Supporting Information

Surface Tethering of Inflammation-Modulatory Nanostimulators to Stem Cells for Ischemic Muscle Repair

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SI METHODS

Synthesis of HA-g-C18

Sodium hyaluronate (FMC BioPolymer) (100 mg, 0.25 mmol of –COOH) was dissolved in formamide (5 mL) by sonication for 30 min and gentle heating. After the solution was cooled to room temperature, two molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (96 mg) and *N*-hydroxysuccinimide (58 mg) were added and stirred for 2 h to activate the carboxylic group. Then, octadecylamine (3.6 mg, 12.5 μmol) dissolved in anhydrous DMF (1 mg mL^{-1}) was added slowly to the solution. To label hyaluronic acid (HA) with rhodamine B, lissamine rhodamine B ethylenediamine (1.6 mg dissolved in DMSO, 50 mg mL^{-1}; Invitrogen™) was added to the reaction mixture. Then, the reaction mixture was stirred at 60 °C under a nitrogen atmosphere for 5 h and an additional 24 h at room temperature. The resultant mixture was dialyzed against excess amount of water/ethanol for 2 days and distilled water for another 2 days. Finally, the solution was filtered with a 0.45 μm filter to remove other impurities, followed by lyophilization. The modified HA is referred to as HA-g-C18. To determine the degree of substitution by ^{1}H NMR analysis, HA-g-C18 was dissolved in deuterium oxide (Cambridge Isotope Laboratory) at a concentration of 10 mg mL^{-1}. ^{1}H NMR (500 MHz, D_2O, 22 °C): δ 3.3-4.0 (m, hyaluronic acid sugar backbone), 3.2 (m, $CH_2(CH_2)_{16}CH_3$), 2.9 (m, $CH_2(CH_2)_{16}CH_3$), 2.05 (s, -$COCH_3$ of hyaluronic acid), 0.85 (t, $CH_2(CH_2)_{16}CH_3$).

Kinetic analysis of liposome tethering to ADSC surface

ADSCs (240,000 cells per group) were mixed with varying concentrations of liposomes labeled with NBD (*i.e.*, 0.1, 0.2, 0.5, 1 mg/mL DPPC). Cell density in the suspension was kept constant at 1 million cells per mL of liposomes suspension. The mixture was incubated for 15 min at 37ºC. The excess liposomes in the suspension were removed by centrifuging the cells and replacing the supernatant with fresh media. The number of liposomes in the supernatant was measured using a microplate reader with a fluorescence setup. To determine the dissociation rate constant, the cell suspension was centrifuged at 5, 10, 15 and 30 min after the replacement with fresh media. Then, the number of liposomes dissociated from the cells was counted using with a calibration curve of fluorescence intensity against number of liposomes labeled with NBD in media.

Preparation of TNFα-releasing HA-liposomes with different TNFα concentrations

Liposomes were prepared by a film hydration method followed by vortex mixing and extrusion. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; Avanti Polar Lipids) was dissolved with chloroform at a concentration of 10 mg/mL in a round-bottom flask. Chloroform was removed by rotary evaporation to form a thin lipid film. Final masses of TNFα ranging from 0 to 2,500 ng per mg liposome (GenScript, USA) was added to HA-g-C18 (2 mg/mL) dissolved in cell culture media. The film was then hydrated with the aqueous mixture of protein and HA-g-C18 at 50 °C, which is above the transition temperature of DPPC. The lipid concentration was kept constant at 1 mg/mL.

Kinetic release of TNFα from liposomes

Liposomes were suspended in PBS at 1 mg mL^{-1} and placed in a cellulose ester dialysis membrane with molecular weight cut-off of 100,000 Da. The bag was submerged into a bottle containing 10 mL PBS (pH 7.4) and placed on a rotary shaker in a 37 °C incubator. At designated intervals (4, 8, 24 h, 2, 3, 4, 5 days), 1 mL of release media was collected and replaced with 1 mL of fresh buffer. TNFα in the release media was measured using an enzyme-linked immunosorbent assay (R&D Systems, USA).

