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Polycationic PAMAM ameliorates obesity-associated chronic inflammation and focal adiposity

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ABSTRACT

As a surging public health crisis, obesity and overweight predispose individuals to various severe comorbidities contributed by the accompanying chronic inflammation. However, few options exist for tackling chronic inflammation in obesity or inhibiting depot-specific adiposity. Here, we report that polycationic polyamidoamine (PAMAM) treatment can improve both aspects of obesity. With the discovery that the plasma cell-free RNA (cfRNA) level is elevated in obese subjects, we applied the cationic PAMAM generation 3 (P-G3) scavenger to treat diet-induced obese (DIO) mice. Intraperitoneal delivery of P-G3 alleviated the chronic inflammation in DIO mice and reduced their body weight, resulting in improved metabolic functions. To further enhance the applicability of P-G3, we complexed P-G3 with human serum albumin (HSA) to attain a sustained release, which showed consistent benefits in treating DIO mice. Local injection of HSA-PG3 into subcutaneous fat completely restricted the distribution of the complex within the targeted depot and reduced focal adiposity. Our study illuminates a promising cationic strategy to ameliorate chronic inflammation in obesity and target local adiposity.

1. Introduction

Obesity and overweight create heavy burdens for the health care systems worldwide. Over the past half century, their rates have tripled and will threaten over 2.5 billion adults by 2025 [1,2]. The high prevalence is particularly alarming given that obesity and overweight predispose affected individuals to a number of serious comorbidities, such as type 2 diabetes mellitus (T2DM), cardiovascular diseases, osteoarthritis, and various cancers. These diseases are major contributors to morbidity and mortality in modern society. A major mechanism underpinning the link between obesity and associated comorbidities is sterile, low-grade inflammation, namely, chronic inflammation, which occurs in multiple tissues, especially white adipose tissue (WAT). Obesity induces a series of pathological events in WAT that trigger chronic inflammation, including macrophage infiltration [3,4] and subsequent proinflammatory cytokine and chemokine secretion [5,6],

hypoxia due to decreased vascular density [7–9], cell death [10], mitochondrial dysfunction [11], endoplasmic reticulum (ER) stress [12–15], etc. The inflammatory state further impairs the metabolic functions of WAT in glucose uptake and lipid storage. As a consequence, excess lipids are deposited into ectopic tissues, such as the liver and muscle, and cause lipotoxicity and worsen insulin resistance, further exacerbating inflammation in WAT in a feed-forward manner. In this regard, obesity and its comorbidities are appreciated as chronic inflammatory diseases. Therefore, improving inflammatory profiles would likely decrease the risk of obesity-associated metabolic disorders [16]. However, despite the tremendous success of numerous anti-inflammatory drugs, the treatment of chronic inflammation in obesity has thus far been lacking [17–22].

Various cationic biomaterials have produced a therapeutic outcome in treating acute inflammation in diverse animal disease models, including acute liver failure [23], trauma [24,25], lupus [26],

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rheumatoid arthritis [27,28], spinal cord injury [29], inflammatory bowel disease [30], and bacterial sepsis [31–33]. However, they have never been tested in metabolic diseases despite this promising anti-inflammatory effect. Polyamidoamine (PAMAM) is the most well-characterized class of dendrimer, possessing a symmetric and highly branched molecular structure. Other than its conventional use as a carrier or for gene delivery, cationic PAMAM can also neutralize negatively charged pathogens [34]. An important class of these pathogens is cell-free nucleic acids (cfNAs), which are released by dead and dying cells but can also be contributed by the gut microbiome, viruses, or even food. These cfNAs are well-established ligands for Toll-like receptors (TLRs), a major class of regulators mediating the innate immune response.

In the present study, we detected strong activation of Toll-like receptors 3 and 8 (TLR3 and 8) by the plasma from diet-induced obese (DIO) mice, which was attributed to increased cell-free RNA (cfRNA). Given that cationic PAMAM generation 3 (P-G3) is able to efficiently scavenge cfRNA, we investigated the potential of this compound to mitigate chronic inflammation, ultimately achieving metabolic improvements in obesity. P-G3 displayed a potent anti-obesity effect, accompanied by improved metabolic status. Finally, we complexed P-G3 with human serum albumin (HSA) to improve the safety profile via a sustained release. Beyond the milder anti-obesity effect of HSA-PG3 by systemic delivery, local delivery of HSA-PG3 to the subcutaneous fat depot restricted the distribution within this depot and specifically reduced the focal adiposity. Therefore, these findings open up new avenues to ameliorate chronic inflammation and local adiposity via polycationic materials.

2. Materials and methods

2.1. Detection of plasma RNA content

The RNA concentration was detected with a Quant-iT RNA assay kit (Q33140, Thermo Fisher Scientific) following the manufacturer's instructions. Murine plasma was harvested from anticoagulated blood by centrifugation at 6000 rpm for 6 min. RNA detection working solution was freshly prepared by diluting RNA reagent in RNA buffer (volume ratio 1:200). 10 μ L of *E. coli* rRNA standards or 2 μ L of murine plasma sample was added to separate wells in a 96-well plate (3915, Corning Costar) loaded with 200 μ L of working solution and mixed well. The fluorescence was measured using a microplate reader at excitation/emission 620/670 nm to determine the RNA concentration according to the standard curve.

2.2. Cell culture

Stable TLR3-, 4-, 8-, and 9-overexpressing HEK-Blue and TLR3 knockout (KO) HEK-Dual Null cells (Invivogen) were propagated in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS, Corning) and maintained in growth medium supplemented with selective antibiotics following the manufacturer's instructions. RAW 264.7 cells (ATCC) were cultured in high-glucose DMEM with 10% (v/v) FBS and then were subcultured by detachment with a cell scraper. C3H10T1/2 cells (ATCC) were cultured in high-glucose DMEM with 10% (v/v) FBS and 1 $\times$ penicillin/streptomycin (Thermo Fisher). 3T3-L1 cells (ATCC) were cultivated in high-glucose DMEM with 10% (v/v) calf serum and 1 $\times$ penicillin/streptomycin to maintain the undifferentiated status. At two days post-confluence, 3T3-L1 preadipocytes were induced to differentiate into adipocytes using an adipogenic cocktail containing 10 μ g/mL insulin, 1 μ M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine in 10% FBS DMEM. Two days post-induction, the cell medium was switched to the maintenance medium of 10% FBS DMEM supplemented with 2.5 μ g/mL insulin. HSA-PG3 or P-G3 treatments (10 μ g/mL) of 3T3-L1 cells were from differentiation Day 0 to Day 6.

2.3. HEK reporter cell treatment and the SEAP assay

HEK-Blue TLR3 cells (5×10^4 cells/well) were seeded and cultured overnight in 96-well plates in DMEM and then stimulated with murine plasma or liver total RNA. After 48 h, the activation of reporter cells was determined with a Quanti-blue medium (Invivogen). For the respective experiments, TLR inhibitor (TLR3/dsRNA complex inhibitor or TLR8 inhibitor CU-CPT9a from Sigma–Aldrich) or cationic P-G3 was added 30 min before the stimulus. In the group with ribonuclease I (RNase I, Thermo Fisher Scientific), plasma or RNA was digested with 5 U of enzyme in a water bath at 37 °C for 60 min. For other TLR reporter lines, different seeding densities were adopted from the product manuals, while the other conditions were the same as in HEK-Blue TLR3 cells.

2.4. RAW 264.7 cell treatment

RAW 264.7 cells at 1×10^5 /cm² were seeded overnight in 12-well plates in 1 mL of DMEM containing 1% (v/v) FBS. Cells were treated with 20 μ g/mL murine liver RNA; the scavenging group was pretreated with 5 μ g/mL P-G3 for 30 min. After 24 h of treatment, the culture medium was harvested to measure the secreted TNF- α .

2.5. ELISA

Secreted TNF- α was determined with an uncoated ELISA kit (Thermo Fisher Scientific). In brief, RAW 264.7 cell culture medium was centrifuged at 5000 $\times$ g for 10 min, and the supernatant was taken for the assay. A Nunc MaxiSorp 96-well plate was coated with 100 μ L/well TNF- α antibody overnight at 4 °C and then blocked with 200 μ L of ELISA/ELISPOT diluent at room temperature (RT) for 1 h. Then, 100 μ L of each standard and sample was incubated at 4 °C overnight. The diluted detection antibody, streptavidin-HRP, and TMB solution were added sequentially according to the manufacturer's manual. Last, 2 N H₂SO₄ was added to stop the reaction, and the plate was read at 450 nm with a subtraction of readings at 570 nm for calculating the concentration.

