescribed by the Institutional Animal Care and Use Committee of BWH. Germ-free C57BL/6 mice were bred and raised in gnotobiotic isolators at Massachusetts Host-Microbiome Center at BWH. Mice were housed in at least two cages per group to minimize cage-related effects.

Antibiotic treatment

SPF and GF mice were treated with a broad-spectrum antibiotic cocktail consisting of vancomycin (0.5 g/L), ampicillin (1 g/L), neomycin (1 g/L) and metronizadole (1g/L) dissolved in drinking water. SPF mice received the antibiotic-containing water *ad libitum* for

2-6 days in all experiments. The antibiotic cocktail was administered by oral gavage for 4 days only in GF and control SPF animals to avoid signs of dehydration seen in GF mice treated with antibiotic-containing water *ad libitum*. All antibiotics were purchased from Sigma except vancomycin (Research Products International).

Preparation of single-cell suspensions from blood, spleen and bone marrow.

Blood was isolated from the facial vein of mice using a 5 mm goldenrod lancet (VWR) and collected in BD Microtainer tubes with lithium heparin (Becton Dickinson). Heparinized-blood was loaded onto Histopaque-1077 (Sigma) and centrifuged for 20 minutes at 900 x g, at 25° C. The interphase containing blood mononuclear cells was collected and stained for monocyte subsets. Single-cell suspensions from spleens were prepared by mechanical disruption using a 70-μm-cell strainer/nylon mesh (Fisher Scientific). Perfusion was not performed before isolation of spleens as the number of contaminating blood monocytes is negligible (Swirski et al., 2009). Bone marrow cells were isolated from both femurs and tibias of animals after centrifugation of bones at 14,000 rpm for 15 seconds. Red blood cells were eliminated from splenic and bone marrow cell suspensions using the ACK lysing buffer (Lonza). Viability and total cell numbers were determined upon staining with Trypan Blue (GE Healthcare Life Sciences) using a hemocytometer.

Flow cytometry and cell-sorting

Single-cell suspensions from blood, spleen and bone marrow were stained with an anti-CD16/32 antibody (BioXCell) to block Fc receptors for 15 minutes on ice. Afterwards, the antibodies described in Key Resources Table were used to stain cell suspesions. Live

monocytes were identified as CD11b^{high} LIN (eFluor660, CD3, B220, NK1.1, CD49b, Ly6G, Siglec-F)$^{-}$ (F4/80, CD11c, I-Ab)^{low} and Ly6C^{high}. Macrophages/dendritic cells were identified as CD11b^{high} LIN (eFluor660, CD3, B220, NK1.1, CD49b, Ly6G, Siglec-F)$^{-}$ (F4/80, CD11c, I-Ab)^{high} and Ly6C^{low}. Monocytes/macrophages/dendritic cell numbers were calculated by multiplying the number of total cells with the percent of the respective gate. To calculate the absolute cell number within the splenic tissue, total monocyte number was normalized to weight (mg) of spleen. Data were acquired on a BD LSRII and BD LSRFortessa (BD Biosciences) and analyzed with FlowJo v.10.5.3. (FlowJo, LLC). For cell sorting, splenic and bone marrow suspensions were pre-enriched in CD11b^{+} cells using the CD11b Microbeads (Miltenyi Biotec) and then stained with the antibodies for sorting. Cells were sorted in a BD FACSAria II (BD Biosciences) using a 100 µm nozzle at 20 psi.