Quantification of VEGF, PEDF and PGE2 in the presence of anti-TNFα antibodies

Anti-TNFα antibodies (Abcam, USA) were added to ADSCs culture media to form a final concentration of 4 μg/mL antibodies. Separately, collagen gels were prepared in a sterile condition. First, 200 μL of type I bovine collagen solution was mixed with 100 μL of culture media. The mixture was pipetted without the formation of air bubbles until the phenol red changed to a homogenous yellow color. Then, 25 μL of reconstituted solution was gently mixed with the collagen solution until the phenol red became pink. The pre-gel solution was quickly transferred to a pre-cooled 96 well plate and incubated at 37 °C for 3 h. To tether the liposomes, 160,000 ADSCs per group were counted and mixed with liposomes by gently shaking at 37 °C for 15 minutes. After 15 minutes, the cells were centrifuged (200 × g, 5 min) and re-suspended with anti-TNFα antibodies-containing media to a concentration of 100,000 cell mL^{-1}. Then, 100 μL of cell suspension was added to the pre-made collagen hydrogels. Cell culture media was collected after 24 h. The amount of VEGF and PDEF were measured using DuoSet® enzyme-linked immunosorbent assay development system (R&D Systems, USA). PGE2 was measured using Prostaglandin E2 Parameter Assay Kit (R&D Systems, USA).

Fabrication of microfluidic device

The design of microfluidic devices was prepared using the AutoCAD software. The microfluidic devices five 200-μm-deep microchannels consisting of a 1000 μm-wide central hydrogel region ③, flanked by 700 μm channels ② and ④. The two outer channels ① and ⑤ are 750 μm-wide. The device was fabricated with polydimethylsiloxane (PDMS; Dow Corning, Sylgard 184) using soft lithography and patterned SU-8 silicon wafers. Inlet and outlet ports were created with biopsy punches. A cover glass was bonded to the PDMS after 60 s of oxygen plasma treatment. The devices were cured at 70ºC for 4 hours and sterilized by UV irradiation before each experiment.

Quantification of VEGF and PEDF from muscle tissues

The amount of VEGF and PDEF in the gastrocnemius muscle were measured using DuoSet® enzyme-linked immunosorbent assay kit (R&D Systems, USA). The entire gastrocnemius muscle was isolated from the leg and weighed. Then, the tissue was homogenized in 1 mL PBS to form a suspension using a rotor-stator homogenizer. The suspensions were either used immediately for the assays or stored at -20 °C. To perform the assay, 96-well microplates

were coated with the primary antibody diluted in PBS. The plates were incubated overnight at room temperature. Free binding sites were blocked with 0.1% bovine serum albumin in PBS. 100 μL of homogenized muscle suspension or standards were added to each well. After incubation for 2 h at room temperature, biotinylated primary antibody was added, followed by the addition of streptavidin-horseradish peroxidase. The plate was washed before each step with 0.05% Tween 20 in PBS. A substrate solution consisting of an equal mixture of Color Reagent containing H_2O_2 and Color Reagent containing tetramethylbenzidine (R&D Systems) was added and incubated for 20 min at room temperature. The reaction was stopped by adding 50 μL of 2 N H_2SO_4. The color intensity was measured immediately using a microplate reader (Tecan M200 Pro) set at 450 nm (with the background correction wavelength set to 570 nm).

Histological analysis of muscle tissues

The presence of macrophages in the gastrocnemius muscles were identified by immunohistochemistry using F4/80 antibodies (Proteintech, USA). Muscle tissues were fixed using paraformaldehyde and embedded in paraffin blocks. The staining was conducted as recommended by the manufacturer. The tissues were counterstained with hematoxylin.

SI EQUATIONS

For FRET analysis

Equation S1: $\text{FRET Efficiency} = 1 - \frac{\text{Intensity of modified liposomes with HA} - \text{g} - \text{C18 or HA at 530 nm}}{\text{Intensity of nitrobenzoxadiazole} - \text{labelled unmodified liposomes only at 530 nm}}$

For kinetic analysis of liposome tethering to ADSC surface

Equation S2: $\frac{C_{L0}}{N_c} = \frac{1}{N_{RT}} C_{L0} + \frac{K_D}{N_{RT}}$

C_{L0}: initial liposome concentration

N_c: number of liposome-cell complexes

N_{RT}: total number of receptors

K_D: dissociation equilibrium constant

Equation S3: $\ln\left(\frac{N_c}{N_{c0}}\right) = -k_{-1}t$

N_c: number of liposome-cell complexes

N_{c0}: initial number of liposome-cell complexes

k_{-1}: dissociation rate constant

Equation S4:

Time required for the concentration to dissociate half of its maximum, $t_{1/2} = \frac{\ln 2}{k_{-1}}$

SI FIGURES

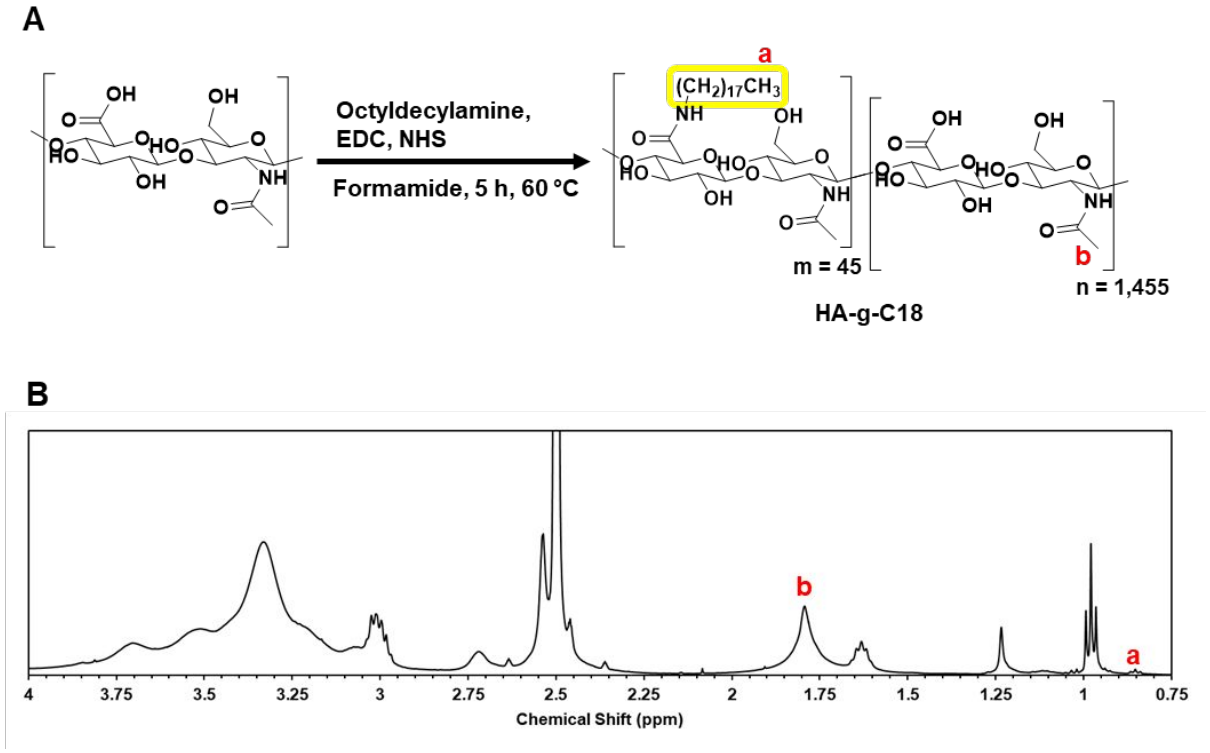

Figure S1. Synthesis and characterization of HA-g-C18. (a) Synthesis of HA-g-C18 formed by amide coupling between the uronic acid groups on hyaluronic acid and amine group of octadecylamine. (b) NMR spectrum of hyaluronic acid before (i) and after (ii) grafting of octydecyl chains.