2.6. HSA-PG3 generation and characterization

Materials and reagents: P-G3 was purchased from Dendritech, Inc. (USA). Human serum albumin (HSA) was purchased from Sigma–Aldrich (USA). Cy5-NHS was purchased from Fisher Scientific (USA). Ultrapure water (18.2 M Ω) was obtained from a Milli-Q water purification system.

Preparation of the HSA-PG3 complex: A methanol solution of 10 mg of P-G3 was vacuum dried, dissolved in 1 mL of water, and lyophilized overnight to remove any remaining methanol. Then, with sonication, the P-G3 solution (10 mg of P-G3 in 1 mL of H₂O) was added dropwise into the HSA solution (100 mg of HSA in 9 mL of H₂O). The mixture was sonicated for another 10 min to obtain the HSA-PG3 complex with an HSA:P-G3 ratio of 10:1. Complexes with different HSA:P-G3 ratios were prepared in a similar way. The Cy5-labeled HSA-PG3 complex was prepared by adding Cy5-labeled P-G3 (Cy5-NHS:P-G3 in a mass ratio of 1:50) into an HSA water solution. The complexes' hydrodynamic diameter, zeta potential, count rate, polydispersity index, and stability were measured using a Malvern Nano ZS90 Zetasizer. The morphology was determined using an FEI Titan Themis 200 TEM. The release curve was measured using the Cy5-labeled HSA-PG3 complex. Briefly, 1 mL of the complex (1 mg/mL) sealed in a cellulose dialysis bag (Spectra/Por 2, MWCO 12,000–14,000, Spectrum, USA) was immersed in 9 mL of PBS in a centrifuge tube. The tube was shaken on a shaking bed at 200 rpm at 37 °C. At different time intervals, the dialysis bag was transferred to a new centrifuge tube with fresh PBS. The fluorescence (Ex/Em: 620/670 nm) of the solution in tubes was measured using a FLUOstar Optima FL microplate reader.

HSA-PG3 characterization – DNA-binding assay: The DNA-binding ability of the complexes was assessed using the Quant-iT PicoGreen

DNA Assay Kit (Fisher Scientific, USA). PicoGreen solutions were diluted 2000-fold in TE buffer (Fisher Scientific, USA). Salmon sperm DNA (Fisher Scientific, USA) was added as a standard DNA with a final concentration of 2 µg/mL. The mixture was incubated at RT for 30 min. Samples were added to a 96-well black plate (50 µL/well). Then, the mixture of PicoGreen and standard DNA (50 µL/well) was added to the same plate. After shaking for 15 min, fluorescence (Ex/Em: 490/520 nm) was measured by a FLUOstar Optima FL microplate reader.

HSA-PG3 characterization – RNA-binding assay: The RNA-binding ability of the complexes was assessed using the Quant-iT RNA assay kit (Thermo Fisher Scientific). Detecting reagents were diluted 1000-fold in TE buffer (Fisher Scientific, USA). *E. coli* rRNA standard was added with a final concentration of 2 µg/mL. The mixture was incubated at RT for 10 min. Samples were added to a 96-well black plate (10 µL/well). Then, the mixture of detecting reagent and standard RNA (200 µL/well) was added to the same plate. After shaking for 15 min, fluorescence (Ex/Em: 620/670 nm) was measured by a FLUOstar Optima FL microplate reader.

HSA-PG3 characterization – Poly (I:C) scavenging assay: HEK-Blue TLR3 cells were seeded and cultured as described above in Section 2.3. P-G3 or HSA-PG3 (1 µg/mL, counting P-G3 in complexed HSA-PG3 unless otherwise stated) was used to treat cells for 30 min before adding 2 µg/mL poly (I:C). After 24 h, the activation of the reporter cells was determined with a Quanti-blue medium.

HSA-PG3 characterization – Cytotoxicity assay: The cytotoxicity of the complexes was measured by the Cell Counting Kit-8 assay (CCK-8, Dojindo, USA). 3T3-L1 cells were seeded in a 96-well plate at 1×10^4 cells/well, kept quiescent overnight, and cultured for another 48 h after adding HSA-PG3 or P-G3. CCK-8 solution (10% in media) was added to each well, and the cells were incubated at 37 °C for 1 h. The absorbance was measured at 450 nm with a FLUOstar Optima FL microplate reader.

2.7. Cy5-labeled P-G3 or HSA-PG3 in vivo distribution imaging

Chow-fed mice were fed a high-fat diet (HFD, 60% kcal from fat, Research Diets) for one week to reduce the possible autofluorescence signal from the chow diet. 200 µg Cy5-labeled HSA-PG3 or P-G3 was administered to mice via the intraperitoneal (IP) route. At Day 1, Day 8, and Day 15 after the IP injection, the mice were sacrificed, and the tissues were imaged by using a PerkinElmer IVIS system. In addition, 200 µg or 50 µg of Cy5-labeled HSA-PG3 was given to mice via the intravenous (IV) route or locally into subcutaneous inguinal WAT, respectively. At Day 3 and Day 7 after the injection, the mice were sacrificed, and the fluorescence signal in the tissues was analyzed using the aforementioned system.

2.8. Animal studies

The animal protocol is reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University. For the treatment of DIO mice with P-G3, 8-week-old male C57BL/6 mice were fed a HFD for 20 weeks to induce obesity and then were given P-G3 (10 µg/g BW) intraperitoneally twice weekly while kept on HFD feeding. For HSA-PG3 used in the obesity prevention study, 8-week-old male mice were fed a HFD and administered HSA-PG3 (10 µg/g BW) intraperitoneally twice weekly. Body weight was monitored weekly. For the glucose tolerance test (GTT), the mice received 20% glucose injection (2 mg/g BW) by IP injection after 16 h of fasting. For the insulin tolerance test (ITT), mice were IP injected with insulin (0.75 U/kg BW) after 4 h of fasting. The blood glucose levels were measured by using an OneTouch glucometer at the indicated time points via tail vein bleeding. For indirect calorimetric analyses, mice were single-housed in metabolic cages (Comprehensive Lab Animal Monitoring System) to monitor the food intake, activity, heat production, O₂ consumption, and respiratory exchange ratio during the 24-h dark/light cycle for 5 days. After 16 h of fasting and 4 h of refeeding, the mice were sacrificed by CO₂

asphyxiation.

For HSA-PG3 local treatment of subcutaneous fat, 26-week-old male mice were treated with HSA-PG3 or HSA once weekly by subcutaneous injection into the inguinal fat while on HFD feeding. For each injection, 50 µg of HSA-PG3 (10:1 ratio) or 500 µg HSA (equivalent to 50 µg of HSA-PG3) was diluted in 300 µL of PBS solution and injected into three spots on one side of the inguinal fat. The vehicle control (PBS solution) was administered to the other side of the subcutaneous fat. Mice were sacrificed at five weeks post-injection.

2.9. Gene expression analysis

Tissues or cells were lysed into TRIzol reagent (Thermo Fisher Scientific), followed by the addition of chloroform for phase separation, and then were purified using a Tri-Isolate RNA Pure Kit (IBI Scientific). 1 µg of RNA was used to synthesize cDNA with a High-Capacity cDNA Reverse Transcription kit from Applied Biosystems. A Bio-Rad CFX96 Real-Time PCR system was used to perform quantitative real-time PCR (Q-PCR) with GoTaq qPCR Master Mix (Promega). The relative gene expression was calculated by using the $\Delta\Delta C_t$ method, and *Rpl23* was used as a reference gene.

2.10. Western blotting

Total protein from eWAT was extracted in the IntactProtein Lysis Buffer (GenuIn Biotech #415) and analyzed using the same protocol as described in a previous study [35]. The antibodies used in this study were FASN (CST #3180), C/EBP α (Santa Cruz, sc-61), PPAR γ (CST #2443), and GAPDH (Proteintech, #HRP-60004).

2.11. Histology and immunohistochemistry

After dissection, tissues were immediately fixed in a 10% formalin buffered solution. Two days after fixation, tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and photographed under a microscope (Olympus IX71). The images were processed using ImageJ software. Frozen eWAT sections were used for the immunohistochemical staining. eWAT sections were incubated with Caveolin-1 (D46G3) (CST, #3267) at a 1:250 dilution overnight, and then anti-rabbit Alexa 488 antibody (Thermo Fisher Scientific, #A27034) was used at a 1:1000 dilution.

2.12. Statistical analysis

The data are presented as the mean $\pm$ SEM. The significance between groups was evaluated using ANOVA for multiple comparisons or *t*-test for comparisons between two groups. A paired *t*-test was used to assess the significance between the subcutaneous fat on the HSA-PG3 treatment side and that on the nontreated side. The significance level was set to a *p* value < 0.05.

