SUPPLEMENTARY MATERIAL for

Polystyrene Nanoplastics Exacerbate HFD-induced MASLD by Reducing Cathepsin Activity and Triggering Large Vacuole Formation via Impaired Lysosomal Acidification

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SUPPLEMENTARY FIGURES

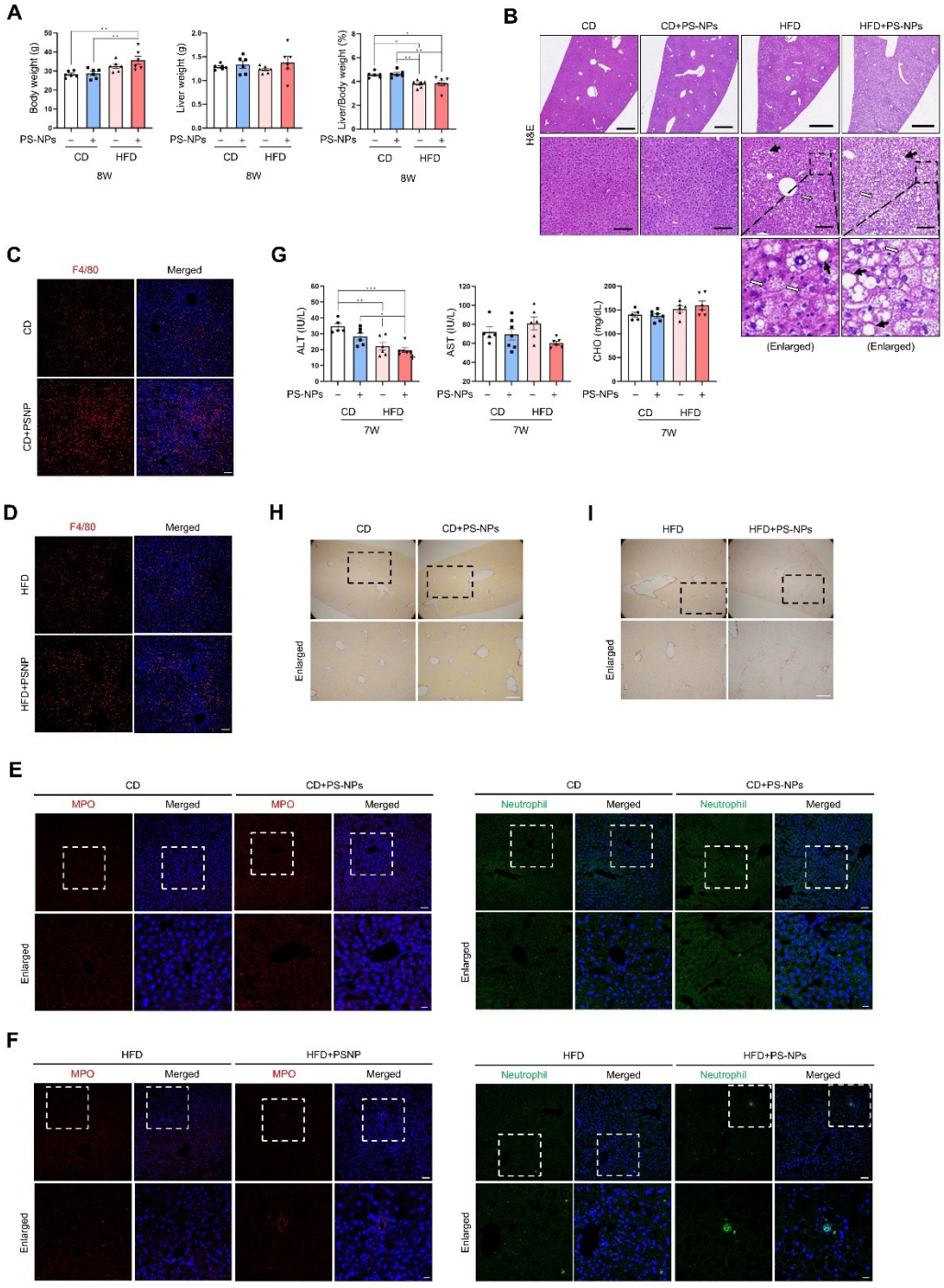

Figure S1. Animal experiments: 8- or 20-week feeding of PS-NPs and CD or HFD. (A) Body weight, liver weight, and liver-to-body weight ratio of mice exposed to 0 or 0.5 mg PS-NPs for 8 weeks. n = 4–6 in each group. (B) Representative images of H&E staining of liver from mice with indicated diet for 20-week (upper, × 40; lower, × 200; and enlarged). Macrovesicular (black arrow) and microvesicular (white arrow) fatty changes observed in the HFD and HFD+PS-NPs groups. Scale bar: upper = 600 μm, lower = 120 μm. (C) Representative images of F4/80 immunofluorescence staining of liver section in mice fed CD or CD+PS-NPs for 20-week. Scale bar: upper = 50 μm, lower = 20 μm. (D) Representative images of F4/80 immunofluorescence staining of liver section in mice fed HFD or HFD+PS-NPs for 20-week. Scale bar: upper = 50 μm, lower = 20 μm. (E) Representative images of MPO and neutrophil immunofluorescence staining of liver section in