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Polystyrene micro- and nanoplastics in a colitis mouse model – effects on biodistribution, macrophage polarization, and gut microbiome

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Abstract

The increasing prevalence of inflammatory bowel disease (IBD) and rising pollution from micro- and nanoplastic (MNP) particles has prompted investigations on their potential interconnection. To elucidate the complex relationship between IBD and exposure to MNPs, we induced colitis in mice using dextran sodium sulfate (DSS) and orally administered a mixture of polystyrene (PS) MNPs (diameter 10, 1, and 0.29 µm). These particles enabled a detailed examination of MNP biodistribution, innate immune cell response and gut microbiome alterations under inflammatory conditions. Specifically, the nanosized PS particles predominantly accumulated in the bloodstream and excretory organs, with enhanced accumulation in the inflamed gut/colon. Proteomic analysis of the colon revealed alterations in molecular pathways related to protein transport, metabolism, and immune responses. Specifically, we found macrophage proteome signatures with pro-inflammatory polarization, highlighting the intricate effects of MNPs on inflammation and immune cell behavior. Moreover, MNPs significantly disrupted the gut microbiome, reducing microbial diversity and shifting bacterial populations towards pro-inflammatory and potentially pathogenic species. These changes suggest that MNP exposure could exacerbate colitis through complex interactions involving MNPs, immune responses, and microbial dynamics. The widespread presence of MNPs underscores the urgent need for comprehensive strategies to address MNP pollution, its implications for disease, and potential impacts on public health.

Introduction

The increasing presence of plastics in our environment, especially micro- and nanoplastics (MNPs), has emerged as a profound concern, impacting the environment and various species, including humans.[1,2] The gastrointestinal (GI) ingestion of plastics, whether directly (e.g. through drinking water) or through trophic level transfer (e.g. contaminated fish), has been shown to cause a variety of mechanical, chemical, and biological impacts in *in vitro* and *in vivo* models.[3] For years, it has been described that the accumulation of large plastic masses can lead to GI blockage and even death in

marine organisms, such as cetaceans, turtles, and birds.[4] An initial pilot study revealed the presence of plastic particles in human stool samples.[5] Since then, the spotlight has been on the GI tract and associated pathologies following exposure to MNPs. However, our current understanding of the toxic profile of MNPs remains limited due to the high complexity of these particles. Variations in chemical composition, additives, shape, size, and other parameters make comparisons between studies challenging.[6] However, it is evident that size matters, as the smaller fractions of plastic particles in the low micro- to nanometer range, pose a more subtle threat due to their potential to accumulate and translocate within the body.[7,8]

Polystyrene (PS) MNPs, a common environmental plastic particle pollutant, have been shown to cross biological barriers,[9,10] leading to immune and inflammatory responses in organisms. Several studies already described the interplay of acute colitis and MNPs.[10–14] The incidence of inflammatory bowel disease (IBD), including conditions like ulcerative colitis and Crohn's disease, is dramatically increasing.[15,16] These complex diseases are influenced by genetic, environmental, immunological, and microbial factors.[17] The dextran sulfate sodium (DSS) mouse model, which closely mimics the clinical and histological features of human IBD, is an invaluable tool for investigating this. It allows for controlled administration of PS MNPs, enabling a systematic exploration of their effects on the disease. Besides the investigation of biodistribution and inflammatory processes, one critical factor is influenced by chronic ingestion of MNPs over long periods: the gut microbiome. The gut microbiome, a vital component of an organism's health, is not immune to the influence of these pollutants. MNPs can alter the gut microbial community composition and function, potentially disrupting the delicate balance of host-microbiome interactions.[18–21] After zebrafish were exposed to polypropylene, a gut microbiome-associated increase in oxidative stress and inflammation was already described.[22] Recently, studies have drawn a connection between gut microbiome and liver toxicity caused by PS MNPs in healthy mice.[23–25] The oxidative stress induced by inflammation can favor the bloom of more resistant bacterial groups, such as the members of *Enterobacteriaceae*, altering the gut's microbial ecosystem. MNPs serve as vectors for microbial dispersal, potentially introducing pathogens into the gut. Forming biofilms on plastic particles can enhance microbial survival and facilitate horizontal gene transfer, increasing the risk of disease transmission. The 'plastisphere'—the microbial communities associated with plastic particles—has recently been recognized for its distinctive taxonomic and functional diversity.[26] However, the implications of the plastisphere as a microbial reservoir for gut colonization and health remain poorly understood.

The DSS-induced colitis model demonstrated that intestinal inflammation is accompanied by major changes in the composition and function of the intestinal microbiota, a key aspect often overlooked in chronic DSS colitis models.[27–29] In parallel, microplastics can alter the gut microbiome, potentially

exacerbating or ameliorating colitis symptoms.[30,31] The gut microbiome has a major and unequivocal dual role in biology. First, it is one of the largest and richest biomes. Second, its evolutionary integration into the host renders its host's physiology, development, resistance to pathogens, and immune response heavily dependent on the healthy state of the gut bacterial communities. Also, the complex relationships between the microbiota and its host suggest that different bacterial species may exert positive or negative effects on different hosts and their physical states. In general, any disturbance that upsets the balance of the gut bacteria causes dysbiosis – a condition commonly triggered by gut inflammation and/or infections.[32] The DSS model provides an opportunity to delve into these interactions and understand how changes in microbial diversity due to microplastics influence intestinal inflammation and immune response. Besides the gut microbiome, macrophage polarization is also crucial to IBD progression and in the IBD inflammatory response.[33] A range of studies have explored the influence of different materials on macrophage polarization. For instance, carboxyl- and amino-functionalized PS or polyurethane nanoparticles can skew macrophage polarization towards an anti-inflammatory phenotype, while biodegradable polyurethane nanoparticles can inhibit pro-inflammatory macrophage polarization.[34]

In this study, we investigated the effects of orally administered PS MNPs in a colitis mouse model, focusing on biodistribution, intestinal morphology, gut proteome, microbiome, and macrophage polarization. Our findings aim to clarify the potential health impacts of microplastic exposure and contribute to understanding its role in inflammatory diseases. Although MNPs are ubiquitous in the environment, their effects on conditions such as inflammatory bowel disease remain poorly defined. Using the DSS-induced colitis model, this study provides insights into how PS MNPs may influence gut health and disease progression.

Material and Methods:

Polystyrene particles and characterization

Commercially available labelled PS particles with a diameter of 10.39 ± 0.13 µm (spherical, aqueous suspension, 5% w/v, blue colored), 1.14 ± 0.03 µm (spherical, aqueous suspension 2.5% w/v, ex/em= 530/607 nm) and 0.293 ± 0.008 µm (spherical, aqueous suspension 2.5% w/v, ex/em= 502/518 nm) were obtained from Microparticles GmbH (Berlin, Germany). According to the manufacturer's information, the particles were delivered in a pure aqueous solution, without any detergents or additives. In order to characterize the particles beyond the protocol of analysis as provide by the manufacturer, PS particle characterization was performed by measuring ζ potential, size, and

polydispersity index (PDI) on a *Zetasizer Pro* device (Malvern Pananalytical, Malvern, United Kingdom).[35] Data analysis was performed by using ZS Xplorer software. ζ potential, size and PDI were measured in deionized (DI) water (conductivity of 0.055 µS/cm) and 1 mM KCl solution, (Supporting Information Table 1 and reference [8]). For clarity, we will use the term *MNP mix* to refer to the mixture of all three particle types in a mass concentration of 0.3 mg/size/dose (total 0.9 mg MNPs/dose) in mice. When referring to individual particle types, the mean size will be specified, for example, MP (1 µm) or NP (0.29 µm). Different particle sizes were chosen on the assumption of differential uptake and penetration abilities in biological systems. Polydisperse PS particles should acknowledge that MNP particles are not present as monodisperse particles in the real world situation.

Microscopy and spectroscopy

A correlative microscopy and spectroscopy were employed to characterize the PS particles. In order to determine the size and surface morphology of the particles, a Scanning Electron Microscopy (SEM) (Zeiss Crossbeam 550) equipped with a Secondary Electrons Secondary Ions (SESI) detector was used at a low voltage of 1 kV to avoid changes caused by electrons. A confocal micro-Raman spectrometer (HORIBA LabRAM HR Evo-Nano) was utilized for spontaneous Raman spectroscopy to confirm the chemical composition and identify the most prominent Raman peak (found at ~1001 cm^{-1}) of the PS particles. For Raman excitation and collection in backscattering geometry, a 785 nm laser with a power of 5.3 mW focused through a 100x objective was applied. Chemical imaging was done applying Stimulated Raman Scattering (SRS) (Leica Stellaris 8) at the selected Raman peak corresponding to a laser excitation of 934.5 nm with a 20x objective. SRS is a resonantly enhanced process giving a much more efficient signal of several orders of higher magnitude than that of a spontaneous Raman scattering.[36] Fluorescence images of particles were taken at a Zeiss LSM 700. Image post processing was performed using StrataQuest software, version 8.0.61 (TissueGnostics, Austria). including analysis parameters, like size, circularity, and fluorescence intensity allow the identification and semi-quantitative assessment of the green particles in tissues with green autofluorescence. For a detailed description see particle quantification in colon and blood.

Fluorescence images of tissue sections and blood smears were taken at a Zeiss Axio Imager M2 microscope (63x objective). Respective image processing was done using Zeiss Zen blue software (version 3.5). For electron microscopy, tissue was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 for 3 to 4 hours. After dehydration, the tissue was embedded in resin (AGAR-100,

Agar Scientific). Ultrathin sections (80 nm) were contrasted with uranyl acetate/lead citrate and studied with a Zeiss EM 900 electron microscope.