3. Results

3.1. Plasma cell-free RNA is increased in obese mice and activates TLR3

To better understand the pathogenesis of chronic inflammation in obesity, which involves multiple organs, we investigated whether proinflammatory factors were released into the plasma during obesity to mediate interorgan communication. Because TLRs play a prominent role in activating the innate immune response, we compared the activation of TLRs by the plasma of obese mice to lean mouse controls (Fig. 1A). Interestingly, plasma from diet-induced obese (DIO) mice activated TLR3 and TLR8 but not TLR4 and TLR9 (Fig. 1B–E). The activation of TLR3 and TLR8 can be blocked by specific TLR3 and TLR8 inhibitors (Fig. 1F and G), reinforcing the presence of their ligands in the obese plasma. Treating the obese plasma with RNase I to deplete single-strand

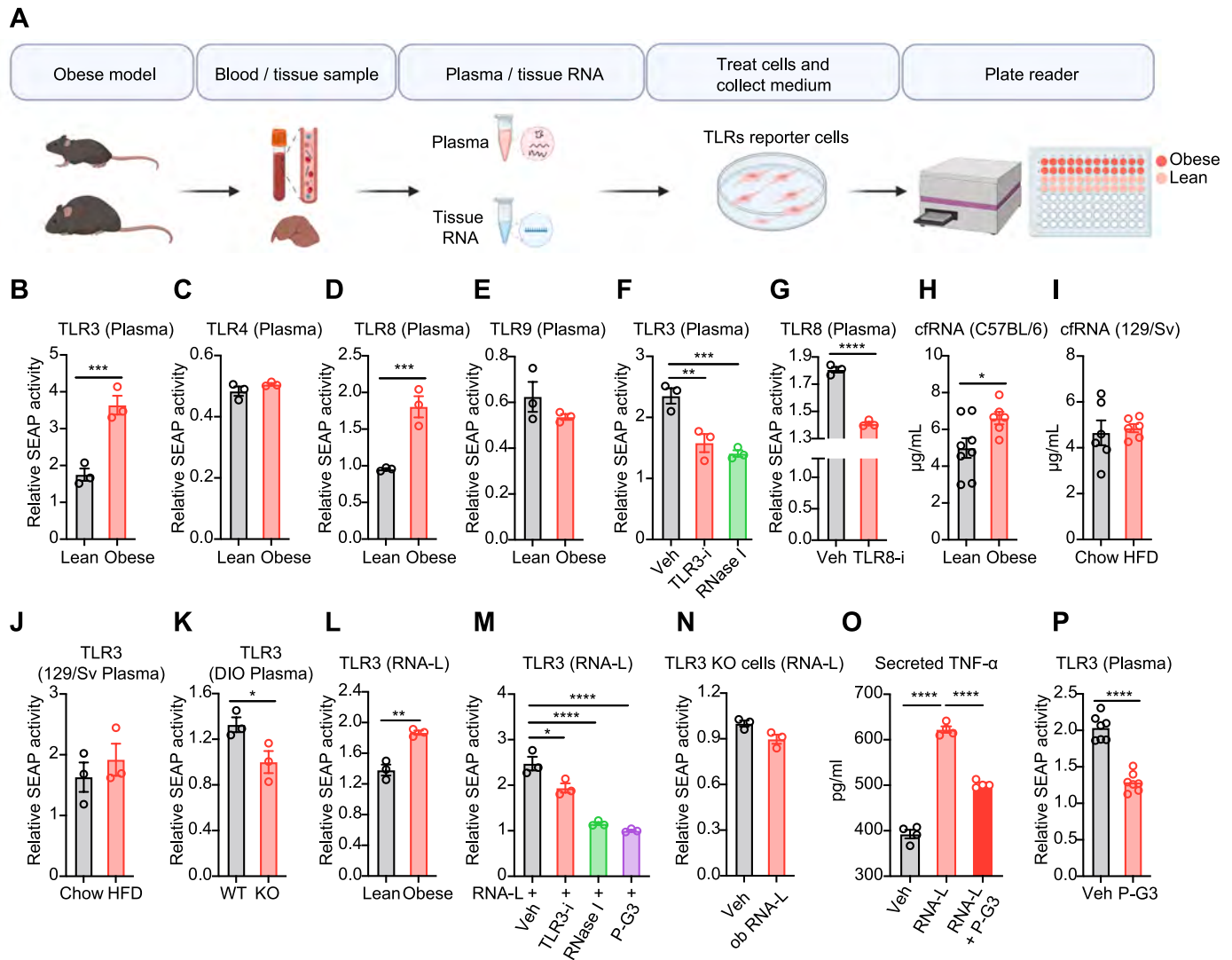


Fig. 1. Plasma cell-free RNA is increased in obese mice and activates TLR3. (A) Schematic diagram of the experimental design. (B–E) TLR-HEK293 reporter cells were treated with the plasma collected from lean or obese mice and measured the activity of TLR3 (B), TLR4 (C), TLR8 (D), and TLR9 (E). (F) The activity of TLR3-HEK293 reporter cells after incubation with obese plasma with or without TLR3 inhibitor or pretreated with RNase I. (G) The activity of TLR8-HEK293 reporter cells after incubation with obese plasma with or without TLR8 inhibitor. (H) cfRNA level in the plasma from lean and DIO C57BL/6 mice. (I) cfRNA levels in the plasma from obesity-resistant 129/Sv mice that received a chow diet or HFD feeding. (J) The activity of TLR3-HEK293 reporter cells after incubation with the plasma from 129/Sv mice fed a chow diet or HFD. (K) The activity of TLR3 after treatment with plasma from wild-type (WT) or PPAR α KO mice on HFD feeding. (L) The activity of TLR3-HEK293 reporter cells after treatment with liver RNA (RNA-L) from obese or lean mice. (M) The activity of TLR3-HEK293 reporter cells after treatment with obese liver RNA in the presence of TLR3 inhibitor, RNase I, or P-G3. (N) The activity of TLR3 KO HEK-Dual Null reporter cells with or without the addition of obese liver RNA. (O) ELISA determination of TNF- α levels in the RAW 264.7 cells culture medium at the indicated treatments. (P) The activity of TLR3-HEK293 reporter cells after incubation with the plasma from P-G3-treated obese mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are presented as the mean $\pm$ SEM.

RNA (ssRNA) surprisingly blocked the activation of TLR3 (Fig. 1F), whose classic ligand is viral double-stranded RNA (dsRNA), suggesting that cell-free ssRNAs exist in the plasma and may form double-stranded structures to activate TLR3 [36]. Indeed, the cell-free RNA (cfRNA) concentration in the obese plasma was increased by $\sim 40\%$ (Fig. 1H). Next, we asked whether the dietary difference accounted for the surge in plasma cfRNA. In the obesity-resistant 129/Sv mice, HFD feeding failed to increase the cfRNA concentration (Fig. 1I) and consequent TLR3 activation (Fig. 1J). To further discount dietary effects, we implemented the genetic model of PPAR α KO mice, which gain less weight when fed a HFD compared to that of wild-type (WT) littermate controls [37,38]. Again, the KO plasma showed less activation of TLR3 reporter (Fig. 1K). Therefore, obesity *per se* rather than the diet likely increases circulating cfRNA to activate TLR3.

To exclude the interference of other plasma components in activating TLR3, we purified total RNA from the liver (RNA-L), one of the major

metabolic organs affected in obesity. Interestingly, the RNA isolated from obese liver displayed stronger activation of TLR3 than that of the lean mouse liver RNA (Fig. 1L). This activation of TLR3 was suppressed by digesting the RNA, blocking the receptor, or ablating the receptor (Fig. 1M and N), confirming the specificity of TLR3. From a functional perspective, RNA isolated from obese mouse livers induced the secretion of TNF- α from murine macrophage RAW 264.7 cells (Fig. 1O). In agreement with the scavenging function of cationic P-G3, the activation of TLR3 by liver RNA and plasma from obese mice was largely inhibited by P-G3 (Fig. 1M, P). Additionally, P-G3 suppressed the liver RNA-augmented secretion of TNF- α from macrophages (Fig. 1O). Together, these results imply positive correlations among obesity, cfRNA, and TLR3 activation, highlighting the potential of P-G3 application to ameliorate chronic inflammation in obesity.