mice fed CD or CD+PS-NPs for 20-week. Scale bar: upper = 50 μm, lower = 20 μm. (F) Representative images of MPO and neutrophil immunofluorescence staining of liver section in mice fed HFD or HFD+PS-NPs for 20-week. Scale bar: upper = 50 μm, lower = 20 μm. (G) Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and cholesterol (CHO) levels in serum of mice treated with or without PS-NPs and/or HFD for 7 weeks. (H) Representative images of Sirius Red staining of liver section in mice fed CD or CD+PS-NPs for 20-week. Scale bar: upper = 500 μm, lower = 20 μm. (I) Representative images of Sirius Red Staining of liver section in mice fed HFD or HFD+PS-NPs for 20-week. Scale bar: upper = 500 μm, lower = 20 μm. Error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. CD-PS-NPs, by ordinary one-way ANOVA and Tukey's multiple comparison tests. CD; chow diet, HFD; high-fat diet.

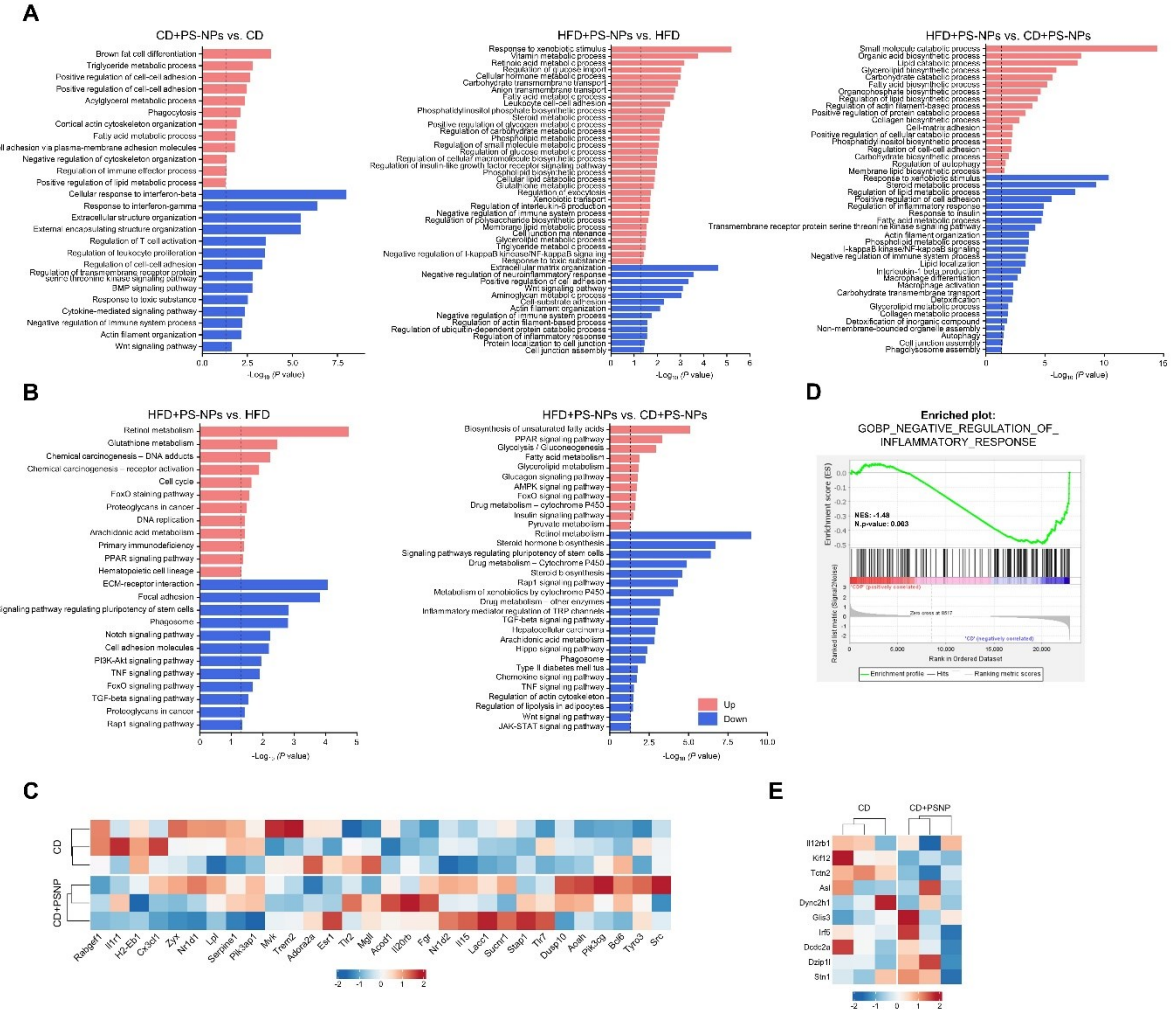
A
CD+PS-NPs vs. CD
HFD+PS-NPs vs. HFD
HFD+PS-NPs vs. CD+PS-NPs
B
HFD+PS-NPs vs. HFD
HFD+PS-NPs vs. CD+PS-NPs
Up
Down
D
Enriched plot:
GOBP_NEGATIVE_REGULATION_OF_
INFLAMMATORY_RESPONSE
NES: -1.48
N.p-value: 0.003
Enrichment score (ES)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
C
CD
CD+PSNP
E
CD
CD+PSNP