Mouse model

Male C57Bl/6J mice were bred in-house and kept under standard conditions (ambient temperature at 12 h light/dark cycle, food and water provided ad libitum). All experiments were conducted according to Austrian animal welfare legislation (license 2022- 0.257.045), and experimental setups were approved by the local animal ethics committee. MNPs were applied as a mixture containing all three sizes at equal weight concentrations, prepared in sterile water, further referred to as MNP mix. MNP mix was applied according to scheme as a 100 µl gavage with a concentration of 0.3 mg/size/dose (total 0.9 mg MNPs/dose), extrapolated from estimates of human upper limit of exposure.[37] (For more details on particle size, type, concentration etc. please see the section "study limitations" at the end of the results section) For proof of MNP uptake into the GI tract, 6 wild-type mice were used (n = 2/group, pilot experiment). Mice were either left untreated (Ctr) or received a single dose of 100 µl MNP mix and were sacrificed after 2 or 4 h. Small intestine (si) and colon (co) were collected and processed for histology and TEM as described in the respective sections. For the main experiment, at the age of weaning (~3 weeks) mice from different litters were randomized into 4 groups (n=8) to compensate for litter specific microbiome effects. Treatment was initiated at 6-8 weeks of age according to the treatment scheme. Group 1 (Ctr) received sterile water, group 2 (MNP) was treated with MNP mix, group 3 (DSS) was treated with DSS and group 4 (DSS+MNP) received both DSS and MNP mix treatment. In detail, mice received a daily gavage of 100 µl sterile drinking water or MNP mix (all three sizes mixed at equal weight concentrations of 0.3 mg/size/dose in sterile water) for 10 days (Figure 1). For the colitis model, groups 3 and 4 were additionally treated for the first 5 days (T0-T4) with 2.5% DSS in their drinking water (ad libitum),[38] parallel to the daily gavage. After these initial 5 days of DSS colitis induction, mice again received regular drinking water (T5-T9) until the end of the experiment. Mice were sacrificed at day 10 (T10 ~ 24 h after the last gavage) and blood (terminal heart puncture under anesthesia, EDTA) and organs were collected. Colon weight and length were recorded before histological processing. For microbiome analysis, stool samples were collected on day 0 prior to treatment start and on day 10 before mice were sacrificed.

Figure 1: Particle characterization and verification of uptake. (A) Spontaneous Raman spectrum of polystyrene (PS). The arrow points at the PS specific peak at ~1001 cm^{-1} that was used for later

sample imaging with stimulated Raman. (B) SRS Raman images of distinct particle sizes taken at the laser wavelength of 934.5 nm. Scale bar 5 µm. (C) SEM image of MNP particle mix. Scale bar 5 µm. (D) Fluorescence, brightfield and merged images of MNP mix. Fluorescence image depicts a maximal orthogonal projection from z-stacks of combined GFP and AF568 channel. Small 0.29 µm nanoplastic particles are shown in green, middle sized 1.14 µm particles are shown in red. Brightfield images (grayscale, no colour image possible) were taken at a single focus plane. Blue arrows point at 10.39 µm spheres, red arrows at 1.14 µm particles. Scale bar 5 µm (E) IF images of small intestine and colon 2h after a single dose MNP mix gavage. Arrows point at 0.29 µm (green) and 1.14 µm (red) PS particles. Scale bar 20 µm. (F) TEM images of MNP particles taken up into the small intestine 2 h after gavage. Images show an intestinal crypt at various magnifications. White rectangle represents zoomed in area. Arrows point at respective micro and nanoplastic particles (green 0.293 µm, red 1.14 µm). For E+F n=2/group.

Histology

The intestine were gently flushed with PBS and 4.5% PFA and fixed as swiss rolls.[39] Organs (heart, lung, liver, kidney, spleen, brain, testicles) were collected and parts were stored in histological cassettes as well. Samples were processed according to a modified isopropanol protocol [40] and embedded in paraffin. 3 µm sections were cut from tissue blocks and used for staining. Fluorescence staining for identification of PS particles were performed according to isopropanol protocol with DAPI nuclear counterstain (Merck Darmstadt, Germany). Blood smears from EDTA blood samples were performed according to standard protocol. Representative fluorescence images of tissue and blood were taken on a Zeiss Axio Imager M2 fluorescence microscope and images were processed with ZEN blue 3.5 software. Immunohistochemistry (IHC) and hematoxylin/eosin staining (H&E) were performed using standard protocols. The following antibodies were used for IHC: F4/80 (Cell Signaling, #70076, 1:120), iNOS (Cell Signaling, #68186, 1:1000) and Arg1 (Cell Signaling, #93668, 1:100). Tissue slides were scanned by a SCAN II digital slide scanner (3d Histech, Budapest, Hungary) using a 20X objective.

Colitis score from H&E scans was evaluated by an experienced pathologist in a blinded fashion as previously reported by Crnčec et al.[38] In brief, colons were scored according to inflammation (0: rare or no inflammatory cells in lamina propria, 1: increased numbers of granulocytes in lamina propria, 2: confluence of inflammatory cells extending to submucosa, 3: transmural extension of

inflammatory infiltrate), crypt damage (0: none, 1: loss of basal 1/3 of the crypt, 2: loss of basal 2/3 of the crypt, 3: entire crypt loss, 4: change in epithelial surface with erosion, 5: confluent erosion), ulceration (0: none, 1: 1–2 ulcers focally, 2: 3–4 ulcers focally, 3: confluent ulceration). Individual scores were added up and multiplied by the score for the affected area (1: 0–25% of the colon, 2: 25–50% of the colon, 3: 50–75% of the colon, 4: 75–100%). Digitized IHC tissue sections were subsequently analyzed using the Definiens® TissueStudio® histomorphometry software or QuPath software (version 0.4.3). Nuclei and cells were identified using the software's cell detection algorithms and categorized as negative or positive based on the staining intensity. The same thresholds were applied to all tissue sections, and the number of positive cells per area of analyzed tissue was determined and compared across groups. The pro/anti-inflammatory ratio was calculated by dividing the normalized number of iNOS+ cells per area by the respective number for Arg1+ cells. Tissue sections of 8 animals per group were stained (6 for DSS, due to dropouts during experiment). In case of bad IHC staining quality (sample wash-off or unequal staining due to modified isopropanol desiccation protocol), single tissue sections had to be excluded from analysis. At least 5 tissue sections per group were analyzed and quantified. Individual data points are shown in the respective graphs.

Particle quantification in colon and blood

Particles were quantified manually in tissue sections from IF-stained whole colon slices on a Zeiss Axio Imager M2 fluorescence microscope (n=8/group, except DSS n=6 due to dropouts during experiment). Only particles within colon tissue were counted and numbers were normalized to assessed colon area. For quantification from blood, 100 µl of EDTA blood (n=5/group) was digested with 10% KOH for 2 h at 56°C at 200 rpm, washed with dH_2O, centrifuged, and concentrated in a small amount of water. Drops were mounted on glass slides and scanned with a TissueFAXS iQ (TissueGnostics, Austria). The samples were analyzed using StrataQuest software, version 8.0.61 (TissueGnostics, Austria). The analysis workflow included initial detection of the particles (FITC channel, dot detection with compactness > 0.5), detection of autofluorescence areas or artifacts in order to eliminate false positive results, reconstruction of the whole bodies of the particles and computing measurements for them in order to separate them into 2 major classes: green particles (FITC+) and red particles (FITC+/TRITC+). Respective size intervals for the detection of 0.29 and 1.14 µm particles were chosen according to pre-measurements on equally processed particle control samples.

FFPE-sample preparation for LC-MS/MS analysis

FFPE-colon tissue blocks (5 mice for condition Control, MNP, DSS, and DS+MNP) were cut in the microtome, collected in 1.5 ml Eppendorf-tubes (6 x 10 µm), deparaffinized and homogenized as described previously.[41] In brief, dewaxing was performed three times by addition of 1 ml xylene, vigorous vortexing for 10 s, heating for 3 min at 50°C, centrifugation for 2 min at RT at 18000x g, incubation on ice for 5 min and removal of the paraffin by careful pipetting. Tissue pellets were then washed twice by the addition of 1 ml ethanol p.a., vortexing, and centrifugation for 2 min at RT and finally transferred to 0.5 ml Precellys tubes containing 1.4 mm zirconium oxide beads and homogenized with 3 cycles of 1 min at 6800 rpm. Following centrifugation, ethanol was air-dried and tissue was lysed by the addition of 4% SDS in PBS for 1 h at 96°C and de-crosslinked at 56°C for 4 h. DNA was sheared in a Bioruptor with 10 cycles for 30 s at maximum power. Insoluble material was pelleted by centrifugation and the protein content was estimated by spotting onto nitrocellulose. Roughly 50 µg were transferred to strip-PCR tubes, reduced (10 mM TCEP, tris(2-carboxyethyl)phosphine), Thermo Scientific) and carbamidomethylated (5 mM, CAA, 2-chlorooacetamide), Merck) by incubation for 10 min at 70°C. One third was transferred to strip-PCR tubes and processed for on-bead digestion using the SP3 method[42] with Lys-C and trypsin at 1:100 enzyme-to-substrate ratios in 50 mM ammonium bicarbonate overnight (12 h) at room temperature. Digests were acidified (1% formic acid, FA), desalted and concentrated with SDB-RPS stage-tips with 2 layers of styrene-divinylbenzene resin (AttractSPE Disk, Affinisep). Peptides were eluted from the SDB-RPS membrane (1% ammonia in 60% acetonitrile (ACN), 15 min at RT) into 0.5 ml tubes, dried in a speed vac, reconstituted in 15 µl resuspension buffer (2% ACN, 5% FA). Peptide concentration was estimated and adjusted based on the UV absorbance of aromatic amino acids at 280 nm with 200 ng HeLa digests as a standard. LC-MS/MS analysis was performed on an Easy nLC1000-QExactive, "Q1" as well as on an Orbitrap Eclipse Tribid Mass Spectrometer, "E1" coupled with an easy nLC 1200 (all Thermo Scientific, Germany). An in-house packed C18 analytical column (15 cm, 75 µm inside diameter, and 1.9-µm ReproSil-Pur C18 beads; Dr. Maisch, Germany) was used with an integrated column oven (50°C; PRSO-V1, Biberach, Germany). Peptides were analyzed using a 60 min gradient (on Q1, pools of condition groups and on E1, individual mice) at a constant flow rate of 250 nl/min using a binary solvent system (A: 0.1% FA in water and B: 80%, initial 3% solvent B, 3-5% B within 1.0 min, 5-30% solvent B within 40.0 min, 30-50% solvent B within 8.0 min, 50-95% solvent B within 1.0 min, followed by washing and column equilibration. Mass spectrometers were operated in a data-dependent acquisition (DIA) mode. For Q1, we used a staggered (overlapping) window pattern to