3.2. Metabolic improvements in diet-induced obesity from P-G3 treatment

Next, we directly tested the metabolic effects of P-G3 treatment in obese mice. Male C57BL/6 mice were fed a HFD for 20 weeks to fully establish obesity. Then, P-G3 treatment was started via the regular IP delivery route (Fig. 2A). An eight-week treatment of P-G3 resulted in a significant anti-obesity effect (Fig. 2B), with a stronger inhibition of epididymal WAT (eWAT) mass than subcutaneous inguinal WAT (iWAT) (Fig. 2C) and no effect on liver mass. In line with the decreased fat mass, adipocytes in the eWAT of P-G3-treated mice were smaller overall (Fig. 2D), accompanied by the downregulation of prominent adipocyte regulators (*Cebpb*, *Cebpa*, and *Pparg*) and their downstream target genes (*Fabp4*, *Adipoq*, *Plin1*, and *Cd36*) (Fig. 2E). Interestingly, immunofluorescent staining showed even distribution of P-G3 signal into eWAT with overlapping with adipocyte marker Caveolin-1 (Fig. S1A), suggesting P-G3 enters adipocytes to function. In addition, key lipogenesis genes (*Fasn*, *Srebf1*, and *Scd1*) were all repressed by P-G3 (Fig. 2E). The decrease in the key regulators PPAR γ , C/EBP α , and FASN was verified at the protein level (Fig. S1B). These data indicate an anti-obesity effect of P-G3.

To understand the anti-obesity effect of P-G3, we subjected mice to metabolic cage analysis to assess energy balance. Without a reduction in food intake or an increase in locomotion activity, P-G3 treatment modestly increased oxygen (O₂) consumption (Fig. 2F–H), indicating a higher metabolic rate. The unchanged food intake and locomotion activity also suggest that P-G3 did not cause sickness in mice at our dose of 10 μ g/g BW. Indeed, P-G3 demonstrated low cytotoxicity in two of the most widely used adipocyte progenitor cell lines, with >90% cell viability at a concentration of 64 μ g/mL (Fig. S1C), which is approximately 6-fold greater than the *in vivo* dose. Furthermore, P-G3-treated mice showed a higher respiratory exchange ratio (RER) (Fig. 2I), which is an indicator of fuel preference between fatty acids and carbohydrates. This higher RER corresponds to higher carbohydrate utilization, counteracting insulin resistance in obesity. Consistently, the obesity-induced impairment of glucose tolerance was rectified by P-G3 treatment (Fig. 2J). Moreover, obesity is associated with dysregulated glucose and lipid metabolism in the liver, whereas P-G3 treatment significantly repressed the key genes involved in hepatic glucose production (*G6pc* and *Foxo1*) and, concurrently, the key genes involved in lipogenesis (*Fasn* and *Acaca*) (Fig. 2K), in line with a mild alleviation of hepatic steatosis (Fig. 2L) as well as the improved glucose homeostasis. Collectively, P-G3 treatment shows systemic metabolic improvements that may aid in combating obesity.

3.3. The anti-inflammatory effect of P-G3 in obesity

After 8 weeks of P-G3 treatment, the surge in plasma cfRNA levels in DIO mice tended to stabilize (Fig. 3A), accompanied by a significant decline in TLR3 activation potency (Fig. 3B). Given that WAT inflammation is a hallmark of obesity, we investigated the effect of P-G3 treatment on inflammation in eWAT. Interestingly, the anti-inflammatory gene *IL-10* was drastically increased, while the proinflammatory markers *IL-6* and *F4/80* remained constant (Fig. 3C). Since the induction of *IL-10* transcription might be secondary to improved adipose tissue health over chronic P-G3 treatment, we tested the direct effect of P-G3 in acute treatment. A single dose of P-G3 treatment significantly upregulated *IL-10* after three days and was maintained after seven days. In contrast, *IL-6* and *F4/80* were unaffected (Fig. 3D). Therefore, P-G3 conveys an expected direct anti-inflammatory role in obesity.

3.4. Controlled release of P-G3 through the formation of a complex with human serum albumin (HSA)

Despite the minimal toxicity of P-G3 we observed in treating DIO mice, we endeavored to improve its translational applicability through

controlled release for the sake of minimizing potential acute cationic toxicity. Albumin was chosen as a viable complexing component because it is abundantly present in the plasma and adipose tissue. Furthermore, it is widely used as a drug delivery carrier [39], such as in FDA-approved Abraxane, where HSA interacts with paclitaxel nanocrystals in a noncovalent manner to form an injectable formulation [40, 41]. We mixed HSA and P-G3 in different ratios and characterized their self-assembly behaviors, including particle size, count rate, polydispersity index (PDI), and zeta potential (Fig. 4A–D). Although both HSA and P-G3 are hydrophilic, dynamic light scattering (DLS) of their mixed solution showed that they contained particulates with a hydrodynamic diameter in the 100–1000 nm range and peaked at approximately 1 μ m at an HSA:P-G3 mass ratio of 10:1 (Fig. 4A), indicating that they can self-assemble into nano- or microparticles. Hydrophilic molecules commonly show a low count rate and high PDI under DLS measurements, and their size and zeta potential are probably not the most accurate. Most importantly, the increase in the count rate and the decrease in the PDI validated the formation of uniform complexes of HSA-PG3 (Fig. 4B and C). The formulation was finally optimized with a mass ratio of 10:1 between HSA and P-G3, resulting in nearly neutral spherical microparticles with a hydrodynamic diameter of 1098 ± 36.2 nm and zeta potential of -3.55 ± 0.25 mV (Fig. 4D–F).

HSA-PG3 complexes showed good stability in water or cell culture media (RPMI media with 5% FBS) for at least 3 days (Fig. 4G and H). They likely associate with one another through charge-charge interactions. We observed that P-G3 was gradually released from the complexes within two weeks (Fig. 4I). HSA-PG3 complexes showed comparable ability in scavenging DNA and RNA as P-G3 (Fig. 4J and K), resulting in the same potency in blocking TLR3 activation as a functional readout (Fig. 4L). Their comparable effect on cell viability at lower doses demonstrated a similar level of cytotoxicity, but HSA-PG3 showed higher cell viability at high doses of 250–500 μ g/mL than P-G3 (Fig. 4M), suggesting its utility in local application. Moreover, HSA-PG3 and P-G3 had similar effects on inhibiting lipogenic genes in 3T3-L1 adipocytes (Fig. 4N), in contrast to the blunted effect of HSA (Fig. S1D). These characterizations highlight the successful complex formation of P-G3 with HSA, which may increase biocompatibility in treating obesity.

3.5. Treating diet-induced obesity with HSA-PG3

Next, we assessed the *in vivo* function of HSA-PG3 complexes in treating obesity. We first compared the biodistribution of HSA-PG3 with that of P-G3. Both showed efficient systemic distribution to the liver, kidney, and the visceral fat depots eWAT and retroperitoneal WAT (rWAT) via IP injection (Fig. 5A and Fig. S2A). However, over time, a lower signal of HSA-PG3 was detected in the liver, while the presence in visceral fat depots persisted at 8- and 15-days post-injection. In addition, this visceral biodistribution of HSA-PG3 was sensitive to the administration route on whether it was IP or intravenous (IV) injection (Figs. S2B and C). We then treated C57BL/6 mice with HSA-PG3 via IP injection twice a week at a P-G3 dose of 10 μ g/g BW and monitored their body weight change on HFD feeding (Fig. 5B). HSA-PG3 showed the delayed inhibition of body weight increase, gradually reaching approximately 10% less weight gain than controls after 7.5 weeks of treatment (Fig. 5C and D). The inhibition of fat mass mainly occurred in eWAT and it was modest (Fig. 5E). Despite the barely affected adipocyte size, there was a strong inhibition of adipogenic genes and lipogenic genes by HSA-PG3 in eWAT (Fig. 5F and G). Again, the anti-inflammatory markers *IL-10* and *Arg1* were markedly stimulated by HSA-PG3 in eWAT without changing the macrophage infiltration marker F4/80 (Fig. 5H), reinforcing its potential to alleviate chronic inflammation in obesity. Furthermore, glucose intolerance and insulin resistance induced by the HFD challenge were both improved by HSA-PG3 treatment (Fig. 5I and J). Collectively, the HSA-PG3 complex showed similar anti-obesity effects and metabolic benefits compared to