Figure S2. DEG analysis. Significantly enriched gene ontology biological process (GOBP) terms (A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms (B) of CD+PS-NPs vs. CD, HFD+PS-NPs vs. HFD, and HFD+PS-NPs vs. CD+PS-NPs groups. The dotted line represents the *P* value threshold of 0.05, with pink bars indicating upregulated pathways and blue bars indicating downregulated pathways. (C) Heatmap of the RNA-seq results showing differences in the expression of inflammatory genes in the liver of CD or CD+PS-NPs groups. The color scale ranges from - 2 (blue) to 2 (red). (D) Gene set enrichment analysis (GSEA) enrichment plot of inflammation-related genes in the liver of CD+PS-NPs vs. CD group. (E) Heatmap of the RNA-seq results showing differences in the expression of fibrogenic-related genes in the liver of CD or CD+PS-NPs groups. The color scale ranges from - 2 (blue) to 2 (red).

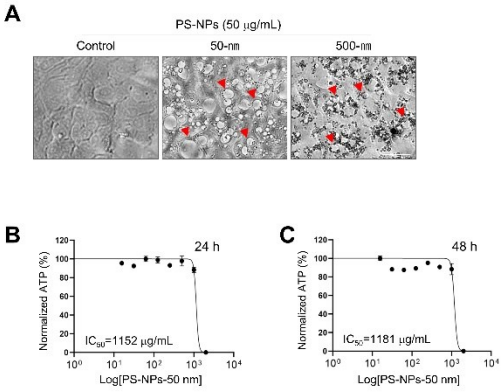
A
PS-NPs (50 μg/mL)
Control
50-nm
500-nm
B
24 h
Normalized ATP (%)
IC50=1152 μg/mL
Log[PS-NPs-50 nm]
C
48 h
Normalized ATP (%)
IC50=1181 μg/mL
Log[PS-NPs-50 nm]

Figure S3. Cytotoxicity of PS-NPs in HepaRG cells. (A) Comparison of vacuoles (red arrowhead) induced by 50 nm and 500 nm PS-NPs. Scale bar: 50 μm. Evaluation of HepaRG cell viability after PS-NP exposure involved treating the cells with serial twofold dilutions of PS-NPs, ranging from 0 μg/mL to 2,000 μg/mL. After 24 h (B) or 48 h (C), cell viability was assessed using ATP content as a control. The graphs depict the half-maximal inhibitory concentration (IC_{50}) of PS-NPs.