Quantitative real-time PCR (Q-PCR)

FACS-sorted splenic Ly6C^{high} monocytes were lysed in RLT buffer and RNA was extracted using the RNeasy® Micro Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qPCR was performed using Taqman Universal PCR Master Mix and Taqman Gene Expression Assays (Applied Biosystems) in technical duplicate wells. All primers and probes were from Applied Biosystems as described in Key Resources Table. All assays were performed using a ViiA7 system (Applied Biosystems). Results were normalized to the housekeeping gene *Gapdh*. The relative expression of each target gene was expressed as ($2^{-\Delta Ct}$) (Livak et al., 2001), where ΔCt is the difference between the mean Ct of the target gene and the mean Ct of *Gapdh*. Gene expression of *Gapdh* was stable between the

antibiotic-treated and control groups.

nCounter gene expression and analysis

FACS-sorted splenic Ly6C^{high} monocytes from experimental and control groups were lysed in RLT buffer and cell lysates were hybridized with reporter and capture probes for nCounter Gene Expression code sets (Mouse Immunology Panel), according to the manufacturer's instructions (Nanostring Technologies). Analysis of expression of 561 genes was performed using the nSolver analysis software, version 4.0.62 (Nanostring Technologies). Data were normalized to spiked positive controls and housekeeping genes. Transcript counts less than 50 were excluded from the analysis as they were considered as background.

Metacore analysis

Data were analyzed using the Metacore software. Differentially expressed genes (with corresponding fold-changes and p values) were uploaded and analyzed by the Enrichment Analysis Tool as well as the Interactome Tool to generate biological networks and predict the top ten transcription factors involved in the regulation of the differentially expressed genes.

Adoptive transfer of splenic monocytes

Splenic Ly6C^{high} monocytes were transferred to recipient mice intrasplenically. Recipient CD45.2 mice were administered water with or without antibiotics for 3 days. Before cell transfer, recipient mice were s.c. injected with 0.6 mg/kg buprenorphine SR and 4 mg/kg

meloxicam SR solutions to minimize post-operative pain (both from Wildlife Pharmaceuticals) and shaved on the middle left side of the abdomen. Then, a skin incision between the last rib and hip joint was made and 5 x 10^4 FACS-sorted splenic Ly6C^{high} monocytes from CD45.1 mice, resuspended in 20 µl sterile PBS, were injected in the subcapsular layer of the spleens of CD45.2 mice. Next, the tissue was sutured using 4.0 chromic gut absorbable sutures. Six hours after the transfer, cell suspensions from blood and spleens of the recipient mice were prepared, stained for the presence of CD45.1$^+$ monocytes and analyzed by flow cytometry.

In vivo phagocytosis

Mice were treated with or without antibiotics for three days. On day three, animals underwent the surgical process described above. Once, spleen was exteriorized, we injected 20 µl of Fluoresbrite Yellow Green Microspheres (0.5 µm; Polysciences) per mouse. Next, the tissue was sutured using 4.0 chromic gut absorbable sutures. Twenty-four hours later, splenic single-cell suspensions were prepared and the frequency of fluorescently labeled splenic Ly6C^{high} monocytes was quantified by flow cytometry.

Detection of cell death *ex vivo*

FACS-sorted splenic Ly6C^{high} monocytes were stimulated with 100 ng/ml LPS for 24 hours. Then, cells were stained with the eBioscience Annexin V apoptosis detection kit and eBioscience Fixable viability dye eFluor780 (all from ThermoFisher Scientific), according to the manufacturer's instructions. Early apoptotic cells were considered as Annexin V$^+$ eFluor780$^-$ whereas late apoptotic cells were Annexin V$^+$ eFluor780$^+$. Necrotic cells,

Annexin V^- $eFluor780^+$, were not detected. For determining the apoptotic rate of monocytes from antibiotic-treated mice, animals were administered water with or without antibiotics for 3 days. On day 3, splenic cell suspensions were incubated with FAM-FLIVO for 60 minutes at 37° C for 60 minutes. Cells were washed twice, fixed and analyzed by flow cytometry.

Ligand injections

Naïve mice were administered water with or without antibiotics for three days. One and two days after initiation of the antibiotic treatment, mice were injected i.p. with ie-DAP (25 µg/mouse) or MDP (25 µg/mouse). Another group of mice received a TLR ligand cocktail consisting of Pam2CSK4 (5 µg/mouse), LPS (5 µg/mouse), CL264 (5 µg/mouse), ODN1826 (5 µg/mouse) and FLAST (1 µg/mouse) only once, one day after initiation of the antibiotics.

