Supplementary Information For

Nanocarriers Targeting Adipose Macrophages Increase Glucocorticoid Anti-Inflammatory Potency to Ameliorate Metabolic Dysfunction

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1. Experimental section

1.1. Histologic Evaluation

Portions of visceral adipose tissue were removed at the time of euthanasia and placed in 10% neutral buffered formalin for 24 h with a volume of at least ten parts formalin to one part tissue. Tissues were then transferred to 80% ethanol for storage until trimming. Tissues were processed in graded alcohol through xylene and then embedded in paraffin blocks. Tissues were sectioned at 5 μm thickness and placed on slides, deparaffinized and stained with hematoxylin/eosin using an automated staining procedure. Slides were evaluated in blinded fashion by a board-certified veterinary pathologist (Dr. Mathew A. Wallig) for the presence or absence of lesions, including adipocyte degeneration / necrosis and interstitial inflammation, characterized by increased interstitial cellularity due to the presence of macrophages, lymphocytes, and/or neutrophils. Hepatic steatosis was scored as described in Table S1, and gonadal adipose steatitis was scored as described in Table S2.

1.2. Culturing RAW 264.7 cells and immunofluorescence staining for NOS2.

RAW 246.7 cells were cultured in DMEM media (Corning, USA) supplemented with 10% FBS in a CO_2 (5%) incubator at 37 ºC. For immunofluorescence staining, 0.3×10^6 cells were seeded onto 18 mm circular glass coverslip and grown until reaching the desired confluence. M1 polarization was induced by addition of 1000 ng/mL LPS followed by overnight incubation, whereas M0 cells were maintained in DMEM. M1 cells were then treated with ND (500 nM) for 24 h or were untreated (control M1 cells). After treatment, the cells were fixed with 4% buffered formaldehyde, washed using PBS, and permeabilized with 0.3% Triton X-100. The cells were then washed with PBS and blocked in a humidified chamber before labeling with a primary anti-NOS2 antibody (1:200) and secondary antibody. Nuclei were stained with Hoechst. The coverslips were then mounted using mounting media before imaging.

2. Supporting Tables and Figures

Table S1. Histopathological scoring system used for evaluating hepatic lipidosis/steatosis[1]

Score	Classification	Description
0	Normal	<3 macrovesicular lipid vacuoles per lobule
1	Minimal	5-10% hepatocytes affected; midzonal location; no hepatocyte swelling; macrovesicular lipid vacuoles
2	Mild	10-15% hepatocytes affected; midzonal location with some centrilobular involvement; no or mild hepatocyte swelling; mainly macrovesicular lipid vacuolation with occasional microvesicular lipid vacuolation
3	Moderate	25-50% hepatocytes affected; midzonal and centrilobular distribution; moderate to marked hepatocyte swelling centrilobular and midzonal; minimal to mild swelling periportal, macro- and microvesicular lipid vacuoles
4	Marked	50-75% hepatocytes affected; midzonal, centrilobular, and some periportal distribution; moderate to severe hepatocyte swelling; macro- and microvesicular lipid vacuolation
5	Severe	75% hepatocytes affected; midzonal, centrilobular, and periportal distribution; severe hepatocyte swelling centrilobular and midzonal; moderate to marked swelling periportal; macro- and microvesicular vacuolation
6	Very Severe	95-100% hepatocytes affected; midzonal, centrilobular, and periportal distribution; severe hepatocyte swelling all zones; macro- and microvesicular vacuolation

[1]Adapted from[29] for greater discrimination of severity.

