**SUPPLEMENT TO**

Metformin modulates microbiota and improves blood pressure and cardiac remodeling in a rat model of hypertension

Wimmer et al.

**Supplementary methods.**

**Metabolic Cages**

Rats from the immune cohort were placed in metabolic cages during the first and the second week of the experimental protocol in metabolic cages (Tecniplast) for 8 hours with access to drinking water. The collected urine was immediately frozen at -80°C.

**Microbiome analysis**

Feces were collected from the animals during the trial (during biweekly weighting) and after sacrifice. Feces was immediately fresh-frozen on dry ice and kept at -80°C. According to the manufacturer's protocol, DNA was isolated from intestinal contents with the Quick-DNA Miniprep Kit (Zymo Research). The samples were stored at -80°C before being shipped on dry ice to Novogene (Cambridge, UK) for sequencing. Novogene performed quality control by assessment of DNA concentration by Qubit (Thermo Fisher Scientific, Waltham, WA), and integrity and purity by 5400 Fragment Analyzer (Agilent, Santa Clara, CA, United States). Subsequently, metagenomic library preparation and sequencing (Illumina NovaSeq 6000 PE150, at minimum of 6G raw data per sample) was performed. Whole-genome shotgun sequencing data were processed using ngless (v1.3.0). Metagenomic reads were quality trimmed (phred score<25), and reads shorter than 45 bp were discarded. Filtered reads were mapped against the rat genome (mRATBN7.2; low complexity regions and regions mapping to the progenomes gene catalog were masked using bbtools). Taxonomic classification was performed using mOTUs module (v2.6.0) within ngless with default parameters. For functional profiling, trimmed and filtered reads were mapped against the genes from Global Microbial Gene Catalog (gmgc v1.0) and summarized additionally on KEGG orthologs (KOs) via eggNOG annotations. The SCFA-pathways (acetate, propionate) were binned manually from KEGG KOs as previously described 1,2.

**Echocardiography**

Echocardiography was performed twice, as per previously published protocols 3. Once during the first week of treatment and the day before or on the day of the sacrifice.

**Heart weight normalization**

Heart weights were normalized by dividing the heart weight through 27.73 plus the cubed mean tibia length (27.73 + ([left tibia + right tibia]/2)3) as per previously published reports 4.

**Immune phenotyping**

Spleens were harvested into PEB buffer (PBS + 0.5% BSA + 2mmol/l EDTA). Spleens were mashed through 70µm strainers (Greiner Bio-one). After hypotonic erythrocyte lysis, splenocytes were filtered through 40µm strainers (Greiner Bio-one). Splenocytes were frozen in RPMI-1640 (Sigma) supplemented with 40% FBS (Sigma) and 10% DMSO (Sigma) using Mr. Frosties or Corning Cool Cell (Corning), according to manufacturer’s protocol.

Kidney and cardiac tissues were minced using sterile scissors and transferred to C-tubes (Miltenyi); tissue dissociation as performed using an Octo MACS Dissociater with Heater (Miltenyi). Kidney tissue was dissociated in 5ml HBSS with Calcium and Magnesium (Thermo Fisher) supplemented with 10mmol/l HEPES (Pan Biotech), 5% FBS, 250U/ml collagenase IV (Sigma) and 200U/ml DNase (Sigma). After mechanical dissociation using the m\_lung\_01 program, 20min incubation at 37°C under constant rotation and second mechanic dissociation using the m\_spleen\_04 program, the enzymatic dissociation was immediately stopped using 5ml ice cold PBS with 10% FBS. Cardiac tissue was dissociated in 5ml HBSS with Calcium and Magnesium (Thermo Fisher) supplemented with 10mmol/l HEPES (Pan Biotech), 5% FBS, 600U/ml collagenase IV (Sigma) and 600U/ml DNase (Sigma). After mechanical dissociation using the m\_heart\_01 program, 30min incubation at 37°C under constant rotation and second mechanic dissociation using the m\_heart\_02 program, the enzymatic dissociation was immediately stopped using 5ml ice cold PBS with 10% FBS.

Single cell solution from the kidney and heart were filtered through 100µm cell strainer (Greiner Bio-one). In this step a sample was taken to normalize leucocytes per percent of total live cells. For this, cells were stained with CD45 PE-Vio770 and DRAQ7 and analysed using volumetric measurement on a BD LSR Fortessa with HTS (BD Bioscience). Cells were afterwards cleaned using a 40/80 percoll (GE healthcare). Live cells were harvested from the percoll and frozen as described for the spleen.