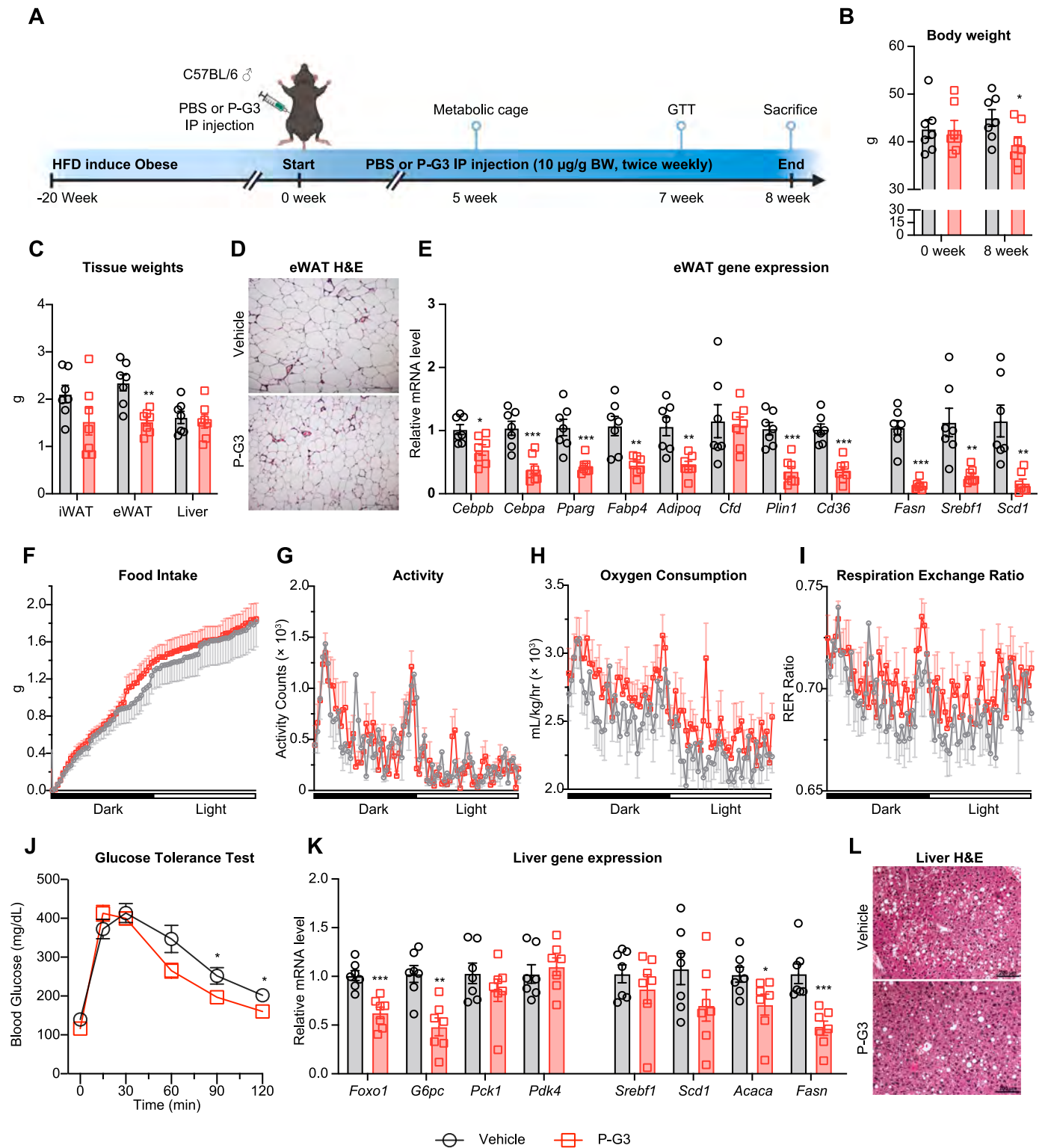


Fig. 2. P-G3 displays anti-obesity effect in DIO mouse model. (A) Schematic diagram of the experimental design. Male C57BL/6 mice were fed HFD for 20 weeks to induce obesity. (B) Body weight before and after P-G3 treatment. (C) Tissue weights at sacrifice. (D and E) Histological analysis (H&E staining) and gene expression of the eWAT. (F–I) Calorimetric analyses of mice with or without P-G3 treatment at ambient temperature. (F) Food intake, (G) activity, (H) oxygen consumption, and (I) respiratory exchange ratio within 1 dark/light cycle. (J) Glucose tolerance test after 7 weeks of P-G3 treatment. (K–L) Gene expression (K) and histological analysis (H&E staining) (L) of the liver. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for vehicle control ($n = 7$) vs. the P-G3 ($n = 7$) group by 2-tailed Student's t -test. Data are presented as the mean $\pm$ SEM.

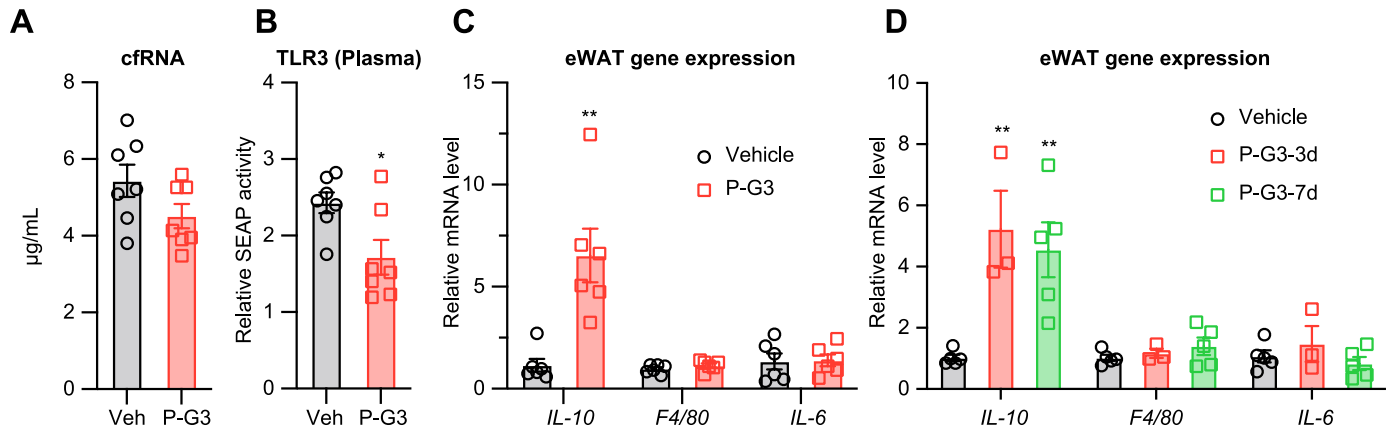


Fig. 3. The anti-inflammatory function of P-G3 in DIO mice. (A) cfRNA levels in the plasma of male DIO mice after 8-wk P-G3 treatment (n = 7, 7). (B) The activity of TLR3-HEK293 reporter cells after exposure to the plasma from mice with or without 8-wk P-G3 treatment (n = 7, 7). (C) Inflammatory gene expression in eWAT from mice in Fig. 2A (n = 6, 6). (D) After 4 weeks of HFD feeding, mice received vehicle or a single dose of P-G3 (10 µg/g BW, IP), and were collected tissues at Day 3 or Day 7 post-injection. Gene expression of inflammatory markers in eWAT (n = 5, 3, 5). *p < 0.05, **p < 0.01 for the P-G3 treatment groups vs. the vehicle control group by 2-tailed Student's t-test. Data are presented as the mean ± SEM.