acquire 25x24 m/z (400-1000 m/z) precursor isolation window DIA spectra (17.500 resolution, AGC target 1e6, maximum injection time 60 ms, 27 NCE, fixed first mass 200 m/z). Full-MS precursor spectra (target range +/- 15 m/z, at resolution 35.000, AGC target 1e6, injection time 60 ms, scan range 385-1015 m/z) were interspersed every 25 MS/MS spectra. For E1, a FAIMS with intra-analysis CV stepping (CV-50 and -70) and vendor-provided settings were used. Raw files were demultiplexed using ProteoWizard[43] with "Apply peak picking," "Demultiplex overlapping spectra," and "Optimization" enabled, and "Intensity encoding precision" set to 64 bit and mzML-files analyzed with DIANN version 1.8.1[44] using the canonical Uniprot mouse fasta database (download 2018, 21757 entries) in library free (predicted) search mode. Missed cleavage and maximum variable modifications (methionine oxidation, N-terminal acetylation, and cysteine-carbamidomethylation) were set to 1, peptide length was set to 7-30 amino acids, precursor charge range 1-4, and m/z range form 300-1800 m/z. Protein inference was on proteins (from Fasta), neural network classifier set to "double-pass-mode," quantification strategy "robust LD (high acquisition)," and "use isotopologues" enabled. At the same time "MBR" was unenabled since we analyzed raw files from two different mass spectrometers. Max.LFQ abundance for protein groups was calculated from the "precursor.translated" column of the DIANN .tsv output with the DIANN R package for MS2-centric methods, filtered for global q-value (Lib.PG.Q.value <0.01 and "count.stripped.sequence" >1. MaxLFQ columns were subsequently analyzed with Perseus (v1.6.15.0)[45] and visualizations done in Instant clue (v0121)[46], GrapPad Prism (v 8.0) and Adobe-Photoshop (CS4).

Microbiome analysis

DNA from stool samples (n=5/group/timepoint) was extracted using the QIAamp Fast DNA stool mini kit automated on the QIAcube. Approximately 200 mg stool was transferred to 0.70 mm Garnet Bead tubes filled with 1 ml InhibitEx buffer. Subsequently, bead beating was performed using a SpeedMill PLUS for 45 s at 60 Hz. Samples were then heated to 95 °C for 5 min and centrifuged for 1 min at 10,000 rpm. 200 µl of the resulting supernatant were transferred to a 1.5 ml microcentrifuge tube placed in the QIAcube for follow-up automated DNA isolation according to the manufacturer's protocol. DNA was eluted from the QIAamp silica-gel membrane with 200 µl TE buffer.

Isolated DNA was used to amplify hypervariable regions V3 and V4 of the 16S rRNA gene using the primer pair 357F-806R in a dual-barcoding approach according to Caporaso et al.[47] 3 µl DNA was used for amplification, and PCR-products were verified *via* agarose gel electrophoresis. PCR products were normalized using the SequalPrep Normalization Plate Kit (Thermo Fischer Scientific, Waltham,

MA, USA), pooled equimolar, and sequenced on the Illumina MiSeq v3 2x300bp (Illumina Inc., San Diego, CA, USA). Demultiplexing after sequencing was based on zero mismatches in the barcode sequences. Initially, an average of 33293.5 ± 10552.0 raw reads were available per sample. After quality filtering, denoising, merging, and removal of chimera, 14272.0 ± 4889.9 reads per sample were used for analysis; the minimum read count and, therefore, rarefaction depth was 9710 reads. The resulting fastq files were used for bioinformatic preprocessing using QIIME2 tools, implemented on a local Galaxy instance (https://galaxy.medunigraz.at/). Amplicon sequencing variants (ASV) were identified using the dada2 inference algorithm; taxonomic assignment was done with a naïve Bayesian classifier trained on the SILVA database V132 with release at 99% identity. Cyanobacteria were regarded as possible contamination and, therefore, removed from the data set. For statistical analysis, count table, taxonomy, and sample data were handed off to phyloseq. Alpha diversity was estimated from an even count table, rarefied to the minimum read count per sample, using the metrics observed OTUs, Shannon index (implemented in the phyloseq::estimate_richness() function), and evenness (calculated as Shannon-index/log(observed OTUs). The influence of DSS-induced colitis and ingestion of MNP mix over time was tested for their statistical significance using a mixed effect model (using the R package lme4). For beta-diversity analysis, ASV was agglomerated at the genus level to avoid extremely sparse data sets using the phyloseq::tax_glom() function. Bray-Curtis dissimilarity was used as the foundation for the distance matrix, calculated with the phyloseq::ordinate() function, and permutational multivariate analysis of variance using distance matrices were used to determine whether there was significant clustering among the test groups, using the vegan::adonis2() function, Principal coordinate analysis (PCoA) was used for low-dimensional visualization (phyloseq::plot_ordination()). Redundancy analysis (RDA) was performed on a Hellinger-transformed abundance table to determine influencing factors on the microbiome composition using the vegan::rda() function. To test whether there were significant differences in the overall change of the microbiome, the pldist R package was used to determine the intra-individual change between timepoints. Bray-Curtis dissimilarities were calculated, and PCoA/adonis and distance-based redundancy analysis (vegan::dbrda()) were performed to identify significant influences on the microbiome changes during the experiment. To describe changes in the microbiome in more detail, multivariate association analysis (MaAsLin2) was applied on microbiome changes over time as obtained with pldist-tools.

In addition to the above-mentioned R packages, the following packages were used: readxl, tidyverse, ggpubr, lmerTest, writexl, ggplotify, ggConvexHull, ggrepel, ggh4x.

Isolation of monocytes from peripheral blood mononuclear cells (PBMCs) of healthy donors

PBMCs were obtained from whole-blood samples of three healthy volunteers using a standard protocol involving LymphoPrep (Stemcell Technologies) and density gradient centrifugation. Subsequently, monocytes were separated from PBMCs by adhering them to ultra-low attachment culture dishes (Corning) and then underwent a 6-day differentiation into macrophages in RPMI-1640 supplemented with 10% FCS (Thermo Fisher Scientific), 1% Penicillin-Streptomycin (Thermo Fisher Scientific), and 20 ng/ml human recombinant M-CSF (Peprotech) with two medium changes. On the sixth day, macrophages were exposed to 5 µg/ml MP (1.14 µm) or NP (0.29 µm) particles and subjected to further analysis after a 24-hour incubation period. All experiments involving healthy subjects were performed upon informed consent and in consensus with "Good Scientific Practice Guidelines" of the Medical University of Vienna, as well as the latest "Declaration of Helsinki". The study protocol was approved by the Institutional Review Board of the Medical University of Vienna (#1374/2014).

***In vitro* macrophage experiments - Confocal microscopy and flow cytometry**

For microscopy imaging macrophages were plated in µ-Slide 8 Wells (ibidi) and exposed to red-labeled MP (1.14 µm) or green-labeled NPs (0.29 µm) for 24 h. Following formalin fixation, slides were rinsed in 0.1% PBS-Tween 20 (PBS-T), permeabilized in 0.3% Triton X-100 in PBS-T and blocked with a blocking solution (10% goat serum in 0.1% Triton X-100 in PBS-T). For NP (0.29 µm) treated cells the primary antibody targeting CD14 (CatNo. # 14-0149-82, Thermo Fisher Scientific) was diluted 1:400 in the blocking solution and incubated overnight at 4°C. Afterward, slides were subjected to secondary antibody incubation (Alexa Fluor 594 goat anti-rabbit, CatNo. A-11012; Thermo Fisher Scientific), followed by counterstaining with DAPI (Serva Electrophoresis). Three representative images were captured using an LSM 5 Exciter (Zeiss) with a 63X oil immersion objective. Image processing was performed using ImageJ.

To quantify the uptake of MNPs, macrophages (from 3 different donors, n=3) were exposed to fluorescently labeled MP (1.14 µm) or NP (0.29 µm) particles at a concentration of 5 µg/ml for 24 h. Concurrently, surface markers indicating macrophage polarization were evaluated in technical triplicates of equivalent samples treated with non-labeled MPs (1.14 µm) or NPs (0.29 µm). Cells were blocked using 40% human AB serum for 5 minutes at room temperature. For phagocytosis quantification, cells previously incubated with fluorescent beads were stained with Zombie Violet™ (CatNo: 423114, Biolegend) as well as an APC-Cy7 conjugated anti-CD11b antibody (CatNo: 101226,

Biolegend). For marker expression, cells previously incubated with non-fluorescent beads were stained with Zombie Violet™, an Alexa Fluor™ 488 conjugated anti-CD206 antibody (CatNo: 53-2069-42, Thermo Fisher Scientific), a PerCP-eFluor™ 710 conjugated anti-CD163 antibody (CatNo: 46-1639-42, Thermo Fisher Scientific), a PE-Cy7 conjugated anti-4-1BBL antibody (CatNo: 311512, Biolegened), an APC conjugated anti-SIRPα antibody (CatNo: 372106, Biolegend), an Alexa Fluor™ 700 conjugated anti-CD45 antibody (CatNo: 368514, Biolegend), the APC-Cy7 conjugated anti-CD11b antibody, a Brilliant Violet 510™ conjugated anti-HLA-DR antibody (CatNo: 307646, Biolegend), a Brilliant Violet 650™ conjugated anti-CD86 antibody (CatNo: 305428, Biolegend) and a Brilliant Violet 785™ conjugated anti-PD-L1 antibody (CatNo: 329736, Biolegend). Flow cytometry was performed using the DxFlex flow cytometer (Beckman Coulter Inc.) and CytExpert software. Compensation was performed using single stainings of respective cell types treated with 5% Tween 20 for 5 min for viability stains, as well as single stainings of UltraComp eBeads compensation beads (CatNo: 01-2222-42, Thermo Fisher Scientific) with respective antibody stains. Gating, compensation matrix calculation, and data visualization were performed in Kaluza analysis (Becton Dickinson).