For analysis, cells were thawed and washed with RPMI 1640 medium (Sigma) with 10% FBS (Sigma). For all samples cells were stained using an overview panel (panel 1) and a T-cell focused panel (panel 2). Furthermore, cells from kidney and spleen were restimulated *in vitro* using with 50ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 750ng/ml Ionomycin (Sigma Aldrich), and 0.75µl/ml GolgiStop (BD Bioscience) for 4 hrs at 37°C and 5% CO2 in RPMI 1640 medium l(Sigma) with 10% FBS (Sigma), 100U/ml penicillin (Sigma Aldrich) and 100U/ml streptomycin (Sigma Aldrich) and stained for cytokine production (panel 3). Dead cells were stained with LIVE/DEAD fixable viability dye for 405nm (Thermo Fisher) for 30min. Afterwards surface antibodies were stained for 30min. Cells were fixed using the FoxP3 Staining Buffer Kit (eBioscience), subsequently antibodies against intracellular antigens were stained for 30min. Data was recorded on a BD LSR Fortessa with HTS (BD Bioscience)and analyzed in Flow Jo (BD Bioscience).

All antibodies used in this study and their respective dilutions and panel assignments are shown in Supplementary Table 1. Gating strategies for the different panels are displayed in Supplementary Figure S8.

**Urine and plasma analysis**

Drawn blood was anticoagulated using Na-EDTA (Sigma). Blood samples were centrifuged at 1500xg for 15min and plasma was collected and immediately frozen and stored at -80°C. Clinical chemistry was performed in urine and plasma samples on an AU480 clinical chemisty analyzer (Beckman Coulter). Plasma BNP levels were measured using Rat BNP 45 ELISA Kit (Abcam). LPS quantification in plasma was performed using the PYROGENT-5000 Assay (Lonza) according to manufacturer protocol. According to manufacturer’s recommendation MgCl2 solution (Lonza) was used to inactivate the EDTA used for plasma collection.

**Histology**

After 24hrs of fixation in 4% PFA cardiac tissue was transferred to 15% sucrose in ddH2O. After 12hrs, samples were transferred into 30% sucrose. Samples were placed in TissueTek and immediately frozen in -40°C Isopentane and subsequently transferred to -80°C for long-term storage. Tissues were cut into 8µm sections.

For WGA staining, slides were rehydrated and permeabilized in PBST (PBS + 0.01% Tween). After blocking in PBST supplemented with 10% normal donkey serum for 2hrs, slides were stained using 1:100 diluted succinylated Wheat Germ Agglutinin (WGA), Fluorescein (Vektor Laboratories) and 1:1000 DAPI for 24hrs. Slides were scanned on a Panoramic MIDI II slide scanner (3D Histech) with the 20x objective. Snapshots in 40x magnification of areas with mainly cross-cut cardiomyocytes in the left ventricle in were taken using CaseViewer (v2.2.1, 3D Histech). After greyscaling and inverting pictures in Fiji (ImageJ2 (v2.9.0/1.53t)), segmentation of cardiomyocytes was performed in Ilastik (v1.4orc6-OSX). Using CellProfiler (v4.2.4), size (MeasureObjectSizeShape, AreaShape), intensity (MeasureObjectIntensity, MeanIntensity) and diameter (MeasureObjectSizeShape, MiniFeretDiameter) of the segmented cardiomyocytes were measured. Based on these measurements, objects were filtered for true cross-cut cardiomyocytes (e.g. objects too small or elongated objects were filtered). Finally, the surface area of each object was measured. For interstitial fibrosis quantification the WGA positive area was quantified and normalized to the number of cardiomyocytes.