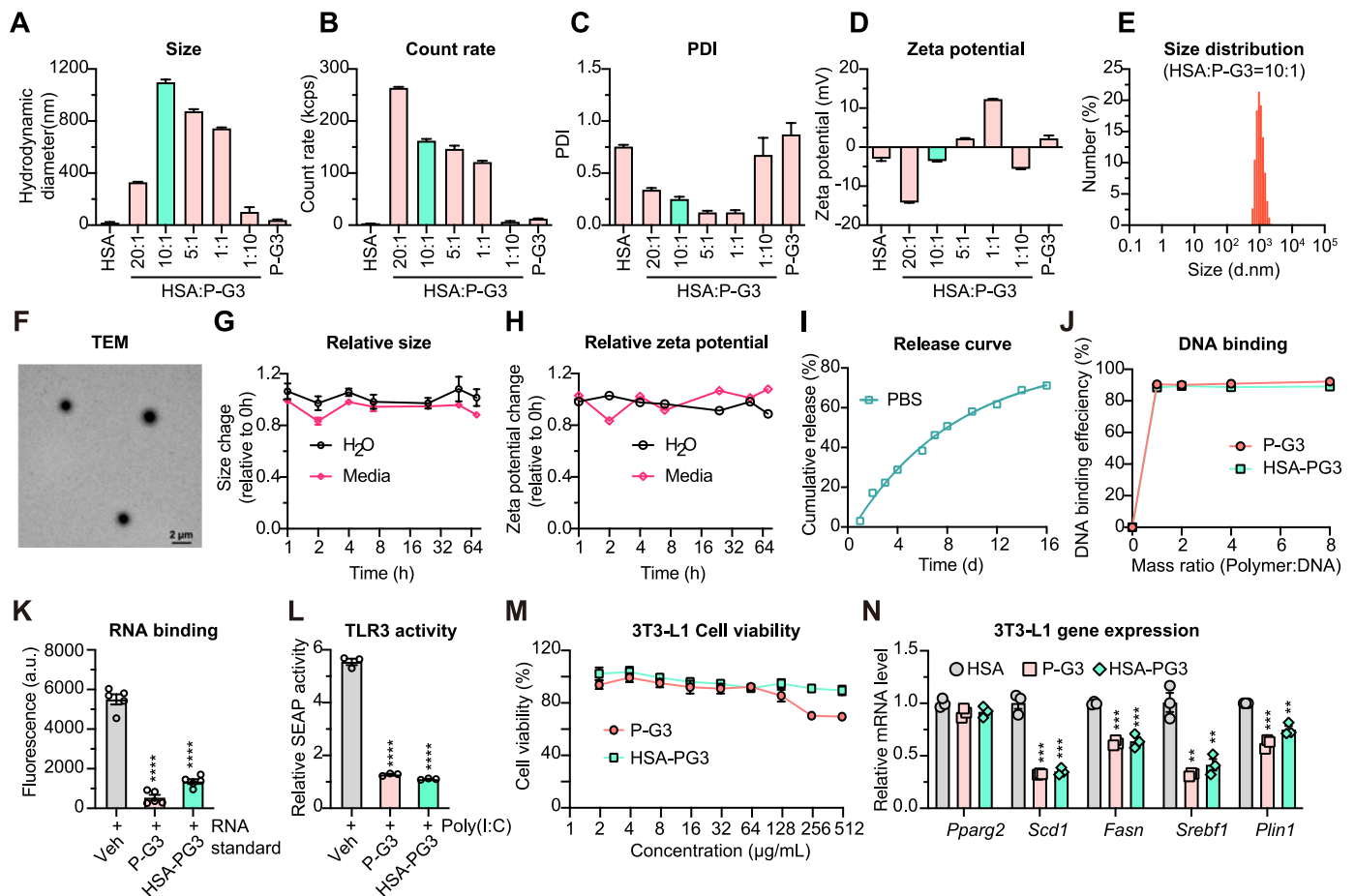


Fig. 4. Complexing P-G3 with HSA and characterizations. (A-D) Optimization of HSA-PG3 complexes with different HSA:P-G3 mass ratios based on (A) hydrodynamic diameter, (B) count rate, (C) polydispersity index (PDI), and (D) zeta potential (n = 3). (E) Size distribution histograms of the optimized HSA-PG3 complex with an HSA:P-G3 mass ratio of 10:1. (F) TEM picture showing the morphology of the optimized HSA-PG3 complex. Scale bar, 2 µm. (G-H) Stability of HSA-PG3 complex in H₂O and cell culture media (RPMI with 5% FBS) based on (G) relative size change and (H) relative zeta potential change (n = 3). (I) Release profile of HSA-PG3 in PBS (n = 3). (J) Comparing the DNA binding efficiency of P-G3 and HSA-PG3 at different polymer:DNA ratios (n = 3, 3). (K) RNA binding efficiency of P-G3 or HSA-PG3 (n = 3, 3). (L) The activity of TLR3-HEK293 reporter cells after incubation with 2 µg/mL Poly (I:C) with or without P-G3 or HSA-PG3 (1 µg/mL) pretreatment (n = 3, 3). (M) 3T3-L1 cell viability upon exposure to different concentrations of P-G3 or HSA-PG3 (n = 6, 6). (N) Comparable effects of HSA-PG3 and P-G3 on the inhibition of lipogenesis in 3T3-L1 adipocytes on Day 6 of differentiation (n = 3, 3, 3). **p < 0.01, ***p < 0.001 for treatment group vs. vehicle control group by 2-tailed Student's t-test. Data were represented as mean ± SEM.

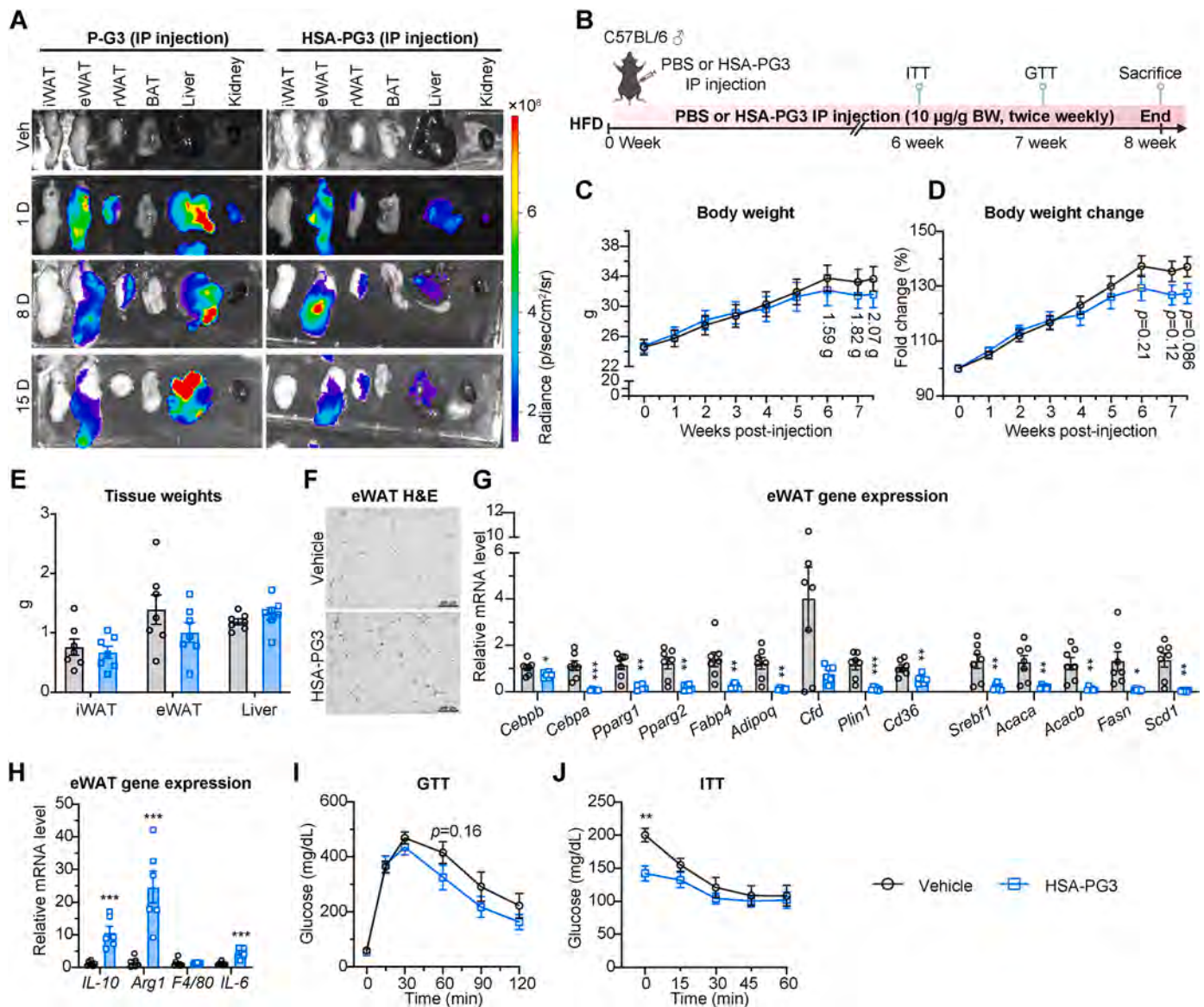


Fig. 5. Treatment of diet-induced obese mice by HSA-PG3. (A) Comparing the biodistribution of Cy5-labeled HSA-PG3 or P-G3 in DIO mice via the IP delivery. Tissue fluorescent signals were determined by using an IVIS Optical Imager at Day 1, Day 8, and Day 15 post-injection of 200 µg polymers. iWAT: inguinal WAT; eWAT: epididymal WAT; rWAT: retroperitoneal WAT; BAT: brown adipose tissue. (B) Schematic diagram of the experimental design. Male C57BL/6 mice were treated with HSA-PG3 or PBS vehicle control at the start of HFD feeding. (C, D) Body weight curve (C) and body weight change (D) during the treatment. (E) Tissue weights at sacrifice. (F) Histological analysis (H&E staining) and (G, H) gene expression in the eWAT (n = 7, 6). (I) Glucose tolerance test. (J) Insulin tolerance test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for vehicle control (n = 7) vs. HSA-PG3 group (n = 7) for metabolic characterizations, n = 6 for qPCR because of the loss of one sample in processing) by 2-tailed Student's *t*-test. Data are presented as the mean $\pm$ SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

P-G3, although the effects were generally milder, likely due to a different pharmacodynamic effect because of the sustained release.