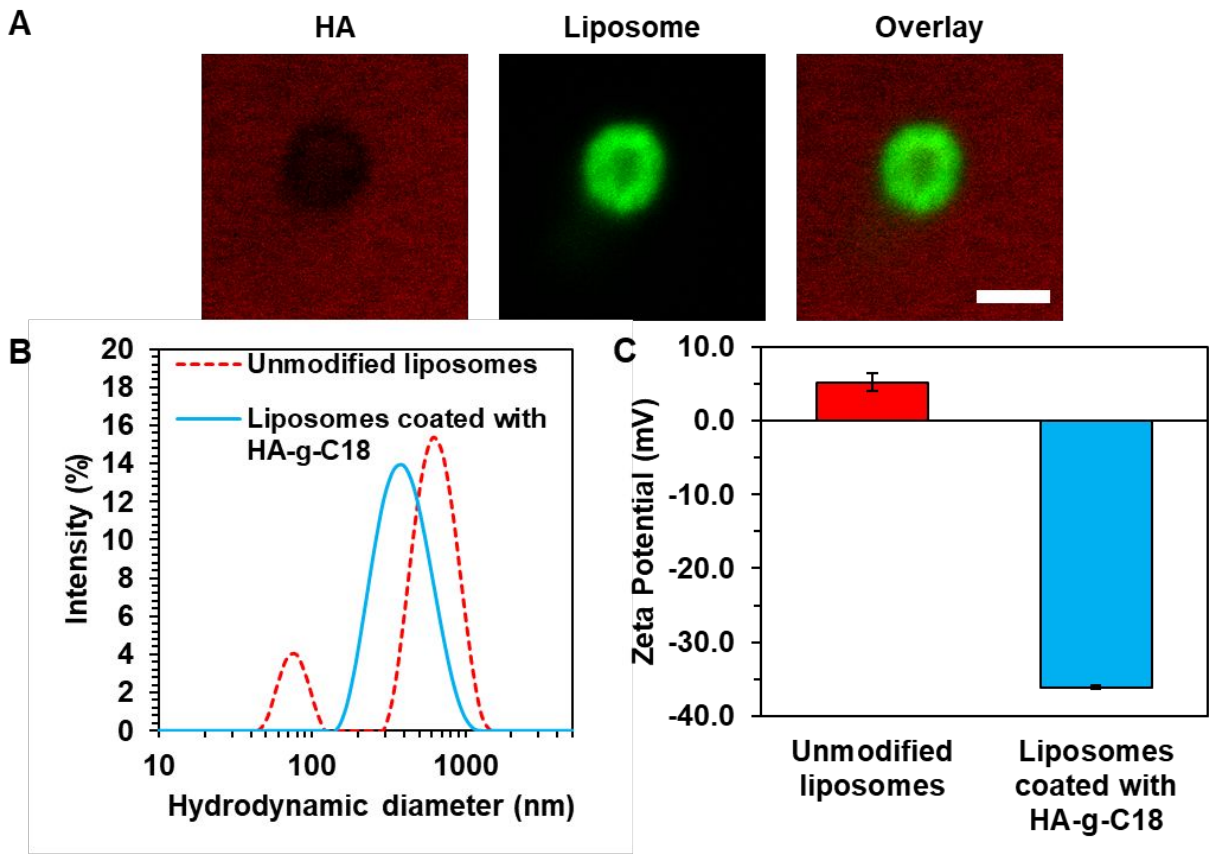

Figure S2. Fluorescence images of liposomes mixed with HA where HA molecules were labeled with rhodamine B (Rh in red) and the lipid layer was labeled covalently with nitrobenzoxadiazole (NBD) (in green). The overlay panel shows the merged images. Images were taken with after mixing HA and liposomes at a mass ratio of 2:1. Scale bars represent 2 μm. (B) Hydrodynamic diameter and (C) zeta potential in deionized water. Data points represent the mean and error bars indicate standard deviations. N=3.