For sirius red staining, slides were rehydrated in running tap water. Afterwards, slides were post-fixed with Bouin solution (Dr. K. Hollborn & Söhne GmbH & Co KG) for 24hrs and stained with Sirius red staining solution (Abcam) for 1.2h and dipped into 0.5% acetic acid (3x), 95% ethanol (2x), and for 30sec in RotiClear, then immediately mounted with RotiMount (Carl Roth). Slides were scanned on a Panoramic MIDI II slide scanner with the 20x objective. Representative screenshots of five segments of the heart (apex, 2x left ventricular septum and 2x left ventricular anterior wall) and two vessels in the left ventricle were taken at 20x magnification. Screenshots were analyzed using Fiji (ImageJ2 2.14.0). For perivascular fibrosis quantification we defined three regions of interest (ROI) using the polygon selection tool: (1) the outer limit of the perivascular fibrosis, (2) the outer limit of the vessel wall and (3) the outer limit of the vessel lumen. Theses ROI were measured using the measuring tool and exported. For statistical analysis the fibrotic area (1) was normalized to the lumen area (3). For interstitial fibrosis quantification , the staining was segmented in Fiji in the green color channel, representing the Sirius red staining the closest. The segmentation was performed using thresholds for the fibrotic area (a) and the freezing artefacts (b) and the percentages of the areas above the threshold were measured in binary images. The fibrosis area was calculated by subtracting the sum of the fibrotic area (a) and the artefact area (b) from 100. For statistical analysis the interstitial fibrotic area was normalized to the cardiomyocyte area.

**RNA analysis**

RNA was isolated using the RNeasy Mini Kit (Qiazol) with minor modifications previously published 3. RNA was transcripted into cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCRs were performed on a Quant Studio 3 (Thermo Fisher Scientific) using SYBR Green PCR Master Mix (Thermo Fisher Scientific) or TaqMan™ Fast Advanced Master Mix for qPCR (Thermo Fisher). All primers and probes used in this study were synthesized by BioTeZ and are shown in Supplementary Table 2. Analyses were performed using a relative standard curve method. Gene expression levels of target genes were normalized by the expression of *18S.* Arbitrary units roughly reflect the ratio of concentrations of target gene mRNA to housekeeping RNA.

**Radiotelemetric blood pressure measurement**

In a subset of animals (telemetry cohort) radiotelemetric blood pressure transducers (TA11PA-C20, Data Sciences International) were implanted according to previously published protocols 3.

**µCT analysis**

In a subset of animals (µCT cohort), microvasculature in the kidney, eye and tongue were investigated by µCT. After perfusion with Vascupaint Silicone Rubber Injection Compounds (MediLumine) and incubation at 4°C for 24hrs, above named organs were removed and fixed in 4% PFA. Rat organs were imaged with SkyScan 1276 (Bruker, Belgium) µCT scanner. The vendor’s software (v.1.4.0.0) was used for image acquisition. Image reconstruction was performed with NRecon (v.1.7.4.6, Bruker, Belgium). Image analysis was done with CTAn (v.1.20.8.0, Bruker, Belgium). A sequence of analysis steps was applied, including filtering, thresholding, morphological operations, and others. This led to image segmentation and subsequent calculation of organ volume, volume of the vascular tree and the distribution of the vessel diameters.

**Determination of metformin plasma levels by LC-MS/MS**

Frozen plasma samples were thawed at room temperature and subsequently 20µl of each was mixed with 380µl of the internal standard d9-trimethylamine N-oxide (d9-TMAO, 52.6nmol/l in acetonitrile) (CDN Isotopes, Pointe-Claire, Canada). Proteins were precipitated by vortexing for 1min, ultrasonication for 5min and subsequent storage at -20°C for 60min. Supernatants were obtained by centrifugation at 16,000 g at 4°C for 10min and subjected to LC-MS/MS metformin quantification applying the multiple reaction monitoring (MRM) approach. Chromatographic separation was achieved on a 1290 Infinity II HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Poroshell 120 EC-C18 column (3.0x150mm, 2.7μm; Agilent Technologies) guarded by a pre-column (3.0x5mm, 2.7μm) of identical material. Water (eluent A) and acetonitrile (eluent B), both acidified with 0.1% formic acid, were pumped with 0.25ml/min, the injection volume was 1 µl. Elution of metformin and the internal standard was achieved with a 5-min linear gradient from 0% to 10% eluent B. Total run-time was 12min including re-equilibration of the LC system. MS/MS analyses were carried out using an Ultivo (6465B) triple-quadrupole mass spectrometer (Agilent Technologies) operating in the positive electrospray ionization mode (ESI+). The following ion source parameters were set: sheath gas temperature, 400°C; sheath gas flow, 12l/min of nitrogen; nebulizer pressure, 30 psi; drying gas temperature, 100°C; drying gas flow, 10l/min of nitrogen; capillary voltage, 2.5kV; nozzle voltage, 0kV. The following mass transitions were recorded (fragmentor voltage [FV] and collision energies [CE] in parentheses): metformin: *m/z* 130.1 → 43.0 (FV: 102V, CE: 40eV), *m/z* 130.1 → 60.0 (FV: 102V, CE: 12eV, quantifier), *m/z* 130.1 → 71.1 (FV: 102V, CE: 24eV); d9-TMAO: *m/z* 85.1 → 46.1 (FV: 101V, CE: 48eV), *m/z* 85.1 → 66.1 (FV: 101V, CE: 20eV, quantifier), *m/z* 85.1 → 68.0 (FV: 101V, CE: 12eV). Peak areas of metformin, as determined with MassHunter Software (Agilent Technologies), were normalized to those of the internal standard d9-TMAO followed by external calibration in the range of 0.001 to 5µmol/l metformin.