3.6. Reduction of focal subcutaneous adiposity by HSA-PG3

Depot-specific fat reduction is an even greater and more important challenge than weight loss *per se*, as all current obesity interventions, whether targeting energy homeostasis or food absorption, lack depot specificity. Several procedures reduce focal subcutaneous adiposity (e.g., liposuction) but most are based on a destructive strategy. Given the controlled release of P-G3 from the HSA complex and its strong repression of lipid synthetic genes, we asked whether HSA-PG3 could be used to overcome the challenge of focal adiposity treatment. To this end, we locally injected HSA-PG3 into iWAT, a major subcutaneous fat depot in mice, and examined its biodistribution. Cy5-labeled HSA-PG3 was

restricted to the iWAT on the injected side without distributing to the iWAT on the other side or any other organs examined, including the adjacent thigh adipose tissue (tAT) [42] (Fig. 6A). The local specificity of the fluorescent signal was strictly retained at least 7 days post-injection (Figs. S3A–C). Hence, we devised a weekly treatment of 50 µg of HSA-PG3 in one side of the iWAT of DIO mice (Fig. 6B), estimating an equivalent P-G3 dose of 10 µg/g-fat mass/day based on the average of 0.7 g iWAT depot size. Treatment of HSA-PG3 for 5 weeks significantly reduced the iWAT mass compared with the vehicle-treated side in the same mouse (Fig. 6C), with an average of a 30% reduction (Fig. 6D). In further support, the adipocyte size in the treated side was reduced (Fig. 6E). Underlying the smaller adipocyte size is the prevalent repression of genes involved in lipid synthesis, including *de novo* fatty acid synthesis (*Fasn*, *Srebf1*, and *Scd1*) and triglyceride synthesis (*Gpat3*, *Agpat2*, and *Dgat2*) (Fig. 6F). Interestingly, adipocyte functions appear to

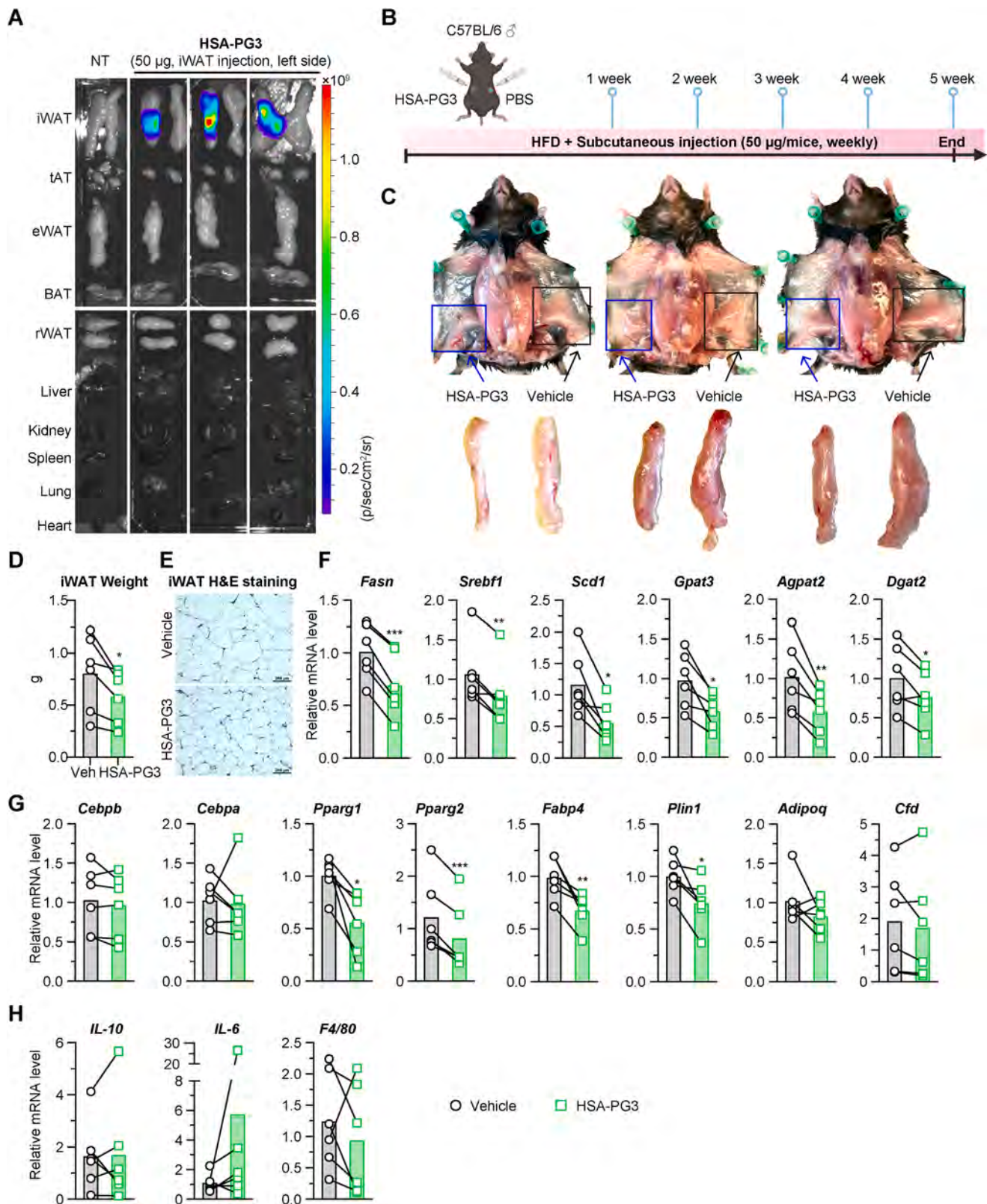


Fig. 6. Focal subcutaneous adiposity reduction by HSA-PG3. (A) At Day 3 after 50 μ g of Cy5-labeled HSA-PG3 injection on one inguinal side, the fluorescent signal in tissues was imaged using an IVIS system. iWAT: inguinal WAT; eWAT: epididymal WAT; rWAT: retroperitoneal WAT; BAT: brown adipose tissue, tAT: thigh adipose tissue (adjacent to iWAT). Non-treated (NT, $n = 1$), HSA-PG3 group ($n = 3$). (B) Schematic diagram of the experimental design. DIO male C57BL/6 mice were injected 50 μ g HSA-PG3 and PBS on each side iWAT weekly. (C) Photos of the mice and iWAT after 5 weeks of HSA-PG3 injection on one inguinal side and PBS on the other side. (D) iWAT weights of the HSA-PG3-treated or control side at sacrifice. (E–H) Histological analysis (H&E staining) (E) and qPCR expression of lipogenic (F), adipogenic (G), and inflammatory genes (H) in iWAT with or without HSA-PG3 treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for vehicle control ($n = 6$) vs. the HSA-PG3 group ($n = 6$) by paired Student's t -test. Data are presented as the mean $\pm$ SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

be largely maintained given the normal expression of housekeeping adipokine genes (*Adipoq* and *Cfd*) and adipogenic transcription factors (*Cebpa* and *Cebpb*), while some genes related to lipid storage (*Pparg1*, *Pparg2*, *Fabp4*, and *Plin1*) were downregulated (Fig. 6G). Moreover, the local inhibition of fat mass did not cause an increase in *F4/80*, a marker for macrophage infiltration and inflammation (Fig. 6H). Of note, HSA alone did not reduce iWAT depot size nor repress most lipid synthetic genes (Figs. S3D and E). However, it did downregulate *Pparg1*, *Pparg2*, and *Fabp4* (Fig. S3F), suggesting the P-G3-dependent repression of lipogenesis. Taken together, HSA-complexed P-G3, as a complex, displays the potential to treat focal adiposity in a targeted and safer manner.

4. Discussion

Harnessing chronic inflammation and focal fat reduction are two prominent challenges in obesity treatment. In this study, polycationic material is applied to alleviate chronic inflammation in obesity, resulting in metabolic improvements, including obesity inhibition. Furthermore, by uncovering the highly restricted local fat distribution of P-G3 in complexes with HSA, we trailblaze a new cationic strategy for treating focal adiposity. These findings signify novel functions and applications of cationic biomaterials in tackling metabolic diseases.

Numerous mechanisms underlie chronic inflammation in obesity and the detrimental development of obesity comorbidities, such as immune cell infiltration and activation, inflammatory cytokine production, lipid toxicity, and microbiome-derived endotoxin. In understanding the triggers of chronic inflammation, we screened the activation of TLRs, the pivotal gatekeepers of the innate immune response, by the plasma of obese mice and found positive responses by TLR3 and TLR8, both of which are receptors of dsRNA and ssRNA, respectively. Although most RNA in mammalian cells is single-stranded, RNA is known to form intra-strand or inter-strand double-stranded structures [36], which may serve as endogenous ligands of TLR3 [43]. The lack of response by TLR4 and TLR9 reporter cells could be explained by the different assay systems (source of stimulants, model cells, sensitivity, etc.) rather than excluding their ligands from the stimulant pool. Nevertheless, the data collected from TLR3 overexpression and KO cells, TLR3/8 inhibitors, and RNA-specific nucleases collectively demonstrate that cfRNA is a pathogen in obesity to intensify inflammation via TLR3/8 signaling and could be used as a surrogate marker of chronic inflammation in obesity. The sources are likely to be apoptotic cells or damaged cells in various tissues from the stress of weight gain, such as the liver, given that total RNA isolated from the obese liver is more potent in inducing TLR3. Moreover, since the minimum length of extracted RNA using our kit was over 200 nt, the possible effect of small RNAs was filtered out. Furthermore, considering cfDNA-activated macrophages through TLR9 are involved in adipose tissue inflammation and insulin resistance [44], P-G3 could unanimously scavenge both cfRNA and cfDNA to alleviate inflammation in obesity.