Table S2. Histopathological scoring system used for evaluating adipose steatitis[1]

Score	Classification	Description
0	Normal	None to rare interstitial aggregates of <3 histiocytes involving <10% of the adipose on the slide
1	Minimal	Focal or multifocal interstitial aggregates of 3-5 histiocytes/ lymphocytes involving <25% of the area of adipose on the slide
2	Mild	Multifocal interstitial aggregates of 3-5 histiocytes/lymphocytes involving >25% of the area of adipose on the slide
3	Moderate	Multifocal interstitial aggregates of 5-10 histiocytes/lymphocytes involving <25% of the adipose tissue on the slide
4	Marked	Multifocal to locally extensive interstitial aggregates of 5-10 histiocytes/lymphocytes involving 25-50% of the adipose on the slide
5	Severe	Multifocal to locally extensive interstitial aggregates of >10 histiocytes/lymphocytes involving >50% of the adipose on the slide

[1]Adapted from[30] for greater discrimination of severity.).

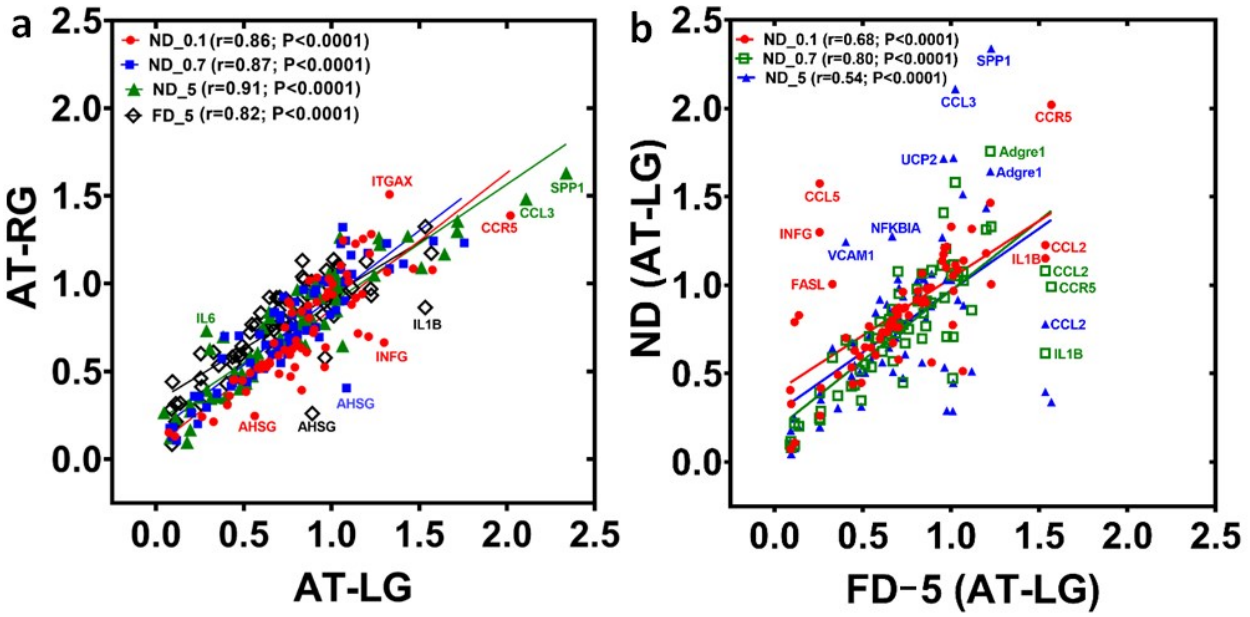

Figure S1. Correlation of gene expression between right and left adipose tissue (AT) depots and between ND and FD. (a) Spearman correlations of left vs. right gonadal AT mRNA expression from DIO mice 24 h after IP injection of ND or FD at indicated dose (0.1, 0.7, or 5 mg/kg). The left and right side of the gonadal AT depot in all the treatment groups show a linear correlation. (b) Comparison of mRNA gene expression between ND vs. FD at the indicated ND dose (0.1, 0.7, or 5 mg/kg).