Gentamicin Protection assay

FACS-sorted splenic $Ly6C^{high}$ monocytes were incubated for 2 hours with *C.rodentium* or *E.coli*, K12 strain, at a multiplicity of infection (MOI) of 20. Next, cells were washed to remove extracellular bacteria and further treated with gentamicin for 1 hour. Then, supernatant was collected to ensure the absence of live extracellular bacteria within the wells and cells were lysed in 1% triton buffer. Serial dilutions of the cell lysates and supernatants were plated on agar plates in triplicates and the number of colonies was counted after overnight incubation at 37° C. Results are presented as absolute CFU counts.

Detection of cytokines in the supernatant

FACS-sorted splenic Ly6C^{high} monocytes from antibiotic-treated and untreated mice were stimulated with or without 100 ng/ml LPS (from E. coli O55:B5, Sigma) for 24 hours. Cell-free culture supernatants were examined for the presence of cytokines using the Legendplex Mouse Inflammation Panel (Biolegend). Quantification of TNF-α, IL-6, IL-1α, IL-1β and IL-10 in samples was also assessed by ELISA kits (DuoSet Kits, R&D Systems), according to the manufacturer's instructions.

Detection of bacterial products in murine sera

C57BL/6 mice were treated with or without antibiotics for 4 days and sera were isolated from both groups before as well as 2 and 4 days after initiation of the treatment. To assess the presence of bacterial products, sera from all time points were incubated with the HEK-Blue TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, NOD1, NOD2 as well as the control cell lines which lack the corresponding PRR (all from Invivogen). Briefly, HEK293 cells stably co-express murine TLRs and an NF-κB/AP-1 inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. In the presence of TLR agonists, activation of NF-κB/AP-1 leads to SEAP secretion in the cell supernatant. Addition of a specific SEAP color substrate in the wells produces a purple/blue color that was measured at 620 nm using a microplate reader. The HEK-Blue cell lines were incubated with HEK-Blue detection medium for sixteen hours before measuring the O.D., according to the manufacturer's instructions.

Microbial Quantification by qPCR

Bacterial DNA was extracted from fecal samples using the DNeasy Powerlyzer PowerSoil kit (Qiagen), according to the manufacturer's instructions. qPCR analysis was performed in duplicate wells by Taqman amplification reactions consisting of genomic DNA, Taqman® Universal PCR Master Mix (Applied Biosystems) and the following primer pair: Forward primer: TCCTACGGGAGGCAGCAGT, Reverse primer: GGACTACCAGGGTATCTAATCCTGTT, Probe (FAM): CGTATTACCGCGGCTGCTGGCAC (Nadkarni et al., 2002). Relative expression of the 16S gene was calculated based on the expression of the gene before treatment in each mouse. Relative expression was normalized to mg of fecal sample.

16s rRNA sequencing and taxonomic microbiota analysis

Fecal DNA was isolated as described above. Amplicons spanning variable region 4 (V4) of the 16S rRNA gene were generated with barcode containing primers and sequenced on Illumina MiSeq. Data processing was performed using an established protocol (Caporaso et al., 2010) using the Qiime software (version 2018.11). Shannon's index and observed OTUs were used to measure alpha diversity whereas beta diversity was expressed as weighted and unweighted UniFrac distance. A Principal coordinates analysis (PCoA) plot was generated to visualize the similarity of the community members. The LefSe algorithm (Segata et al., 2011) was used to identify differentially abundant features that are statistically different among different groups.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical data analysis was performed with Prism software version 8.0 (GraphPad Software). For comparisons of two groups, two-tailed Student's t-test was used. Comparisons of multiple groups were made using one-way ANOVA, as described in the figure legends. Data represent mean ± standard error of the mean (SEM) or mean ± standard deviation (s.dev.), as indicated in the figure legend. $P < 0.05$ was considered significant.

SUPPLEMENTAL REFERENCES

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