Statistical data analysis

Statistical analysis for histological samples was performed with GraphPad Prism 8.0. Individual groups were compared using one-way ANOVA (with Sidak's multiple comparisons test), and the effect of MNP treatment (+/-MNP) and colitis (+/-DSS) was assessed additionally using two-way ANOVA. Outliers were identified using the ROUT method (Q=1%) and excluded from analysis.

The principal component analysis (PCA) on sample variance for proteomic analysis was done after filtering for proteins detected in each sample (1242 proteins) using the interactive tool SRplot.[48] We then drew custom Venn diagrams from proteins identified in each treatment cohort after filtering for at least 3 valid values in each group to find proteins that only present a specific condition (http://bioinformatics.psb.ugent.be/webtools/Venn/). The Perseus-implemented annotation database was used to functionally annotate proteins with GOBP. For tissue and cell-type-based annotations, we utilized single-cell transcriptomics data from mouse intestinal epithelium[49], regulatory T-cell (Treg) proteomic signatures reported in an animal model of inflammatory bowel disease[50], an in-house proteomics dataset from M1 (IFNg+LPS) and M2 (IL4+IL13) polarized mouse BMDM and a meta-analysis comparing *in vitro* and *in vivo* macrophage polarization marker genes[51] was utilized. For analysis of tumor-infiltrating immune cell signatures, we utilized TIMER2.0 (http://timer.cistrome.org)[52] using the "Immune Estimation" explorative tool. Protein expression

(abundance) heatmaps were prepared by unsupervised hierarchical clustering of protein´s mean and z-normalized (row) abundance values with missing values replaced by 0. Linkage was based on average Euclidian distance, k-means set to 300 and 10 iterations. To find differentially expressed proteins (DEPs) between treatment groups, we considered only proteins with 3 valid values in each treatment group (Ctrl vs MNP (4817 proteins), Ctrl vs DSS (5179 proteins), Ctrl vs DSS+MNP (4343 proteins) and DSS vs DSS+MNP (4541 proteins) as visualized in Venn diagrams and performed Student´s T tests reporting conservative, (-) log10 transformed p-values and permutation-based multiple-testing corrected (FDR) q-values. DEPs were visualized in Volcano plots with p-value cutoffs ($p<0.01$ corresponding to (-) log10 >2). Heatmaps summarizing significant fold chances (FC (lg2), $p<0.05$) were prepared from the union of proteins in respective pairwise comparisons. Missing values (nan) caused by the absence of specific proteins in one sample group and thus no possible comparative abundance analysis was included and colored in grey. Venn diagrams of significantly ($p<0.05$) up- or down-regulated proteins were prepared as described above. To evaluate the impact on MNP on colitis specifically, we performed a 2-way ANOVA (group -/+ MNP and group -/+ colitis) and performed 1-way ANOVA with Post hoc Tukey´s HSD FDR correction on proteins with interaction p-value ($p<0.05$. 487 proteins). Pathway- and GO-enrichment analysis were performed with Webgestalt (www.webgestalt.org) based on ranks (T-statistics of DSS+MNP vs DSS).To evaluate the impact on MNP on colitis specifically, we performed a 2-way ANOVA (group -/+ MNP and group -/+ colitis) and performed 1-way ANOVA with Post hoc Tukey´s HSD FDR correction on proteins with interaction p-value ($p<0.05$. 487 proteins). For categoric analysis of M1 or M2 signatures, the raw dataset was annotated with Orecchioni´s Table S5, “in vivo”[51] and matching protein counts in relation to the total number of identified proteins in each sample (%) were visualized and analyzed in GraphPad Prism (v.8.0) using the one-way-ANOVA on ranks (Kruskal-Wallis-test) with “each comparison stands on its own” option enabled. Significance was considered at $p<0.05$. All source data are provided in supplementary data 1,2 and 3. All raw data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PDX059763.[53]

Results

MNP characterization and verification of gastrointestinal tissue uptake

For this study, three different sizes of PS particles were used which were supposed to have different interaction and uptake properties according to their size (10.39 ± 0.13 µm spherical, aqueous suspension, 5% w/v, blue colored; 1.14 ± 0.03 µm spherical, aqueous suspension 2.5% w/v, ex/em= 530/607 nm; 0.293 ± 0.008 µm spherical, aqueous suspension 2.5% w/v, ex/em= 502/518 nm) - 10

µm particles, which are most likely not taken up through the intestinal barrier, 1 µm particles, that can interact with tissues and cells and 0.29 µm particles that are more likely to be taken up by cells and distributed systemically.[54] Particles were thoroughly characterized by their chemical composition, fluorescence properties, surface texture, and charge. The distinct Raman spectrum (Figure 1A) of the employed pristine particles showed the highest characteristic peak at ~1001 cm^{-1}) for PS that was used for specific scanning of the particle samples using SRS at 934.5 nm laser excitation. SRS images of 10.39 µm and 1.14 µm particles gave a clear PS-specific signal, and individual particles were distinguishable (Figure 1B). 0.29 µm nanoparticles tend to aggregate in hydrophilic solutions and due to methodological limitations, single particles were not resolved. Particle size and surface morphology were further analyzed by SEM. As depicted in Figure 1C, pristine particles were equally round-shaped spheres of respective sizes and had a smooth surface. All PS particles (Figure 1D) were either colored or fluorescently labelled. 10.39 µm particles were blue-colored spheres, whereas the two smaller PS particles were fluorescently labelled. The nanometer particles (0.29 µm) had distinct signal in the green channel (ex/em 488/529-555 nm), while the small microplastic particles (1.14 µm) had signal in the orange/red channel (ex/em 488/> 560 nm) as well as some slight crosstalk into the green channel (not shown). All particles exhibited a negative ζ-potential in 1 mM KCl solution, whereas the fluorescently labelled particles showed a higher negatively charged surface than the 10.39 µm particles (Supporting Information Table 1). A mixture of all three PS particle sizes was prepared for application in mouse experiments to mimic a rather natural exposure to different-sized particles in water. The daily gavage dose was extrapolated from estimates of human MNP exposure[37] , and the three different-sized PS particles was mixed in equal weight concentrations (0.3 mg/size/day, total dose 0.9 mg MNP mix/day). To investigate the potential uptake of our experimental PS MNPs mix into the GI tract, we performed a pilot study where healthy C57Bl/6J mice were gavaged with a single dose of the MNP mix. Si and co were collected after 2 and 4 h. Immunofluorescence microscopy revealed an uptake of both 1.14 and 0.29 µm PS particles already after 2 h into GI tissues (Figure 1E). Although sparsely distributed, nanoplastic particles (0.29 µm) seemed more frequently taken up into the tissue than 1.14 µm particles, potentially caused by the higher amount of nano- than microparticles as equal mass concentrations were applied and not equal particle concentrations. Regular brightfield microscopy did not detect the bigger 10 µm particles within the tissue. This aligns with TEM analysis of small intestine tissue and the observed appearance of PS particles at the brush border of enterocytes after two hours of gavage. The accretion of 1.14 and 0.29 µm PS predominantly takes place (Figure 1F)

The DSS+MNP model showed distinct signs of colitis

With the initial proof that MNP particles with sizes < 1.2 µm penetrate the GI tissue, we investigated their distribution throughout the body as a key factor of toxicokinetics. Given the growing number of studies describing the inflammatory effects of MNP in tissue, we utilized a colitis mouse model (DSS-induced colitis) alongside a healthy control group to examine the distribution under both healthy and pathological conditions. In the diseased cohort, colitis was induced by applying 2.5% DSS in drinking water over five days, followed by an additional five days of recovery, during which the mice continued to exhibit signs of colitis. Both healthy and colitis cohorts received oral gavage for ten days of either vehicle (drinking water as negative control) or a PS MNP mix (0.3 mg/size/day). The exact treatment scheme for these four groups is shown in Figure 2A.

Figure 2: MNP treatment exhibits a trend toward exacerbating the symptoms of colitis. (A) Application scheme of MNP mix in a colitis model. Healthy or DSS-induced colitis mice were treated either with H_2O (untreated control) or with 0.9 mg/day MNP mix (equal mixture of 3 sizes; 10, 1, and 0.25 µm) over 10 days. (B) H&E of colon of Ctr, MNP, DSS and DSS+MNP treated mice. Arrows point at inflammatory spots. E = erosion of mucosal epithelia; e SM = edema in sub-mucosa. Scale bar indicates 100 µm. (C) Combined colitis score for all treatment groups. n=7; DSS vs DSS+MNP p=0.1931. (D) Colitis score of single parameters comparing DSS treated colitis mice +/-DSS: inflammation (p=0.462), crypt damage (p=0.093), ulceration (p=0.194) and affected area (p=0.429); ns = not significant. For B-D n=8/group (except for DSS n=6).

Histo-morphological analysis revealed structural changes in the colon tissue of mice in the control MNP, DSS, and DSS+MNP groups (Figure 2B). A series of pathological changes occurred in the DSS and MNP+DSS groups compared with the control and MNP groups. Overall, a significant impact in physiology of the colon in terms of a decrease in length and increase in weight for both DSS treated cohorts were observed (Supplementary Figure 1). In the colon mucosa of DSS and DSS+MNP treated mice, the number of goblet cells was decreased, the inflammatory cells, including macrophages, lymphocytes, eosinophils, and occasional neutrophils, infiltrated into the stroma (see arrows), and changes of crypt architecture were observed. These findings were more pronounced in the DSS+MNP mice, where infiltrating lymphocytes were also found in the mucosal epithelial cells. Furthermore, in DSS+MNP mice, the colon tissue presented with more focal erosions (indicated by E) in the mucosa and submucosal edemas (indicated by eSM), primarily due to local inflammatory cell infiltrations. Accordingly, the mean values for combined colitis scores, assessed from tissue sections of the colon from DSS+MNP treated mice, were almost doubled compared to DSS-only treated mice

(18.5 vs. 10.14) (Figure 2C). The control group (Ctr) and the mice treated only with MNP mix showed no signs of colitis, severe tissue damage, or inflammation (Figure 2C). A similar trend was observed in the individual parameters of the colitis score, including inflammation, crypt damage, ulceration, and affected area (Figure 2D). Colitis mice treated with the MNP mix tended to show higher scores across all four parameters relative to the DSS-only cohort. While the MNP mix alone did not cause marked GI effects during short-term exposure (10 days), it may have modestly aggravated histopathological signs of colitis. Nevertheless, these trends did not reach statistical significance, possibly reflecting the limited sample size (n=7) and heterogeneity of the DSS model.