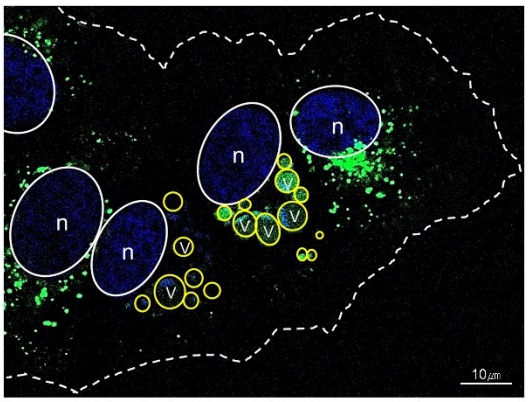
n
n
n
n
V
V
V
V
V
V
10 μm

Figure S4. Internalization analysis of PS-NPs using fluorescein-conjugated dextran and vacuole formation. Representative image of HepaRG cells after incubated with 50 μg/mL PS-NPs and 0.1 mg/mL fluorescein-conjugated dextran (green) for 24 h and 30 min, respectively. The image was captured using a confocal microscope with ×100 objective magnification is shown. Scale bar: 10 μm. n; nucleus, v; vacuoles.

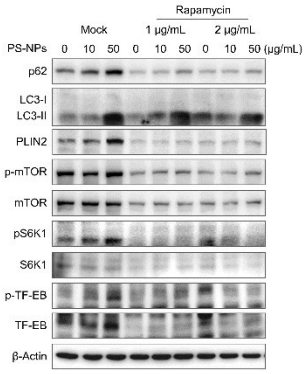
Rapamycin
Mock
1 μg/mL
2 μg/mL
PS-NPs 0 10 50 0 10 50 0 10 50 (μg/mL)
p62
LC3-I
LC3-II
PLIN2
p-mTOR
mTOR
pS6K1
S6K1
p-TF-EB
TF-EB
β-Actin

Figure S5. Rapamycin recovered autophagy disruption induced by PS-NPs. (A) HepaRG cells were pretreated with 1 or 2 μg/mL rapamycin, an mTOR inhibitor and autophagy enhancer, for 3 h and subsequently exposed to PS-NPs at the indicated concentrations.

SUPPLEMENTARY TABLES

Table S1. List of reagents used in the study

Reagents	Company	Catalog No.	Stock Cont.	Working Cont.
Dimethyl sulfoxide	SantaCruz	sc-358801		
Bafilomycin A1	InvivoGen	tlrl-baf1	100 μM	10 nM
3-Methyladenine	Sigma	M9281	100 mM	5 mM
5-(N-ethyl-N-isopropyl) amiloride	Sigma	A3085	100 mM	3 μM
Chlorpromazine hydrochloride	Sigma	31679	200 mM	5 μM
Cytochalasin B from *Drechslera dematioidea*	Sigma	C6762	10 mM	1 μM
Genistein	Sigma	G6649	20 mM	100 μM
Rapamycin	Sigma	37094	1 mg/mL	2 μg/mL
Sodium oleate	Sigma	O7501	5 mM	0.5 mM
BODIPY™ 493/503	Invitrogen	D3922	3.8 mM	5 μM
Nile Red	Invitrogen	N1142	0.5 mg/mL	5 μg/mL
Dextran	Invitrogen	D1845	25 mg/mL	50 or 100 μg/mL
ER-Tracker™ Red	Invitrogen	E34250	1 mM	1 μM
MitoTracker™ Deep Red FM	Invitrogen	M22426	1 mM	1 μM
LysoTracker™ Red DND-99	Invitrogen	L7528	1 mM	1 μM
LysoSensor™ Yellow/Blue DND-160	Invitrogen	L7545	1 mM	1 μM
Hoechst 33258	Invitrogen	H3569	10 mg/mL	10 μg/mL
Oil Red O	Sigma	O1391	0.5%	
Crystal violet	Sigma	C0775	0.5%	0.5%
Acetic acid, glacial	Sigma	A6283	99%	
Ethanol	Merck	1.00983.1011	99.9%	
2-Propanol	Merck	1.09634.1011	99.8%	
Methanol	Merck	1.06009.1011	99.9%	
Xylene	Junsei	25165S0480	85%	