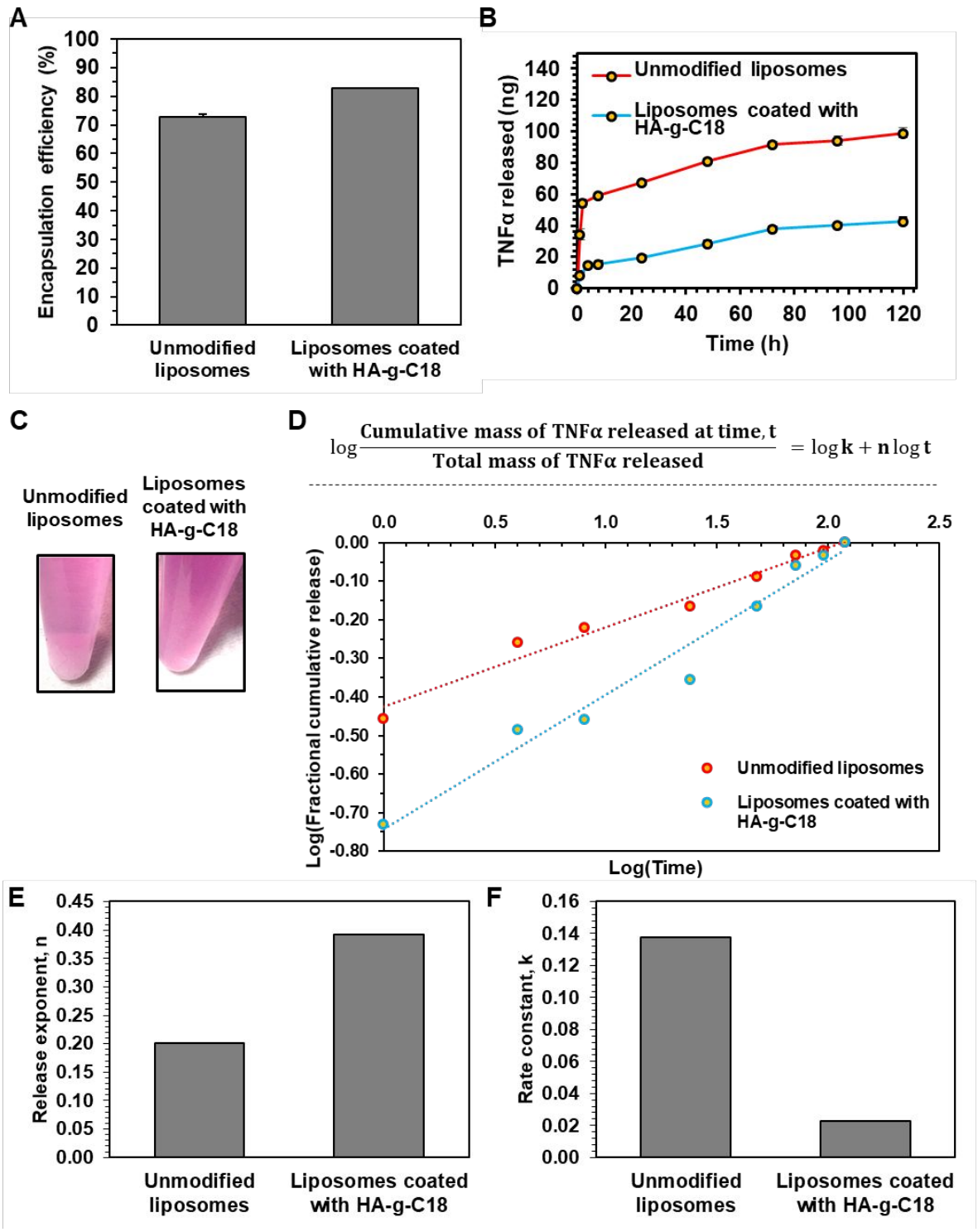

Figure S3. Characterization of TNFα-loaded liposomes. (A) Encapsulation efficiencies of unmodified liposomes and liposomes coated with HA-g-C18. (B) Cumulative release of TNFα protein molecules from liposomes with HA-g-C18 (in blue) or without HA-g-C18 (in red) after incubation at 37 °C. The released proteins were quantified using ELISA. Data points represent the mean and error bars represent standard deviations. N = 3. (C) Photographs of unmodified

liposomes and liposomes coated with HA-g-C18 suspended in media for 4 h. (D) TNFα release profile from unmodified liposomes (in red) and HA-liposomes (in blue) against the empirical model for TNFα transport from liposomes. *k* represents the release rate constant, and *n* represents the release exponent. For spherical liposomes, $n \leq 0.43$ corresponds to Fickian diffusion, $0.43 < n < 1.00$ corresponds to non-Fickian transport, $n = 1.00$ corresponds to zero-order release. The plot shows the data points from the release profile from t = 8 to 120 h. (E) Release exponent, *n*, and (F) release constant, *k*, of unmodified liposomes and liposomes coated with HA-g-C18.