MNPs are distributed to blood and organs

Toxicokinetics is facilitated by the uptake of MNPs in the bloodstream, followed by subsequent particle distribution throughout the body. Qualitative fluorescence evaluation of blood and tissue sections was performed to evaluate the uptake of MNP mix across the intestinal lining into the blood and potential distribution across various organs. As described in the previous experiment, MNPs were found to be taken up into the colon tissue of the MNP mix-treated mice (Figure 3A), however, at much lower numbers compared to the previous experiment (see Figure 1E). As mice were sampled in this experiment 24 h after the last gavage (10 days), the lower amount observed within the tissue might be either since MNPs have naturally moved through the GI tract and were excreted *via* feces or MNPs might have surpassed the intestinal barrier and were distributed throughout the body. No signs of MNP accumulations within the colon have been observed.

Figure 3: MNP mix uptake into blood and other organs. (A+B, E) Fluorescence images of colon (A), EDTA-blood smears (B), liver and kidney (E) tissue after 10 days treatment. Representative images per treatment group (n=8, for DSS n=6) are shown. (A+E) Nuclear counterstain: DAPI. Arrows point at 0.29 µm (green) and 1.14 µm (red) PS particles. Scale bar 20 µm. (C+D) Quantification of particles in colon (C, n=8/group) and blood (D, n=5/group). Differences between groups were compared by mixed-effects analysis using Sidak's multiple comparisons test; ns = not significant, *p < 0.05. (* one data point for DSS+MNP for 0.29 µm PS particles was excluded as an outlier)

To test the hypothesis of uptake of MNPs into the bloodstream, smears of EDTA sampled blood were performed and evaluated under the microscope for the specific fluorescence signal of the 0.29 and 1.14 µm MNP particles. 0.29 µm NPs were observed in the blood smears in both groups treated with the MNP mix, while 1.14 µm MPs were primarily present in the DSS+MNP group (Figure 3B). The biggest 10 µm particles were not detected in the blood.

To better understand how many particles could penetrate the tissue and pass through the intestinal barrier into the bloodstream, we quantified tissue resident particles in the colon and blood. Figure 3C depicts similar amounts of 0.29 µm particles in colon tissues 24 h after the last gavage in both MNP and DSS+MNP treated groups (0.71 and 0.67 particles/mm²). For the 1.14 µm particles, only a single particle was detected in one out of 8 (1/8) MNP mix colon samples, while in the DSS-MNP group, 5/8 colon tissues contained these particles at an average number of 0.28 particles/mm². Quantification of particles from blood samples (Figure 3D) showed increased numbers of 0.29 and 1.14 µm particles in the DSS+MNP cohort compared to the MNP group.

While in the healthy mouse model, only a few particles were observed in the blood samples (equal amounts of 0.29 and 1.14 µm particles at 19.1 and 18.6 particles/µl respectively), in the DSS-treated colitis mice exposed to MNP mix increased numbers of 1.14 µm MPs (65.7 particles/µl) and strongly elevated number of 0.29 µm NPs (416.1 particles/µl) were detected in the blood samples. This suggests that significantly more NPs and even larger-sized MP particles could potentially cross the compromised intestinal barrier in the colitis cohort and circulate in the bloodstream.

As MNPs were observed in the blood, we further analyzed peripheral organs that MNPs can reach *via* the bloodstream. Figure 3E shows representative pictures from liver and kidney tissues (for closeup and better contrast see also Supplementary Figure 2a+b), but NPs were also detected in other organs like the brain, heart, lung, spleen, and testicles (data not shown). Except for brain tissues, 0.29 µm NP particles were observed in healthy and DSS-induced colitis mice. Bigger 1.14 µm MPs were not observed in peripheral organs, highlighting the invasiveness of the nanoparticle fraction.

MNP treatment promotes macrophage infiltration and pro-inflammatory phenotype

Based on the IHC results and the respective increase in the inflammatory parameter in the MNP and DSS-treated cohort, we analyzed the colon of all four treatment groups for further inflammatory parameters, with a special focus on macrophage infiltration. The F4/80+ macrophages increased slightly in the wild type and were more pronounced in diseased models following the administration of MNPs (Figures 4A and 4B). Two-way ANOVA analysis showed that aside from colitis (+/-DSS, p=0.004), MNP mix treatment significantly increased macrophage numbers (p=0.03). Further analysis evaluated the shift in macrophage polarization phenotype following exposure to MNP mix in colon tissue. While anti-inflammatory Arg1+ cells were only slightly increased in DSS-treated mice (Figure 4C and 4D), pro-inflammatory iNOS+ cells increased primarily in the MNP mix-treated colitis mouse model (Figure 4E and 4F) - two-way ANOVA significance of p=0.04 for MNP mix effects on pro-inflammatory iNOS. Overall, the numbers of pro- and anti-inflammatory macrophages were

almost similar in the control and DSS groups, with slightly increased numbers of Arg1+ cells in the latter. In both MNP mix-treated groups, there was no change in overall infiltration of anti-inflammatory Arg1+ cells, while the counter iNOS+ cell phenotype increased.

Figure 4: MNP mix treatment promotes inflammatory processes in colon. (A+D+F) Immunohistological staining of representative colon sections from Ctr, MNP, DSS and DSS+MNP treated mice, stained for (A) F4/80, (D) Arg1 and (F) iNOS. Scale bar indicates 100 µm. (B+C+E) Quantification of immunohistological stains for (B) F4/80, (C) Arg1 and (E) iNOS for total colon area. Bars indicate mean number of positive cells per mm² of total colon tissue +/- SD. Signs show individual values per mouse. Individual groups were compared using multiple comparison one-way ANOVA, effect of colitis (+/-DSS) and MNP mix treatment (+/-MNP) were analyzed by two-way ANOVA. (B) F4/80: Ctr vs MNP (p=0.801), Ctr vs DSS (p=0.487), DSS vs DSS+MNP (p=0.076); two-way ANOVA: colitis (p=0.004), MNP (p=0.03). (C) Arg1: Ctr vs MNP (p=0.999), Ctr vs DSS (p=0.411), DSS vs DSS+MNP (p=0.679); two-way ANOVA: colitis (p=0.227), MNP (p=0.503). (E) iNOS: Ctr vs MNP (p>0.999), Ctr vs DSS (p=0.992), DSS vs DSS+MNP (p=0.073); two-way ANOVA: colitis (p=0.236), MNP (p=0.040). (G) Pro/Anti-inflammatory macrophage ratio in colon sections, calculated from positive cell numbers per area of iNOS divided by Arg1. Values above 1 highlight rather pro-inflammatory phenotype, values below 1 a rather anti-inflammatory phenotype. Ctr vs MNP (p=0.988), Ctr vs DSS (p=0.999), DSS vs DSS+MNP (p=0.183)); two-way ANOVA: colitis (p=0.326), MNP (p=0.122); ns = not significant; *p < 0.05; **p < 0.01. Tissue sections of at least 5 animals per group were analyzed. Each datapoint represents the measurement of a single individual.

When calculating the “pro/anti-inflammatory”ratio (- “M1/M2” ratio) for each mouse (Figure 4G), we observed that for the wildtype and DSS-induced colitis control mice, the ratio was relatively balanced and close to 1. In contrast, for the DSS+MNP treated mice, the ratio was shifted towards the pro-inflammatory side (6 out of 8 mice had a ratio > 1.5), which was less pronounced in the non-treated control mouse model and stronger in the DSS-induced colitis model.

Furthermore, qPCR analysis revealed a significant increase in the biomarkers Ifit1 (innate immune activation),[55] IL-1 (marker of acute and chronic inflammation), and Nqo1 (cellular defense against oxidative stress)[56] for the MNP mix-treated cohort compared to control (Supplementary Figure 3). A similar effect was not observed within the colitis model, which may be caused by the highly pronounced progress of the colitis potentially interfering with RNA expression. Ifi205a[57], associated with the innate immune response, showed a significant decrease comparing the control groups (Ctr and DSS) to the respective MNP mix-treated groups (MNP and DSS + MNP). These biomarkers

further underline the interplay of MNP mix exposure and inflammation, oxidative stress and the immune system.