Mayer's Hematoxylin	Dako	S3309
Eosin	Dako	CS701
Triglyceride (TG)	Beckman-Coulter	OSR61118
Alanine aminotransferase (ALT)	Beckman-Coulter	OSR6107
Aspartate aminotransferase (AST)	Beckman-Coulter	OSR6109
Cholesterol (CHO)	Beckman-Coulter	OSR6116
Triglyceride assay kit	Abcam	ab65336
Cathepsin B activity fluorescence-based assay kit	Abcam	ab65300
Picro-Sirius Red	Abcam	Ab246832

No., Numbers
Cont., Concentrations

Table S2. List of primary antibodies used in this study

Antibody	Host species	Source	Catalog No.
β-actin	Mouse	Enogene	E12-041
C/EBPα	Mouse	Santa Cruz	sc-365318
C/EBPβ	Mouse	Santa Cruz	sc-7962
Cathepsin D	Mouse	Abcam	ab6313
EEA1	Rabbit	CST	3288
LAMP1	Rabbit	CST	9091
LAMP1	Mouse	Santa Cruz	sc-20011
LAMP2	Mouse	Santa Cruz	sc-18822
LC3A/B	Rabbit	CST	4108
LC3B	Mouse	Santa Cruz	sc-376404
LGP85	Mouse	Santa Cruz	sc-55570
mToR	Rabbit	CST	2983
Myeloperoxidase	Rabbit	Abcam	ab208670
Neutrophil	Rat	Abcam	ab2557
PLIN2	Rabbit	Abcam	ab108323
p38α	Mouse	Santa Cruz	sc-81621
p62	Rabbit	CST	8025
PPARα	Mouse	Santa Cruz	sc-398394
PPARγ	Rabbit	CST	2435
RAB7	Rabbit	CST	9367
S6K	Rabbit	CST	9202
TFEB	Rabbit	CST	4240
MYC	Mouse	Santa Cruz	sc-40
Phospho-mToR	Rabbit	CST	5536
Phospho-p70S6K	Mouse	CST	9206
Phospho-TFEB	Rabbit	CST	37681

CST, Cell Signaling Technology

Table S3. List of secondary antibodies used in this study

Antibody	Company	Catalog No.
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Invitrogen	A11001
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Invitrogen	A11008
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	Invitrogen	A11032
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	Invitrogen	A11012
Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor ™ 488	Invitrogen	A11006
Goat Anti-Mouse IgG -HRP	AbFrontier	LF-SA8001
Goat Anti-Rabbit IgG-HRP	AbFrontier	LF-SA8002

Table S4. List of primer sequences

Symbol	Forward primer (5'→3')	Reverse primer (5'→3')
Albumin (*ALB*)	AGAATGCGCTATTAGTTCGT	ACTTACTGGCGTTTTCTCAT
CD36	AGATGCAGCCTCATTTCCAC	GCCTTGGATGGAAGAACAAA
CEBPA	GTGGACAAGAACAGCAACGA	GTCATTGTCACTGGTCAGCTC
CEBPB	CGCTTACCTCGGCTACCAG	TTGTACTCGTCGCTGTGCTT
CYP3A4	TTTTGTCCTACCATAAGGGC	CATAAATCCCACTGGACCAA
FABP1	GCAGAGCCAGGAAAACTTTG	TCTCCCCTGTCATTGTCTCC
HO-1	CAGTGCCACCAAGTTCAAGC	TTGAGCAGGAACGCAGTCTT
HNF4a	GCTCGGAGCCACCAAGAGAT	CGTATGGACACCCGGCTCAT
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
LC3B	GTCAGCGTCTCCACACCAA	TTTCATCCCGAACGTCTCCT
P62	AGGCGCACTACCGCGAT	CGTCACTGGAAAAGGCAACC
MYC	GAGGAGGAACGAGCTAAAAC	GAGTTCCGTAGCTGTTCAAG
PGC-1A	AAGGATGCGCTCTCGTTCAA	AAGGGAGAATTTCGGTGCGT
PLIN2	GCTGAGCACATTGAGTCACG	TGGTACACCTTGGATGTTGG
PPARG	GATGTCTCATAATGCCATCAGGT	TCAGCGGACTCTGGATTCAG
RPL13A	CATAGGAAGCTGGGAGCAAG	GCCCTCCAATCAGTCTTCTG