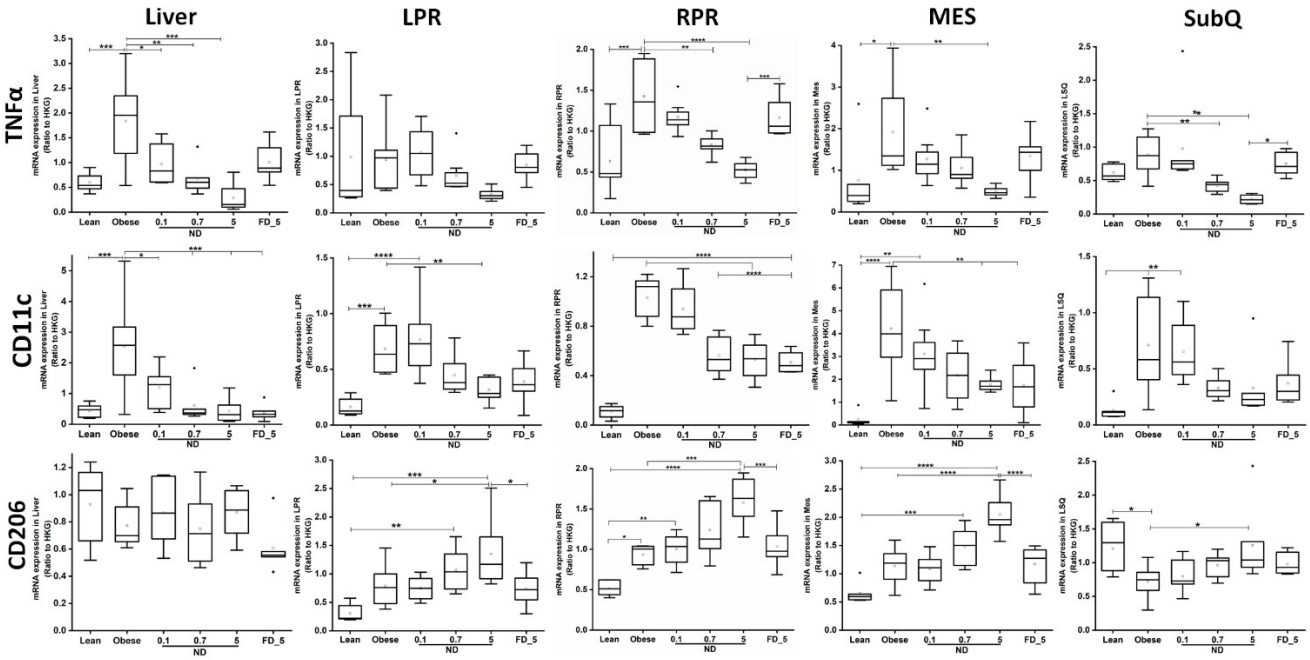

Figure S2: **ND treatment improves inflammatory gene expression profile in adipose tissues (AT) and liver.** (a) Dose-dependent down-regulation of TNFα and CD11c mRNA expression and up-regulation of CD206 across different adipose depots and liver of DIO mice 24 h after intraperitoneal administration of ND or FD in comparison to DIO and lean controls. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, groups were compared using ANOVA (n=7-8; LPR = left perirenal AT; RPR = left perirenal AT; MES = mesenteric AT; SubQ = subcutaneous AT).

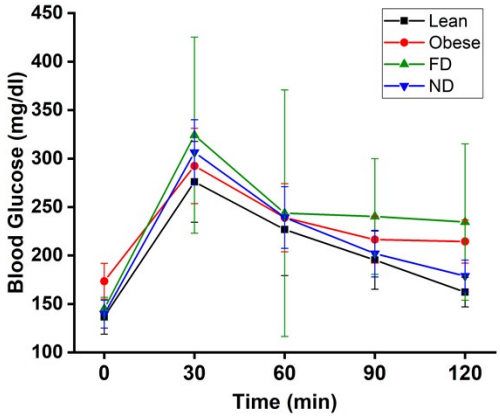

Figure S3: Blood glucose levels following glucose tolerance test 2 wk after ND (0.7 mg/kg) treatment ceased (wk 4 of study), showing improvement compared to DIO controls.