Unbiased shotgun-proteomic profiling of colonic FFPE tissue sections

To elucidate the molecular mechanisms of DSS-induced colitis and the effects of MNP mix treatment in greater detail, we performed unbiased proteomic profiling of FFPE colon sections. This method complements IHC analysis by providing a comprehensive and quantitative overview of the proteome, allowing simultaneous identification and quantification of thousands of proteins, including changes in abundance, post-translational modifications, and cell-specific markers often beyond the scope of targeted IHC. Our analysis identified 6319 proteins in FFPE colonic tissue. Principle component analyses (PCA) on the raw data without imputation revealed an expected separation in mice receiving DSS alone or in combination with MNP mix (DSS+MNP) with a notable higher variability in the presence of MNP mix (Figure 5A), however, colitis samples (DSS and DSS+MNP) were clearly separated from control samples (Ctrl and MNP). A key challenge in unbiased shotgun-proteomics is the occurrence of missing values resulting from the extensive dynamic range of protein abundance. In contrast to transcripts, protein abundances are influenced by synthesis and post-translational modifications and degradation, which can hinder detection by MS. As a result, proteins with missing values are excluded from comparative statistical analyses. To address this problem, we analyzed the qualitative intersections of proteins that only occur in certain treatment groups, as shown in the Venn diagram in Supplementary Figure 3A. Accordingly, we found 4163 proteins in all conditions and 52, 43, 613, and 16 proteins in Ctrl, MNP, DSS, or DSS+MNP only (Source data Supplementary data 01). Since we were interested in whether MNP mix impacts DSS-induced inflammatory and cancer-driving processes, we analyzed tumor-infiltrating immune cell signatures using the explorative Immune Estimation web platform TIMER2.0. According to this bioinformatic tool, our proteomics dataset confirmed DSS-induced inflammation as etiologic to cancer development, as our dataset was identified/scored as COAD (colon adenocarcinoma) (Supplementary Figure 3B). Unsupervised hierarchical clustering based on protein abundance in the four treatment groups revealed distinct protein clusters enriched in GOBP terms, terms including macromolecule and xenobiotic metabolic processes, protein modification and response to stress, immune – and DNA damage response, as denoted to profile plots shown in Supplementary Figure 3C. In addition, we found enrichment of proteins assigned to dedicated cell types and typically present in the gut, such as stem cells (Stem), enterocytes (Ent), goblet cells (Gob), enteroendocrine cells (EEC), Paneth-cells (Pan), tuft cells (Tuft) and fibroblasts (Fib). Anticipating that the acute colitis induced by DSS and perhaps also after MNP uptake, not only changes the gut microbiota but also

profoundly alters the composition of the gastrointestinal innate and adaptive immune system, we categorized our dataset for the presence of B-cells (B), T-cells (T), regulatory T-cells (Treg), neutrophils (Neu) or macrophages (Mac), *in vitro* differentiated and polarized M1 and M2 (BM-M1, BM-M2) and *in vivo* polarization proteotypic marker proteins (labelled M1*, M2*).[51] Indeed, we found all cell types differentially enriched in protein abundance clusters (Supplementary Figure 3C). We found a profound disturbance of the colonic proteome following DSS treatment compared to the control. MNP mix also had significant, albeit more moderate, effects, as outlined in volcano plots and Venn diagrams shown in Supplementary Figures 3D-G.

Figure 5: Unbiased proteome profiling of colon tissue and macrophage M1/M2 polarisation signatures. A) Principal component analysis on sample types (ctrl= control, MNP=micro-/nanoplastic mix, DSS=dextran sulfate sodium, D+M= DSS and MNP). B, C) Qualitative analysis of M1 and M2 proteotypic signatures reported in-vivo with protein counts assigned M1 or M2 in each sample in relation to total protein counts identified (%) shown in boxplots. The one-way ANOVA on ranks (Kruskal-Wallis test) was used to determine statistically significant differences between sample groups, p-values for significant pairs are denoted. D) Venn diagrams denoting overlaps of significantly up-regulated or down-regulated proteins ($p<0.05$) in pairwise comparisons. Corresponding Volcano-plots and source data are provided in Supplementary Figure 3D-G) Heatmap of differential expressed proteins with M1 and M2 signatures in pairwise comparisons with significant differences ($p<0.05$) indicated by a yellow dot. Color code denotes lg2 fold change (FC), missing values (nan) are in grey. . Source data are provided in Supplementary tables 01 and 02. F) Volcano plot of 487 proteins with significant interaction p-value (-/+ MNP vs -/+ DSS) with significant regulated proteins indicated (one-way ANOVA $p<0.05$ with Post Hoc Tukey´s HSD correction). G, H) Enriched pathways (KEGG and Panther) and cell types based on ranks (T-statistics), source data are provided in Supplementary table 03.

The primary cell types driving early inflammation are neutrophils and inflammatory macrophages. We analyzed the relative counts of respective M1 or M2 marker proteins. Boxplots of some candidates showing individual values as well as their expression profiles in immune cells according to HPA is provided in Supplementary Figure 4A and B. As depicted in boxplots in Figure 5B and C, relative counts for pro-inflammatory M1 marker proteins were significantly increased upon DSS-treatment as expected and we found a trend towards even more M1 proteins in presence of MNP mix, while the M2 phenotype remained heterogenous. In addition to the qualitative analysis, we performed quantitative, pairwise statistical comparisons to understand how MNP, DSS, or a combined DSS+MNP treatment affected the colon proteome. A summary of up- or downregulated proteins is shown in Venn diagrams Figure 5D **,** and respective source data can be found in Supplementary data 2. To visualize quantitative protein abundance differences in treatment groups, we subset significantly regulated proteins across conditions on the basis of marker proteins reported

for *in vivo* M1 and M2 polarization as described by Orecchioni et al.[51] Indeed, we find significant quantitative differences in M1 signatures (98 proteins) or M2 signatures (88) as illustrated in protein-annotated heatmaps shown in Figure 5E, with M1 and M2 marker proteins significant in any of the pairwise comparisons marked with a yellow dot. These include typical pro-inflammatory and interferon regulated M1 proteins such as Icam1 Nfkb1, Nfkb2, Rela, Sod2, Mmp14, Stat1, Stat2, Ifitm3 or Gbp2 for example (Supplementary data 2, sheet 10) or proteins assigned to an anti-inflammatory, synthetic M2 phenotype including fatty acid metabolism, mTorc1 and Il-2/Stat5 signaling proteins including Hadhb, Echs1, Idh1, Phgd, Cd9, Add3, Rnh1, Ahnak or Ckap4 for example (Supplementary data2, sheet 11). To investigate if treatments (MNP and DSS) display synergistic or antagonistic effects, a two-way ANOVA (-/+ MNP vs -/+ DSS) was performed. The 487 proteins with significant interaction were subsequently subjected to one-ANOVA comparing DSS+MNP vs DSS (Supplementary table 03). We found 34 proteins higher and 105 lower expressed in DSS+MNP as compared to DSS alone as shown in the volcano plot in Figure 5F. Among the 34 upregulated proteins, we found Ceacam1, which is implicated in colon-cancer[58] and enriched in eosinophil and neutrophils (human protein atlas, HPA), as well immune-cell enriched Ptma, Raph1, Znf593, B4galt1, Raph1, Tor1aip2, Znf593, Vmac, Crip1 and Ptma, proteins with secretory functions in enteroendocrine cells (Chga, Chgb), cell cycle regulator Cdkn1b and oxidative stress-response proteins Crip, Gstt2, Ftl1 and Txnl1. In pathway enrichment analyses, we find lysosome, tuberculosis, transcriptional misregulation in cancer as well as Parkinson disease enriched in DSS+MNP colonic samples, while hypertrophic cardiomyopathy, metabolic pathways, motor proteins, sphingolipid-, gonadotropin-releasing hormone receptor- inflammation by chemokine and cytokine- and Wnt-signaling pathways enriched in DSS as shown in Figure 5G. Cell type enrichment analysis showed an enrichment of myeloid- microglial-, endocrine, macrophage and neutrophil cells in DSS+MNP as shown in Figure 5D. Differential enriched GOBP-terms and transcription factor targets are shown in Supplementary Figure 3C and D. Top terms were “regulation of DNA-binding transcription factor activity” and zinc finger transcription factor binding sites in DSS+MNP samples, while in DSS samples, “positive regulation of cell cycle” and AREB6 transcription factor binding sites were enriched. In summary, we find exposure of MNP in an inflammatory setting affects the gut´s cellular composition via transcriptional and posttranscriptional protein processing pathways.

MNPs trigger a polarization shift in human PBMC-derived macrophages

Further *in vitro* studies were conducted to assess the impact of MPs and NPs on macrophage polarization in naïve human PBMCs. Isolated cells were exposed to single sized MP or NP fractions of either 1.14 µm or 0.29 µm to investigate if macrophage polarization response differs due to particle

size. The 10.39 µm fraction was not used as literature reports that macrophages are not capable to fully ingest such big particles.[59,60] At an exposure concentration of 5 µg/ml, MPs and NPs were taken up by the macrophages in a size-dependent way (Figure 6A and 6B). Macrophages exhibited a pronounced preference for the 0.29 µm NPs, with 99.88 % of macrophages demonstrating uptake of these particles, while the 1.14 µm MPs were only taken up by 83.97 % of macrophages. As mass concentrations were applied, this can be a dose or size dependent effect, or a combination of both. Additionally, the expression levels of markers linked to both anti-inflammatory and pro-inflammatory states of macrophage activation were examined. The anti-inflammatory macrophage markers CD206 and PD-L1 were significantly downregulated following treatment with both particle sizes (Figure 6C). Both plastic treatment groups showed a decrease in anti-inflammatory markers, with no significant differences observed in the downregulation between them. Conversely, the pro-inflammatory macrophage marker 4-1BBL showed significant upregulation compared to the control only when incubated with the 0.29 µm NPs (Figure 6D). CD86, an additional marker associated with pro-inflammatory macrophage activation, remained unchanged in response to MPs and NPs (Figure 6D). These results can partially explain the tendency for M1 pro-inflammatory differentiation observed in vivo. Of note, at the used exposure conditions (5 µg/ml for 24h) we didn't observe any toxic effects on macrophage viability as shown by FACS gating for the viable cell population (see Supplementary Figure 6).

Figure 6: Influence of MP and NP on the polarization of human PBMC-derived macrophages.
(A) Confocal microscopy of macrophages incubated with fluorescent 1.14 µm MPs (left, red: MPs) red) or 0.29 µm NPs (right; red: CD14, green: NPs). Blue: DAPI; original magnification, 64x; scale bar, 20 µm. (B) Overall uptake of fluorescently labeled MPs and NPs by macrophages quantified by flow cytometry, with distinct bars corresponding to varying particle sizes. Depicted values are presented as percentages of MNP^+ macrophages relative to the total population, in three different healthy volunteer samples. Statistical significance was calculated by unpaired t-test. ***$p < 0.001$; (C and D) Expression of anti-inflammatory (C) and pro-inflammatory (D) macrophage markers shown as means ± SEMs for median fluorescence intensity (MFI) for the given marker in three different healthy volunteer samples. Distinct bars correspond to the untreated, and varying MP or NP treated macrophage samples. Differences between groups were compared by one-way ANOVA using Tukey's multiple comparisons test; ns = not significant; *$p < 0.05$; ***$p < 0.001$. Each experiment was conducted with 3 different PBMC donors (n=3/treatment group).