**SCFA quantification by NMR**

From each specimen a total of 30mg of feces were homogenized in 37.5μl of 70% 2-propanol by vortexing for 1min. The resulting homogenate was then diluted with an additional 37.5μl of 70% 2-propanol and subjected to homogenization once more. Subsequently, nicotinic acid was added as an extraction standard giving in the final NMR samples a concentration of 1mmol/l. Fecal specimens were dried and resuspended in 600μl of double distilled water. Resuspension was achieved through alternate vortexing and sonication. The samples then underwent three rounds of centrifugation at 12,000g at 4°C for 10min each, effectively removing particulate matter followed each time by subsequent transfer of supernatant to a fresh vial. Of the final fecal supernatant 400μl were thoroughly mixed with 200μl of 0.1 mol/l phosphate buffer (pH 7.4) and 50μl of a 0.75(wt) solution of 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP; Sigma-Aldrich, Taufkirchen, Germany) in deuterium oxide, serving as an internal standard for NMR measurements. All NMR experiments were performed on a 600 MHz Bruker Avance IIIHD spectrometer, using a triple resonance (1H, 13C, 15N, 2H lock) helium cooled cryoprobe with z-gradient. Handling of samples was done by an automatic Bruker SampleJet sample changer (Bruker Biospin GmbH, Ettlingen, Germany). Before measurement, each sample was allowed to equilibrate for 300 seconds in the magnet, and the probe was automatically locked, tuned, matched, and shimmed. One-dimensional 1H NMR spectra were obtained at 298K using a nuclear Overhauser enhancement spectroscopy pulse sequence with solvent signal suppression by presaturation during relaxation and mixing time. For each spectrum, 640 scans were collected into 65,536 data points over a 20-ppm spectral width using a relaxation delay of 4seconds, an acquisition time of 2.66seconds, and a mixing time of 0.01seconds. Spectra were automatically Fourier transformed, phase and baseline corrected. In each spectrum short-chain fatty acids were semi-automatically identified and relative to the TSP reference signal quantified using the CHENOMX 9.02 (Chenomx Inc, Edmonton, Canada) software suite.

**Macrophage differentiation**

Macrophage differentiation was performed as previously published5. During the 24hrs incubation with LPS for M1-like and IL-4 + IL-13 for M2-like differentiation, 1mmol/l sodium sodium acetate (NaC2), sodium propionate (NaC3), sodium butyrate (NaC4) or sodium chloride (NaCl) were added. For M1(LPS), all experiments were additionally performed with 0.1mmol/l. After 24hrs medium was taken off and cells were immediately frozen in Qiazol at -80°C for further mRNA isolation.