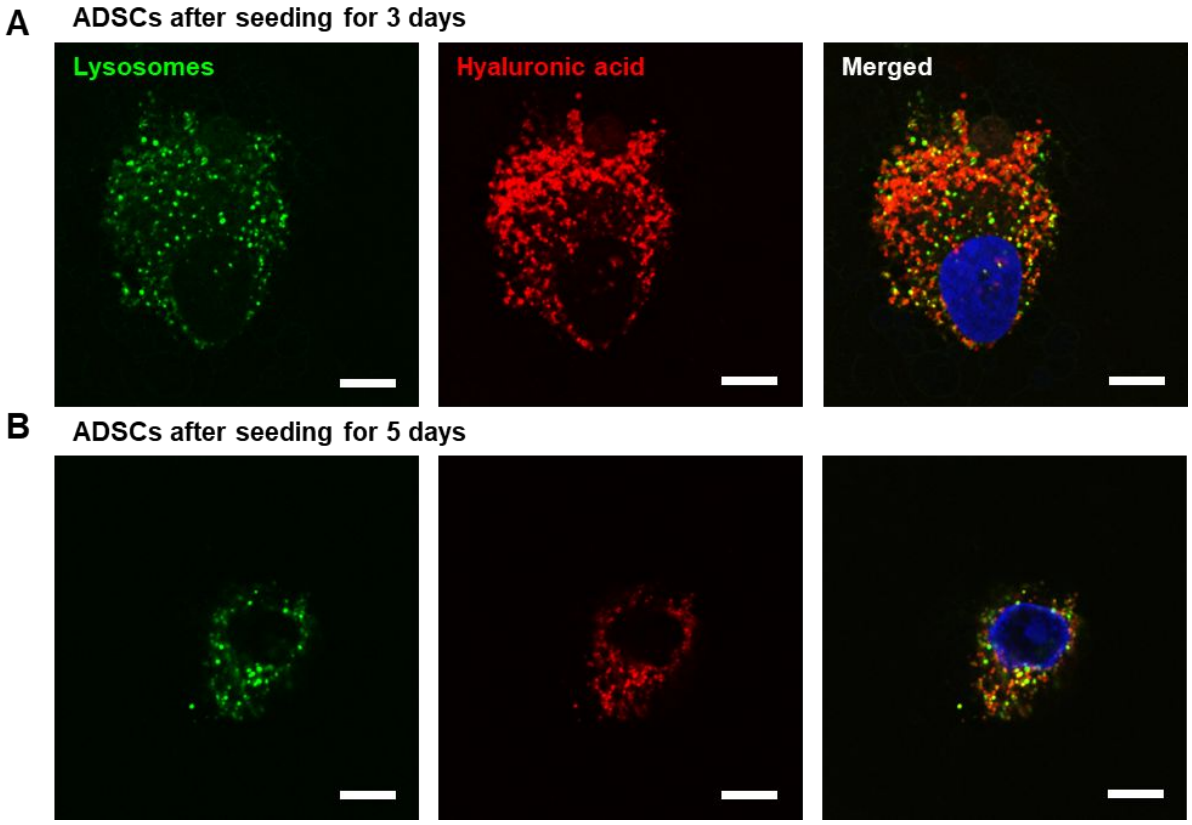

Figure S4. Analysis to determine cellular internalization of liposomes coated with HA-g-C18. Lysosomes were labeled with LysoTracker Green while liposomes were labelled with rhodamine B-conjugated HA-g-C18 (red). ADSCs were incubated on collagen hydrogels for (A) 3 days and (B) 5 days. The last column shows the merged images of the first two columns. Scale bars represent 10 μm.