MNPs modulate the microbial communities in DSS-induced colitis model

Finally, the influence of MNP ingestion on the gut microbiota in a healthy state and during colitis, stool microbiomes of the four treatment groups were profiled *via* 16S rDNA sequencing. 5172 distinct ASV, 113 genera, and 9 phyla were detected across all samples.

Analysis of the microbiome composition indicated that the microbial richness decreased significantly during DSS treatment ($p<0.001$), while the ingestion of MNP mix did not show a significant effect on this alteration ($p=0.583$). Similar patterns were observed for the Shannon index (DSS: $p<0.001$; MNPs: $p=0.912$) and Evenness (DSS: $p<0.001$; MNPs: $p=0.950$), see Figure 7A. When analyzing the development of alpha diversity estimators in each group separately, microbial richness and Shannon index significantly decreased in both DSS-treated groups with and without MNP mix. In contrast, evenness was significantly reduced in mice treated with DSS+MNP. Evenness decreased in the DSS-group without microplastic, comparable to the DSS+MNP group, even though the decrease did not reach statistical significance ($p=0.056$; Figure 7A). Exploratory network analysis, redundancy analysis, and principal coordinate analysis indicated that DSS treatment significantly altered the gut microbiome composition irrespective of the ingestion of MNP mix (Figure 7B-C, Supplementary Figure 7). Next, we aimed to investigate which bacterial taxa were differentially represented in the four groups. The DSS-treatment (in the presence or absence of MNP mix) increased the representatives of *Bacteroides* and *Parabacteroides* (Figures 7D and 7E). For *Bacteroides*, the presence of MNP mix alone (DSS-untreated) also led to a significant increase in this genus compared to untreated controls (Figure 7D).

Figure 7: MNP exposure modulates the microbial communities in DSS-induced colitis model. (A) Alpha diversity metrics across groups and timepoints, expressed as richness, Shannon index and evenness. (B+C) Taxa plot for all treatment groups and timepoints on phylum (B) as well as the 20 most common genera (C). (D-I) Abundances of bacterial genera that showed bigger changes from baseline in either group compared to their untreated control (T0). Bacteroides (D), Parabacteroides (E), Clostridium sensu stricto 1 (F), Turicibacter (G), Enterococcus (H) and Escherichia-Shigella (I); ns = not significant; *$p < 0.05$; **$p < 0.01$ ***$p < 0.001$. For all data graphs shown in this figure n=5/group.

Furthermore, the abundance of *Clostridium sensu stricto 1* and *Turicibacter* significantly increased in mice treated with DSS; however, the effects appeared to be less pronounced for the cohort treated additionally with MNP mix (Figure 7F-G). Notably, the *Enterococcus* and *Escherichia-Shigella* generally displayed significantly higher abundance in the DSS-treated mice in the presence of MNP mix (Figure 7H and I). While the abundance of *Escherichia-Shigella* was also significantly higher in the

DSS-treated group without MNP mix, the abundance was markedly higher in mice simultaneously treated with MNP mix (Figure 7I). However, for all of these 4 taxa (*Clostridium sensu stricto 1*, *Turicibacter, Enterococcus* and *Escherichia-Shigella)* the differences between DSS and DSS+MNP treated groups were not statistically significant. Of note, certain bacterial genera were below the level of detection in one or more groups of mice (Supplementary Table 2). For example, in the mouse group with induced colitis, the presence of MNP mix reduced the levels of the representatives of *Lachnospiraceae* below detection, unlike the other three groups. On the other hand, the group treated only with MNP mix had representatives of *Odoribacter* and the RC9 group below the limit of detection.

Discussion

In recent years, inflammatory diseases of the GI tract have been on the rise.[16] As the GI tract is the leading organ group exposed to increasing levels of MNP exposure, we focused our study on the interconnection of colitis and MNP toxicity for short- and medium-term exposure.

An investigation of the effects of PS-MNP mix on wild-type mice and the DSS-induced colitis mouse model revealed that short-term exposure to MNP mix showed a trend toward higher combined colitis scores, although being not statistically significant at the conditions studied in this cohort. This potential exacerbation was noted across all evaluated histopathological colitis sub-parameters (inflammation, crypt damage, ulceration, and affected area), with DSS+MNP-treated mice displaying heightened severity compared to DSS-only counterparts. Although Schwarzfischer et al.[61], reported no histopathological difference at lower PS concentrations of ~ 0.2 mg/day in a similar colitis setting, other studies report exacerbated MNP-associated colitis in mice.[12–14] Notably, we detected no histopathological alterations in healthy mice subjected to MNP mix treatment for ten days, suggesting MNP mix alone do not induce overt tissue damage. In contrast, other researchers could show histopathological changes to the colon at longer treatment times (> 28 days). Various effects (e.g., change in goblet cell number, increased permeability, reduced mucin, inflammatory infiltrates) were observed for PS particles[62]and PVC[63], PE[64], and PP[65]. In addition to the trend of an elevated colitis scores with MNP mix, we observed an increased infiltration of macrophages into colon tissue, including a shift towards higher inflammatory macrophage phenotypes. These data align with the findings from Collin-Faure et al.[66] and Merkley et al.[67], showing increased pro-inflammatory phenotypes when treating mouse macrophages with MNP mix. Along these lines, we observed in human PBMC-derived macrophages a preferential high uptake of small NPs (0.29 µm), leading to significant downregulation of anti-inflammatory and a moderate modulation of pro-inflammatory markers. This effect can stem from either the size or the higher applied concentration, or a

combination thereof. Similar effects were previously demonstrated in pre-polarized macrophages by Fuchs et al. [34] However, one should note that donor variability, as well as particle characteristics (size, concentration, type of plastic particles, treatment time, corona) and polarization methods used for PBMCs, could affect the magnitude of change of surface marker expression and, thus, results from *in vitro* macrophage studies could differ strongly.[68–70]

To gain deeper insights into the molecular mechanisms underlying these effects, we performed unbiased shotgun proteomics of FFPE sections of the colon and identified 6319 proteins. This comprehensive analysis revealed distinct clusters of proteins enriched in inflammation-related processes and cell-specific markers, including stem cells, goblet cells, and fibroblasts. Notably, proteins characteristic of immune cell types such as macrophages and neutrophils were enriched in the DSS and DSS+MNP groups, consistent with the increased pro-inflammatory macrophage signatures observed in the colitis model. Quantitative comparisons revealed a profound disruption of the colonic proteome triggered by DSS and exacerbated by MNP mix, confirming a synergistic effect on inflammation. These results support our hypothesis that DSS-induced colitis promotes MNP uptake and distribution and enhances inflammatory responses through proteome dysregulation. Furthermore, our proteomic data revealed significant quantitative differences in macrophage polarization markers, including increased M1 (pro-inflammatory) and M2 (anti-inflammatory but tumor-promoting) protein signatures. This suggests a dual effect of MNP mix exposure in inflammatory situations, with potential implications for chronic inflammation and tumor development. These proteomic findings complement our IHC results and provide a more comprehensive understanding of the molecular landscape underlying MNP-induced exacerbation of colitis.

A rapid uptake of MNPs by gut tissues was observed already two hours after exposure to particles below the size of 1.2 µm. Long-term distribution of MNPs after ten days of treatment showed a different distribution of the MNPs within the MNP-treated compared to the DSS model. Translocation into the bloodstream of the MNPs was observed at low concentrations, whereas MNP levels in the DSS model were considerably higher, especially for the nano-sized particles. Additionally, MNPs were mainly found in the excretory organs, like the kidney and liver, in the untreated mice. In contrast, due to the facilitated uptake under inflammatory conditions, MNPs were also observed in other peripheral organs, like the brain. These data align with the findings from Jani et al.[71], who also showed that size-dependent differential uptake rates for PS particles are higher for smaller particles. They reported that particles could be detected in the liver, spleen, bone marrow, and blood, pinpointing a systemic uptake and distribution of orally ingested particles from the GI tracts. Although Jani et al. detected no particles >300 nm in the blood of healthy female rats,

Doyle-McCullough et al.[72] reported uptake of 2 µm PS microparticles at low rates (0.1-0.3% of dose). In general, uptake of particles up to 130 µm across the GI tract was postulated to occur *via* persorption.[73] However, most authors suggest a size dependency with higher efficiency for smaller particles[74]. Also, in cell culture systems of intestinal cell monolayers, size-dependent uptake rates have been reported[75] , and for nanocarriers, increased retention times in the mucus have also been observed for smaller particles (200-500 nm). Furthermore, Lamprecht et al.[76] also studied the deposition of PS particles in the context of inflammation and observed size-dependent retention rates being up to almost 7-fold increased for 0.1 µm PS particles in colitis vs control rats. This effect, in the context of colitis, is also referred to as the epithelial enhanced permeability and retention (eEPR) effect—similar to the effect used in cancer for nanoparticle and liposome delivery—and it is often used for the specific deposition of certain drugs in inflamed areas or to increase the uptake of nano-sized particles by immune cells present in these areas.[77] Hence, existing colitis facilitates the uptake of MNPs into the blood and distribution in distant organs and underscores the potential of MNPs to aggravate inflammatory conditions, as evidenced by an increase in pro-inflammatory iNOS+ and a decrease in anti-inflammatory Arg1+ macrophages in the DSS colitis model. Our findings from shotgun proteomics further substantiate this by revealing molecular disturbances linked to macrophage polarization, inflammation, and proteome-wide dysregulation, emphasizing the critical role of MNPs in exacerbating inflammatory pathways. Our data align with previously published results for similar experiments using 5 µm PS particles in larger mouse cohorts or longer MNP treatment regimens.[12,13] For mice treated with MNP mix alone for 10 days, no histopathological changes were observed when compared to H&E sections of the control group. However, when analyzing the number of macrophages present in colon tissue, we observed increased amounts in both MNP-treated and DSS-colitis mice when treated with MNP mix. This pro-inflammatory shift reflected in the elevated M1/M2 ratio in MNP-treated mice, particularly under DSS-induced conditions in a size-dependent manner, emphasizes the critical role of nanoparticle characteristics and the physiological state in determining their biological impact. Our findings necessitate a nuanced understanding of nanoparticle-macrophage interactions, underlining the importance of considering both nanoparticle properties and the host's physiological condition in evaluating the health implications of nanoparticle exposure, especially in inflammatory conditions with compromised barrier functions.