**Supplementary Table 1.**

Antibodies used for flow cytometry. Different panels are indicated as 1 (overview), 2 (T cell focused), 3 (restimulated cells).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Antigen** | **Fluorophore** | **Clone** | **Dilution** | **Manufacturer** | **Panel** |
| CD3 | VioBlue | REA223 | 1:50 | Miltenyi | 1, 2, 3 |
| CD4 | PE-Cy5 | OX-35 | 1:400 | BD | 1, 2, 3 |
| CD8a | BV786 | OX-8 | 1:100 | BD | 3 |
| CD8a | PerCP-Vio700 | REA437 | 1:20 | Miltenyi | 2 |
| CD25 | BV650 | OX-39 | 1:100 | BD | 2 |
| CD43 | APC-Vio770 | REA503 | 1:200 | Miltenyi | 1 |
| CD44 | BV711 | OX-49 | 1:400 | BD | 2 |
| CD45 | PE-Dazzle594 | OX-1 | 1:200 | Biolegend | 1, 2, 3 |
| CD45R (B220) | BV605 | HIS24 | 1:100 | BD | 1, 3 |
| CD62L | PE | HRL1 | 1:200 | BD | 2 |
| CD68 | PE-Vio770 | REA237 | 1:10 | Miltenyi | 1 |
| CD86 | PE | 24F | 1:400 | Biolegend | 1 |
| CD11b/c | BV711 | OX-42 | 1:400 | BD | 1, 3 |
| CD103 | BV786 | OX-62 | 1:100 | BD | 2 |
| CD127 | Alexa Fluor 700 | 717519 | 1:100 | R&D Systems | 2 |
| CD134 | PE-Vio770 | REA540 | 1:10 | Miltenyi | 2 |
| CD161 | R718 | 10/78 | 1:400 | BD | 1 |
| CD163 | Alexa Fluor 647 | ED2 | 1:200 | BioRad | 1 |
| FoxP3 | Alexa Fluor 700 | FJK-16s | 1:100 | Thermo Fisher | 3 |
| FoxP3 | APC | FJK-16s | 1:200 | Thermo Fisher | 2 |
| HIS48 | FITC | His48 | 1:200 | BD | 1 |
| IFNγ | eFluor 660 | DB-1 | 1:100 | Thermo Fisher | 3 |
| IL 17A | PE-Cy7 | eBio17B7 | 1:100 | Thermo Fisher | 3 |
| IL-10 | PE | A5-4 | 1:100 | BD | 3 |
| IL-22 | PerCP-eFluor710 | IL22JOP | 1:100 | Thermo Fisher | 3 |
| RORγt | FITC | NR1F3 | 1:500 | Novus Biologicals | 2 |
| RP1 | BB700 | RP-1 | 1:50 | BD | 1 |
| RT1b (MHCII) | BV786 | OX-6 | 1:200 | BD | 1 |
| Tbet | BV605 | 4B10 | 1:50 | BioLegend | 2 |
| TCRγδ | APC-Vio770 | REA547 | 1:50 | Miltenyi | 2, 3 |
| TNFα | FITC | TN3-19.12 | 1:100 | Thermo Fisher | 3 |

Supplementary Table 2. Primer sequences used for RT-PCR.