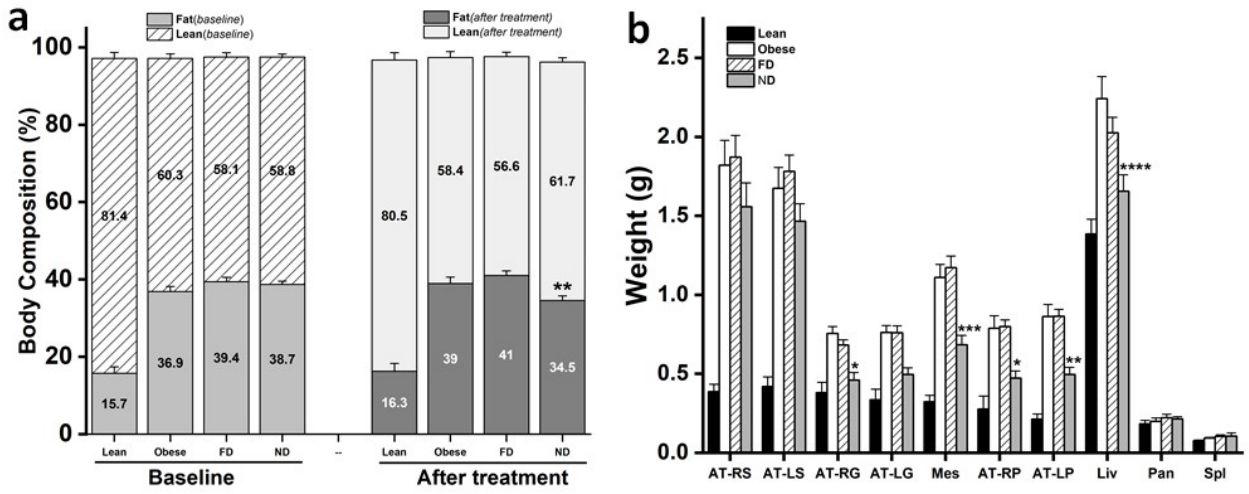

Figure S4: Weight loss due to ND treatment in DIO mice is associated with loss in total fat mass that persisted despite ND cessation. (a) Body composition of mice as determined by EchoMRI showing a significant reduction in the fat content ND-treated group compared with obese controls. (b) Measured weights of organs and adipose tissue depots were lower in the ND treated group compared with obese control and FD groups. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, groups were compared using ANOVA; n= 7-8; AT-LG = left gonadal adipose tissue; AT-RG = right gonadal adipose tissue, MES=Mesenteric adipose tissue, AT-LP = Left Perirenal adipose tissue, AT-RP = Right Perirenal adipose tissue, Liv = Liver, Pan = Pancreas, Spl = Spleen.