The gut microbiome's response to MNP mix exposure, particularly in the context of DSS-induced colitis, revealed significant dysbiosis characterized by reduced evenness and shifts in specific bacterial genera, including *Bacteroides*, *Parabacteroides*, *Clostridium*, *Turicibacter*, *Enterococcus*, and

Escherichia/*Shigella*. Microbiome changes due to MNP mix alone were only subtle after this 10 days exposure and would probably only develop over longer time periods of MNP mix application as already described by other authors.[78,79] The functional changes in the distal colon caused by DSS treatment, where particularly more pronounced in the mucus-associated microbiota than in the microbiota present in the distal colon content, underscore the complexity of these interactions and the need for comprehensive analysis approaches.

In detail, there were two types of changes in the microbiota. The first type was induced only by DSS, and the microbial upshift was observed in the DSS-treated mice in the presence and absence of MNP mix. The landmark of the inflamed gut was represented by the bloom of the members of *Escherichia/Shigella*, the increased levels of which have been implicated in a pro-inflammatory immune response.[32] The effect was more evident when the mice were treated with DSS in the presence of MNP mix. The increase in *Bacteroides*, *Parabacteroides,* and *Clostridium*, core components of the gut microbiota, might indicate a compensatory mechanism, i.e., an adaptive response to the altered environmental conditions caused by these pollutants. In the animal intestine, most members of the *Clostridium* group, one of the richest gut bacterial lineages, are commensals, which positively affect their host.[80] An increase in *Parabacteroides* has been shown to exert an anti-inflammatory effect on their host, while the over-abundance of *Bacteroides* may also be linked to the degradation of mucus released during the DSS treatment and can potentially serve as a reservoir for extraintestinal infections.[81–83] We detected a decrease in *Bacteroides* when the mice were exposed only to MNP mix, previously associated with gastroenterological pathologies due to either inflammation or infection.[82–84] The second type of change was observed only in the group of DSS-treated mice in the presence of MNP mix and is exemplified by the Gram-positive bacteria *Turicibacter* and *Enterococcus*. Normally, the members of *Turicibacter* are low abundant, and they have been demonstrated to have a protective effect against infections, e.g., with *Citrobacter rodentium* and to be one of the main regulators of the bile acid metabolism and 5-hydroxytryptamine production.[85–87] Interestingly, one of the few studies showing significantly endogenously elevated levels of these bacteria is linked to CD8+ T-cell deficiency [88], suggesting that the MNP mix and the DSS might negatively impact cytotoxic T-cells. The members of the genus *Enterococcus*, also found to be significantly increased in the DSS-treated mice in the presence of MNP mix, are notoriously known for their multidrug resistance and ability to cause extraintestinal infections.[89] Moreover, some representatives of this genus are implicated in inflammatory processes, e.g., colitis in immunocompromised hosts, such as $Il10^{-/-}$ mice.[90]

The 'diversity begets diversity' (DBD) hypothesis, as explored by Calcogno et al.[91] and Schluter et al.[92], proposes that higher levels of community diversity can increase the rate of speciation or

diversification. This is particularly relevant in our study, where the introduction of MNPs could disrupt these ecological interactions, potentially altering the course of microbial evolution and diversification within the gut. The contrasting 'ecological controls' (EC) hypothesis posits that competition for limited niches at high levels of community diversity negatively affects further diversification, a concept supported by metabolic models[93] and empirical data from the Earth Microbiome Project.[94] Our findings, indicating a reduction in microbial diversity due to MNP exposure, could be interpreted through the lens of these hypotheses. MNPs may create new selective pressure and niches, potentially leading to eco-evolutionary feedback that is yet to be fully understood. This is particularly relevant considering the more substantial support for DBD in animal guts compared to more diverse microbiomes like soils and sediments.[94] The fine-scale strain-level variation within microbial communities, as discussed by Goyal et al.[95] and Martiny et al.[96], highlights the complexity of these interactions and the potential for microplastics to influence microbial evolution in ways that are not immediately apparent.

Study limitations

We are aware that the investigated plastic particles differ in variety, shape, and dose to adequately reflect an environmentally realistic situation. At the time of the study, only PS round-shaped particles were commercially available at the relevant particle sizes, which reflect mainly the primary microplastics fraction. However, biologically more relevant fragmented or fiber-like particles, or weathered particles, might even show more potent effects due to greater physical (damage of cell membranes by irregularly shaped particles), chemical (diverse reagents used during production processes, leachates, or chemical modification during weathering of particles) or even biological (microbes on the surface of rough particles) toxicity.[97–99] We agree with, Gouin et al. that there are significant differences between PS MNP manufactured for biomedical and analytical purposes and environmentally generated PS MNP,[100] and that we have to improve the availability of relevant MNPs for future studies. Due to the absence of real-to-world particles in this study, we tried to account for the mentioned limitations by at least applying polydisperse PS, instead of monodisperse one.

The applied dose was extrapolated from Senathirajah et al. who estimated an upper limit of human MNP exposure of 5 g/week for human.[37] The estimated human upper exposure limit of 5 g/week (≈0.714 g/day for a 62 kg adult, ~11.5 mg/kg/day) was scaled to mice using body surface area, resulting in an equivalent dose of ~0.288 mg/day for a 25 g mouse. Accordingly, a dose of 0.3 mg/size/mouse/day was used. Since then, several studies have stated a much lower amount of MNP

exposure for the majority of people (e.g. 0.01-0.1 mg of MNPs/week estimated by Nor et. al.[101]). As the ground truth is currently not available and varies based on the environmental setting, we decided to study the upper limit of exposure to reflect the "worst case scenario". This setting enables the detection and quantification of the amount of particles, especially as only short term exposure scenario of 10 days was studied and in a realistic situation chronic exposure over years or decades is a reality. We chose to use an MNP mix of three different sizes at equal weight concentrations, assuming that bigger macro- and microplastic particles will degrade over time into multiple smaller sized particles at almost constant weight.[102] However, we are aware of the fact and also want to pinpoint, that although weight concentrations might be the same for different sized particles, the particle number and also the surface to volume area of the particles to interact with various (biological) entities increases strongly if particle sizes get smaller. (e.g. 10.4 µm vs 0.29 µm: # of particles differs by a factor of 46024, total surface area by a factor of 1286).It should be noted that both control and DSS-treated mice were not entirely free of MNP contamination, exhibiting a natural background level of environmental MNPs. Therefore, any effects described in this study can be attributed specifically to the additional exposure to experimentally administered PS particles. As the PS particles were fluorescently labelled, these could be distinguished from the background plastics. All animals and samples were consistently handled under conditions designed to minimize the risk of cross-contamination with experimental particles. Handling always began with the non-MNP-exposed groups, and, whenever feasible, separate equipment was used for each experimental group.

Conclusion and future prospects

Our findings contribute to the expanding body of knowledge regarding the impact of PS MNPs on macrophage polarization and gut microbiome diversity in the context of GI inflammatory processes. Our results underscore that spherical PS MNPs mildly exacerbate existing colitis but also lead to increased MNP biodistribution, reduced microbial evenness, and downregulation of anti-inflammatory macrophage markers. The observed decrease in microbial diversity by PS MNPs particles in the DSS mouse model highlights the necessity for further research to comprehensively grasp the effects of MNPs on gut health and disease. This study clarifies the complex interaction between MNP exposure, gastrointestinal inflammatory diseases, microbial interactions in ways that may not be immediately apparent. Proteomic profiling also revealed several protein clusters and immune cell signatures associated with inflammation and macrophage polarization. Notably, MNP exposure enhanced DSS-induced pro-inflammatory M1 macrophage signatures, while the M2

phenotype remained heterogeneous. The proteomic data also confirmed inflammation-related signaling pathways associated with cancer progression. This highlights the need for future studies to identify biomarkers of spherical PS MNP exposure and to develop therapeutic strategies to mitigate adverse effects.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Competing interests:

No, I declare that the authors have no competing interests as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Authors' contributions

Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; and Writing - review & editing (first author) Verena Kopatz Investigation, formal analysis, data curation, visualization, writing – original draft; (co-first author) Ulrike Resch investigation (proteomics), formal analysis, data curation, visualization; Kristina Draganic investigation, writing original draft; Angela Horvath formal analysis, visualization, writing – original draft; Janette Pfneissl investigation; Julijan Kabiljo investigation; Bernadette Mödl methodology; Gerald Timelthaler formal analysis, software; Julia Wallner investigation; Zeynab Mirzaei methodology, investigation. Saule Beratlyte Formal analysis; Michaela Schlederer investigation; Stefan Sarbu investigation; Simina Laslau investigation; Oldamur Holloczki validation, writing – review & editing; Martin Raigel methodology; Elisabeth S. Gruber validation; Joachim Widder resources, writing-review and editing; Iris Kufferath investigation; Marion Pollheimer investigation and validation; Wolfgang Wadsak writing – review & editing, resources; George Sarau methodology, investigation, writing-review&editing; Silke Christiansen methodology, resources; Nikola Zlatkov Kolev investigation; Marcus Krueger resources, reagents and instrumentation for mass spectrometric analysis; Robert Eferl methodology; Gerda Egger resources, supervision, writing-review and editing; Vanessa Stadlbauer formal analysis, resources, writing – review&editing; (corresponding) Verena Pichler conceptualization, data curation, resources, supervision, writing – original draft, writing – review&editing; (senior, corresponding) Lukas Kenner conceptualization, resources, formal analysis

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Synopsis

This study examines the impact of micro- and nanoplastic pollution on the exacerbation of colitis in mice, highlighting the environmental implications of plastic waste on public health.

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