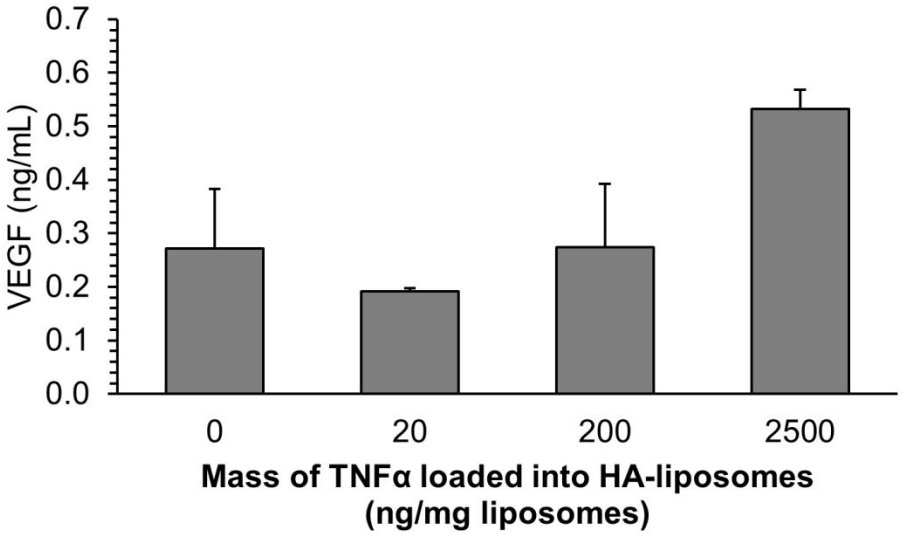

Figure S5. Quantification of VEGF secreted by ADSCs tethered with TNFα-releasing HA-liposomes containing TNFα ranging from 0 to 2,500 ng per mg liposomes. The measurement was made after incubating the tethered cells in the cell culture media over 24 h. Data points represent the mean and error bars represent standard deviations.

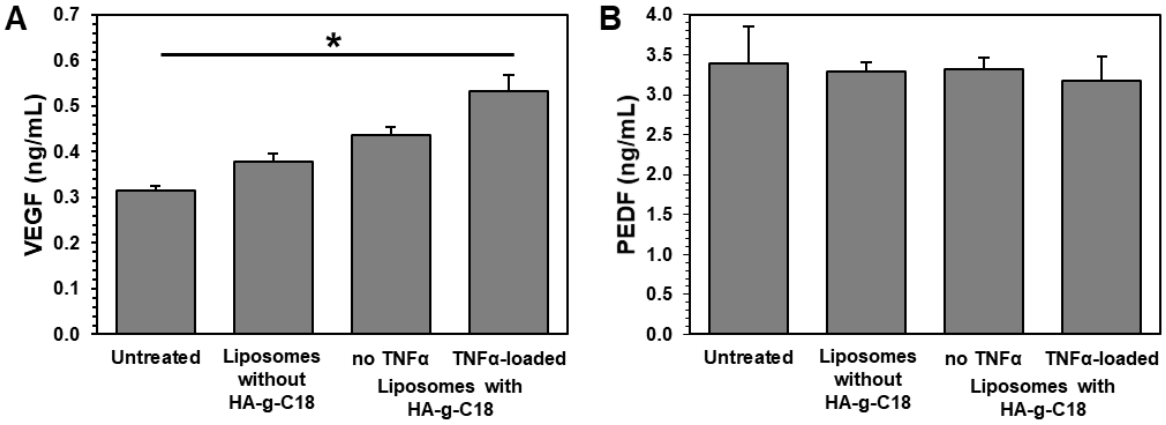

Figure S6. Quantification of (A) pro-angiogenic VEGF and (B) anti-angiogenic PEDF secreted by ADSCs using ELISA after 24 h. Data points represent the mean and error bars indicate standard deviations. N = 3, * represents the statistical difference between ADSCs tethered with TNFα-releasing HA-liposomes and untreated ADSCs. $p < 0.05$.

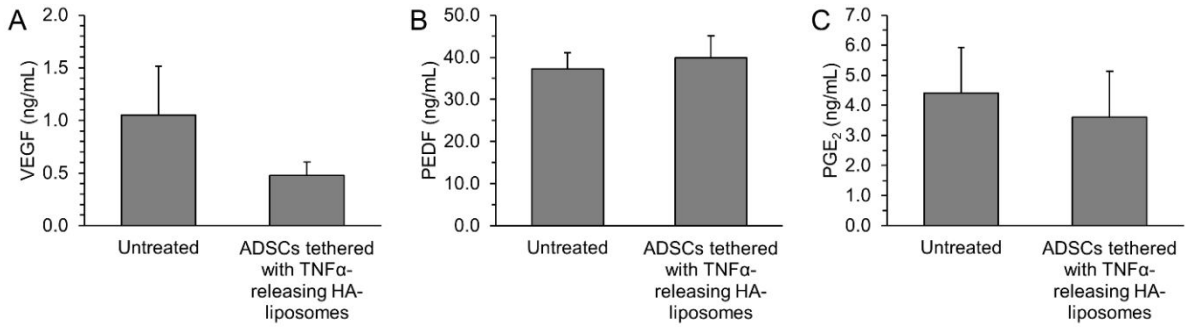

Figure S7. Quantification of (A) pro-angiogenic VEGF, (B) anti-angiogenic PEDF, and (C) immunomodulating PGE_2 secreted by ADSCs using ELISA after 24 h incubation in the presence of anti-TNFα antibodies. Data points represent the mean and error bars represent standard deviations. N=3. There is no significant difference in protein concentration between untreated ADSCs and ADSCs tethered with TNFα-releasing HA-liposomes.