The scavenging effect of P-G3 on cfRNA mainly depends on its cationic surface that interacts with negatively charged molecules. This cationic nature of PAMAM function is linked to the main concerns regarding its toxicity, which is believed to arise from the binding to and destabilizing of anionic cell membranes, eventually leading to cell lysis [45]. Cytotoxicity largely depends on the concentration, charge density, and structure and varies across models [46]. The half maximal inhibitory concentration (IC₅₀) of P-G3 to induce significant mortality in zebrafish embryos is 2 mg/mL [47], whereas the same polycation shows an IC₅₀ of over 10 mg/mL in L929 mouse fibroblasts [48]. Higher generations of PAMAM have better efficiency in gene or drug delivery but also higher cytotoxicity. Hence, a balance between its efficacy and cytotoxicity should be carefully evaluated when applied to obesity treatment. In the present study, the chosen dose of 10 µg/mL P-G3 did not lead to viability change in the 3T3-L1 and C3H10T1/2 cell lines, nor did it cause any noticeable toxicity *in vivo*, such as changes in food intake

and feeding behavior, locomotion activity, and lean body mass. Therefore, we believe that the anti-obesity effect of P-G3 is not due to the complications or consequences of toxicity. Furthermore, neither the enriched distribution of P-G3 in eWAT nor the local injection of HSA-PG3 into iWAT caused inflammatory macrophage infiltration. In contrast, P-G3 stimulated an anti-inflammatory response to alleviate chronic inflammation in the obese adipose tissue microenvironment.

Nonetheless, we are fully cognizant of the safety concerns of polycationic PAMAM in treating obesity. The pharmacokinetic and pharmacodynamic properties and efficacy can be improved by modifying the exterior of PAMAM with various chemicals or biomolecules or further fabricating them into nanoparticles [49,50]. For example, we have previously shown that cationic nanoparticles can be more efficiently distributed into inflamed tissues to more effectively block the TLR9 pathway [31]. Here, we introduced a new manipulation of PAMAM by forming microspheres in complex with HSA. Albumin is produced by the liver and is highly present in the serum and is recognized as an excellent drug delivery system due to advantages such as high drug loading capacity, nontoxicity, and low immunogenicity. It can be easily prepared into well-defined sizes from nanoparticles to microspheres for different application contexts. Albumin microspheres ranging from several micrometers to several hundred micrometers hold benefits such as the controlled release of cargo, specific targeting of a desired tissue or organ, protection of the payload from degradation or clearance, and increased biocompatibility [51–53]. In our study, HSA was employed as the carrier of P-G3 to form microspheres with a diameter of approximately 1 µm. This modification improved the adipose tissue distribution and retention of P-G3, showing a potent and highly specific effect on reducing focal adiposity in our proof-of-concept study. Future engineering of polycationic materials with albumin may further improve the potency and reduce the administration frequency in treating adiposity.

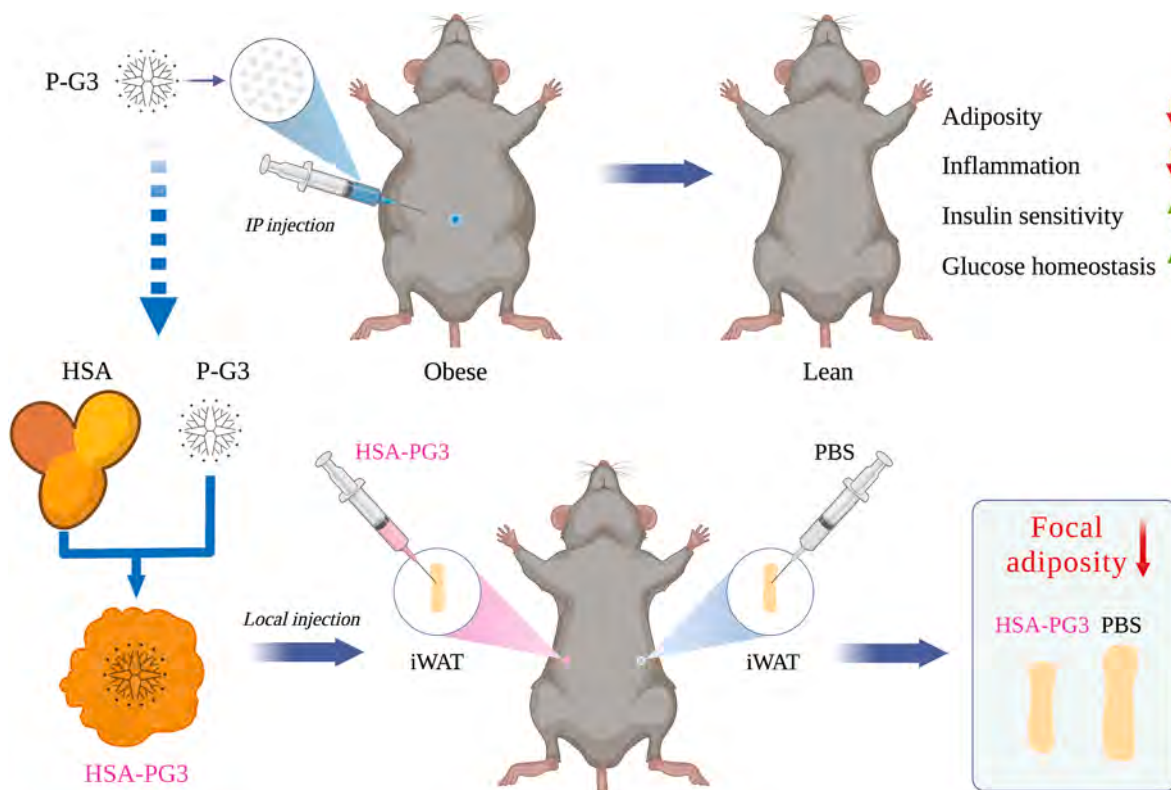
The preferential enrichment of systemic IP delivery into visceral fat depots, together with the specific distribution of local injection into focal fat depots, is an intriguing characteristic of polycationic PAMAM. In a parallel study, we demonstrate that the cationic charge is required for interacting with the highly negatively charged extracellular matrix of adipose tissue, accounting for the adipose-specific distribution. The cationic charge-dependent distribution to adipose tissue can be leveraged to address the challenges of obesity treatment. PAMAM has been extensively studied as delivery vehicles, and the globular interior of high-generation PAMAM is suitable for loading bioactive compounds [54]. Using P-G3 to deliver fat-manipulating reagents and gene therapies into a targeted location may achieve the additive benefit of inhibiting adiposity. Further, the strong suppression of the lipogenesis program by P-G3 may facilitate the discovery of novel mechanisms to manipulate adipocytes. In summary, the present study documents the potential of polycationic materials in treating metabolic diseases, with the dual benefits of alleviating chronic inflammation and inhibiting adiposity, particularly in a depot-specific manner.

Credit author statement

K.W.L. and L.Q. conceived and supervised the study. B.H. and Q.W. together with L.Q. designed the study and wrote the manuscript. T.L. designed and characterized the polycationic materials. B.H., Q.W., and L.Y. performed the experiments. W.D. performed the immunohistochemistry staining. K.W.L. and C.C. revised the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kam W. Leong, Li Qiang, Tianyu Li, Baoding Huang, Qianfen Wan has patent Polycation complexing with HSA to treat adiposity pending to N/A.



Scheme 1. Systemic intraperitoneal administration of P-G3 was first found to reduce adiposity, alleviate inflammation, and improve metabolic functions in DIO mice. Further on, when complexed with HSA, the resultant HSA-PG3 attained controlled release and the local injection of the microparticles potentially inhibited the enlargement of subcutaneous iWAT.

Data availability

Data will be made available on request.

Acknowledgments

Cartoons in Scheme 1, Figs. 1A, 2A and 5B, and 6B were created with BioRender.com. This research was supported by the Blavatnik Foundation for SIRS (L.Q. and K.W.L.), the Russell Berrie Foundation (L.Q. and Q.W.), the National Institutes of Health grant R01AR073935 and USAMR W81XWH1910463 (K.W.L.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2022.121850>.

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