|  |  |  |  |
| --- | --- | --- | --- |
| **Species** | **Gene** | **Primer** | **Sequences (5’→3’)** |
| Universal | *18S* | For | ACATCCAAGGAAGGCAGCAG |
| Rev | TTTTCGTCACTACCTCCCCG |
| Probe | CGCGCAAATTACCCACTCCCGAC |
| Rat | *Mhy7* | For | GCCAAGACAGTTCGGAATGATAA |
| Rev | CCTGTTGCCCCAAAATGG |
| Rat | *Lcn2* | For | CAGGGCAGGTGGTTCGTT |
| Rev | AGCGGCTTTGTCTTTCTTTCTG |
| Probe | TCGGCCTGGCAGCGAATGC |
| Rat | *Col1* | For | AGAGCGGAGAGTACTGGA |
| Rev | CTGACCTGTCTCCATGTTGCA |
| Probe | CAAGGCTGCAACCTGGATGCCATC |
| Rat | *Tgfb1* | For | GCCTGAGTGGCTTTTTGA |
| Rev | CCTGTATTCCGTCTCCTTGGTT |
| Probe | CACTGGAGTTGTCCGGCAGTGGC |
| Rat | *Col4* | For | CCAAGAGGTTATTCGGGAGA |
| Rev | GACAAGACAAAGAGGCGACA |
| Probe | TCCTGCAGGACCTGGTCTCCC |
| Rat | *Fn1* | For | GGACCTGCACGCCAATAGCT |
| Rev | TCCCCCACGACGTAGGAA |
| Probe | AGAAATGTTTTGATCACGCTGCTGGGA |
| Rat | *Ccn2* | For | CGCCAACCGCAAGATTG |
| Rev | CACGGACCCACCGAAGAC |
| Probe | CACTGCCAAAGATGGTGCACCCTG |
| Mouse | *Arg1* | For | CCACAGTCTGGCAGTTGGAA |
| Rev | GCATCCACCCAAATGACACA |
| Probe | TGGCCACGCCAGGGTCCAC |
| Mouse | *Cox2* | For | CAGGTCATTGGTGGAGAGGTGTA |
| Rev | GGATGTGAGGAGGGTAGATCATCT |
| Probe | CCCCCCACAGTCAAAGACACTCAGGT |
| Mouse | *Nos2* | For | GGGCAGCCTGTGAGACCTT |
| Rev | TGCATTGGAAGTGAAGCGTTT |
| Probe | TCCGAAGCAAACATCACATTCAGATCCC |
| Mouse | *Mgl2* | For | GAGACAGACTTGAAGGCCTTGAC |
| Rev | GCCACTTCCGAGCCATTG |
| Mouse | *Chil3* | For | TCCTACTGGAAGGACCATGGAGCA |
| Rev | TCCTGGTGGGCCAGTACTAATTGT |
| Mouse | *Retnla1* | For | CGTGGAGAATAAGGTCAAGGAACT |
| Rev | CACTAGTGCAAGAGAGAGTCTTCGTT |
| Probe | TTGCCAATCCAGCTAACTATCCCTCCACTG |
| Mouse | *Il1b* | For | CGTGGACCTTCCAGGATGAG |
| Rev | GAGGATGGGCTCTTCTTCAAAG |
| Mouse | *Il6* | For | GTTGCCTTCTTGGGACTGATG |
| Rev | GGGAGTGGTATCCTCTGTGAAGTCT |
| Probe | TGGTGACAACCACGGCCTTCCC |
| Mouse | *Egr2* | For | CTACCCGGTGGAAGACCTC |
| Rev | AATGTTGATCATGCCATCTCC |
| Mouse | *Irf4* | For | CGGGCAAGCAGGACTACAA |
| Rev | TCGGAACTTGCCTTTAAACAATG |
| Mouse | *Nlrp3* | For | GGAGTCTAGCAGACCTGATTGTCA |
| Rev | GGCTTGCGCAGGATCTTG |
| Probe | CTGCTGGCCTGACCCAAACCCA |
| Mouse | *Mrcl* | For | AATACCTTGAACCCATTTATCATTCC |
| Rev | GCATAGGGCCACCACTGATT |
| Probe | CGATGTGCCTACCGGCTGCCC |
| Mouse | *Tnf* | For | GGTCCCCAAAGGGATGAGAA |
| Rev | TGAGGGTCTGGGCCATAGAA |
| Probe | TTCCCAAATGGCCTCCCTCTCATCA |
| Mouse | *Ccl5* | For | GCAGTCGTGTTTGTCACTCGAA |
| Rev | GATGTATTCTTGAACCCACTTCTTCTC |
| Probe | AACCGCCAAGTGTGTGCCAACCC |

**Supplementary Figures.**

**Supplementary Figure 1**

**Ein Bild, das Diagramm, Design enthält.

Automatisch generierte BeschreibungFigure S1.** **A)** Body weight analysis over time. **B)** Body weight at the endpoint (week 7) normalized to tibia length3. **C)** Plasma metformin levels were analyzed by LC-MS/MS. **D)** Plasma glucose and **E)** lactate were analyzed.

**Supplementary Figure 2**

**Ein Bild, das Diagramm, Plan enthält.

Automatisch generierte Beschreibung**

**Figure S2.** **A)** Pielou’s evenness was analyzed in longitudinal shotgun metagenomic sequencing data. **B)** Fecal butyrate and **C)** 3-hydroxyisobutyrate were analyzed by NMR. **D)** Acetate and **E)** propionate split into the three cohort. **F)** Cuneiform plot of SCFA-associated gene pathways in week 5, 6, and 7 (endpoint). **G)** Fold change increase (baseline vs endpoint) of the phylum Proteobacter. **H)** Lipopolysaccharide (LPS)-associated gene pathways and **I)** LPS plasma levels at the endpoint.

**Supplementary Figure 3**

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Automatisch generierte Beschreibung

**Figure S3.** **A)** Heart rate analysis over time. **B-D)** Heart rate analysis of weekly mean heart rate measured by normalized area under the curve (AUC). **E-F)** Correlations of mean systolic blood pressure over the complete experimental time (analyzed as normed AUC) with fecal levels of acetate **(E)** and propionate **(F)**. Spearman coefficients (*R*) and p values are shown, lines represent linear regression with 95% confidence interval in grey.