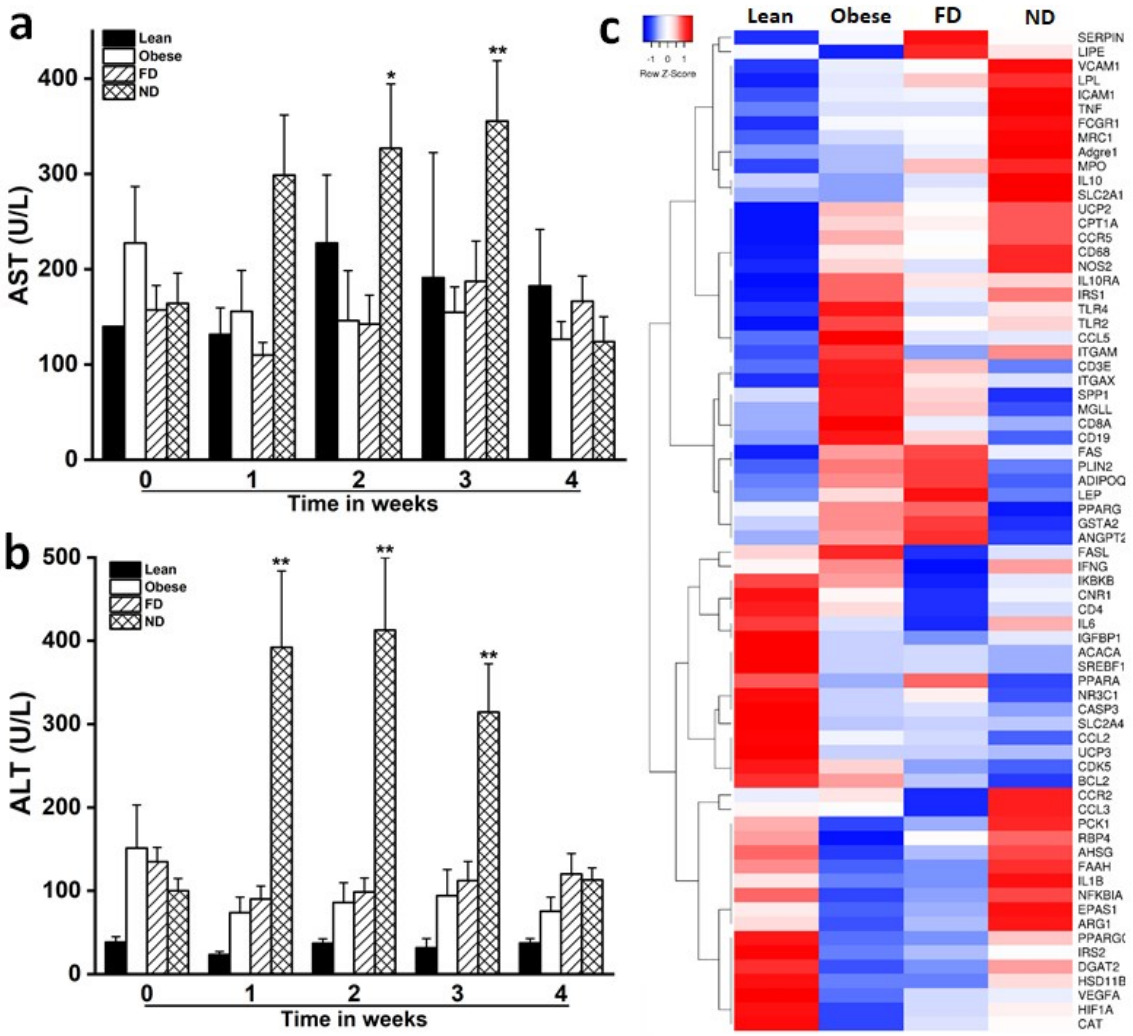

Figure S5: Long-term ND injections in DIO mice altered circulating hepatic enzymes and hepatic mRNA expression. (a) Increase in circulating aspartate aminotransferase (AST) and alanine aminotransferase (b) (ALT) concentrations in DIO mice during the two weeks of treatment with intraperitoneal (IP) injections of ND (0.7 mg/kg) compared to DIO mice receiving IP injections of saline (obese control) or dextran or compared with lean mice. Stress observed during treatment and subsequently declined after ND withdrawal. (c) Heatmap of mRNA expression from liver of lean control mice (treated with saline) and DIO mice treated with saline, ND (0.7 mg/kg), or dextran. Values represent the log(2) ratio over housekeeping genes with scaling to obtain relative expression among samples within each gene. Each row represents a specific gene of interest and each column represents the mean of each treatment group. The 69 genes in rows are clustered based on correlation of fold change values. All up-regulated genes are colored red, while down-regulated genes are colored blue.