**Supplementary Figure 4**

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Automatisch generierte Beschreibung

**Figure S4.** **A)** Correlation of plasma BNP and heart weight (normalized to tibia length3). Spearman coefficients (*R*) and p values are shown, lines represent linear regression with 95% confidence interval in grey. Sirius red staining was used to quantify **B)** interstitial and **C)** perivascular fibrosis. **D-H)** Expression of fibrosis genes *Col1*, *Col4*, *Fn1, Ccn2* and *Tgfb1* in the heart. **I)** Five echocardiographic features most distinguishing the group separation in PC2. **J-M)** Global longitudinal, circumferential, radial strain, and cardiac output (CO) at the endpoint. **H)** Correlation of end-diastolic volume (EDV) with **N)** fecal acetate and **O)** propionate levels.SD shown for reference.

**Supplementary Figure 5**

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Automatisch generierte Beschreibung

**Figure S5.** **A)** Analysis of the body weight development in the week following metformin treatment start, for all three cohorts. **B)** Urine volumes in ml per 8 hours for the cohorts which underwent metabolic cages. **C)** Drinking volume in ml normalized to bodyweight (bw) for the first four treatment days. Measurements were taken every 3-4 days to adjust metformin concentration in the drinking water.Comparison of echocardiography results from the different cohorts of animals in D) LV mass, E) Ejection fraction and F) End diastolic volume.

**Supplementary Figure 6**

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Automatisch generierte Beschreibung

**Figure S6.** **A)** Analysis of blood urea nitrogen and **B)** plasma creatinine at the endpoint. **C)** Longitudinal analysis of albuminuria quantified as albumin-to-creatine ration after half a week of treatment (week 4.5), a week later (week 6.5) and at the endpoint. **D)** Albuminuria quantified at the endpoint with SD as reference. **E)** Inflammatory response quantified as CD45+ cells as percent of live cells in the flow cytometry. Gene expression for **F)** Lcn2, **G)** Col1, **H)** Tgfb in kidney tissue. Microvascular structure was quantified by µCT. Histograms indicate the distribution of vessels with different diameters in the **I)** kidney, **J)** tongue and **K)** eye.

**Supplementary Figure 7**

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Automatisch generierte Beschreibung

**Figure S7.** **A)** PCA of immune cell data from the spleen, kidney, and heart. **B)** Heatmap of all immune parameters analysis from splenic immune cells. dTGR were clustered by k-means clustering, SD serve as reference. **C)** same as B) for the kidney.

**Supplementary Figure 8**

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Automatisch generierte Beschreibung**

**Figure S8.** Gating strategies for the different flow cytometry panels **A)** Common gating strategy. **B)** Non-T cell panel gating strategy. **C)** T cell panel and **D)** T cell restimulation panel gating strategies.

**Supplementary Figure 9**

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Automatisch generierte Beschreibung

**Figure S9.** Cardiac T cell phenotype. Abbreviviation and relevant markers per subtype are indicated in brackets. **A)** T cells (T, CD45+ CD3+) **B)** T helper cells (Th, gdTCR- CD8- CD4+ Tcells) **C)** cytotoxic T cells (Tc, gdTCR- CD4- CD8+ Tcells) and **D)** gdT cells (gdT, gdTCR+ Tcells). **E)** regulatory T cells (Treg, FoxP3+ T helper cells) and **F)** conventional T cells (Tconv, FoxP3- T helper cells). **G)** RORgt+ cytotoxic T cells (RORgt+ Tbet- Tc) and **H)** RORgt+ gd T cells (RORgt+ Tbet- gdT). **I)** Tbet+ regulatory T cells (Tbet+ RORgt- Treg), **J)** Tbet+ conventional T cells (Tbet+ RORgt- Tconv), **K)** Tbet+ cytotoxic T cells (Tbet+ RORgt- Tc) and **L)** Tbet+ gd T cells (Tbet+ RORgt- gdT).

**Supplementary Figure 10**

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**Figure S10.** Effect of 100µM dose of sodium acetate (NaC2), sodium propionate (NaC3), sodium butyrate (NaC4) or sodium chloride (NaCl) on mouse bone marrow derived macrophages (BMDM) which underwent differentiation towards an M1-like phenotype using LPS. **A)** Heatmap showing log2-transformed fold changes (log2FC) for typical marker genes for M1-like differentiation (cyclooxygenase-2 (*Cox2*), interleukin (IL)-6 (*Il6*), chemokine (C-C motif) ligand 5 (*Ccl5*), IL-1β (*Il1b*), inducible nitric oxide synthase (*Nos2*), NLRP3 (*Nlrp3*), tumor necrosis factor (*Tnf*)). **B) – D)** Boxplots showing gene abundance of *Nos2, Cox2, and Il1b* as M1 markers.

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