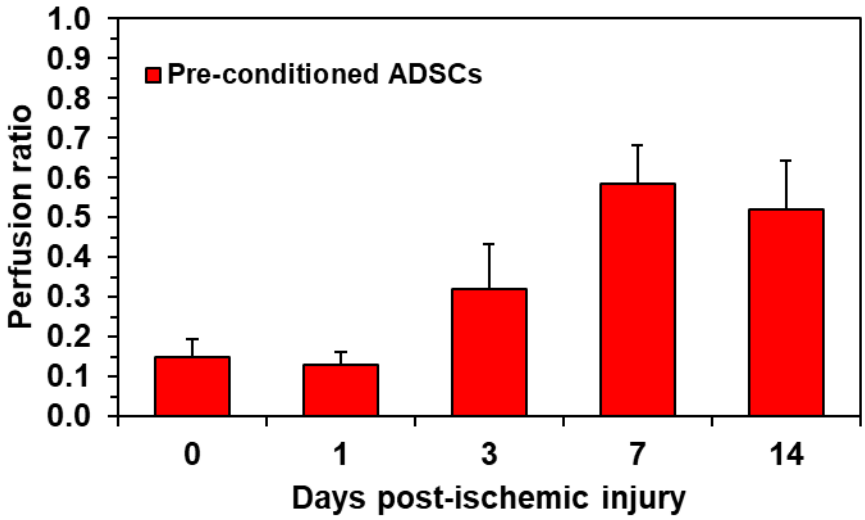

Figure S8. Quantification of the mean perfusion ratio defined as the perfusion in the ischemic limb divided by the perfusion in the non-ischemic limb. (N=5 mice).

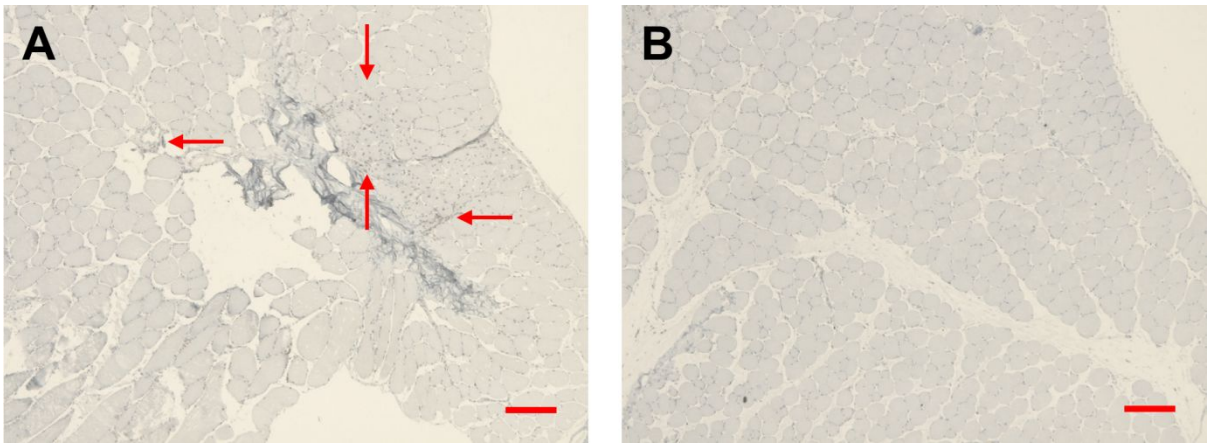

Figure S9. Immunohistochemical analysis of ischemic tibialis anterior 14 days after ischemic injury and injection of (A) PBS and (B) ADSCs tethered with TNFα-releasing HA liposomes. Brown colored cells are marked by antibodies against mouse F4/80 which is a glycoprotein only expressed on the surface of macrophages (pointed with red arrows). Muscle tissue are counter-stained with hematoxylin. Scale bars represent 200 μm.

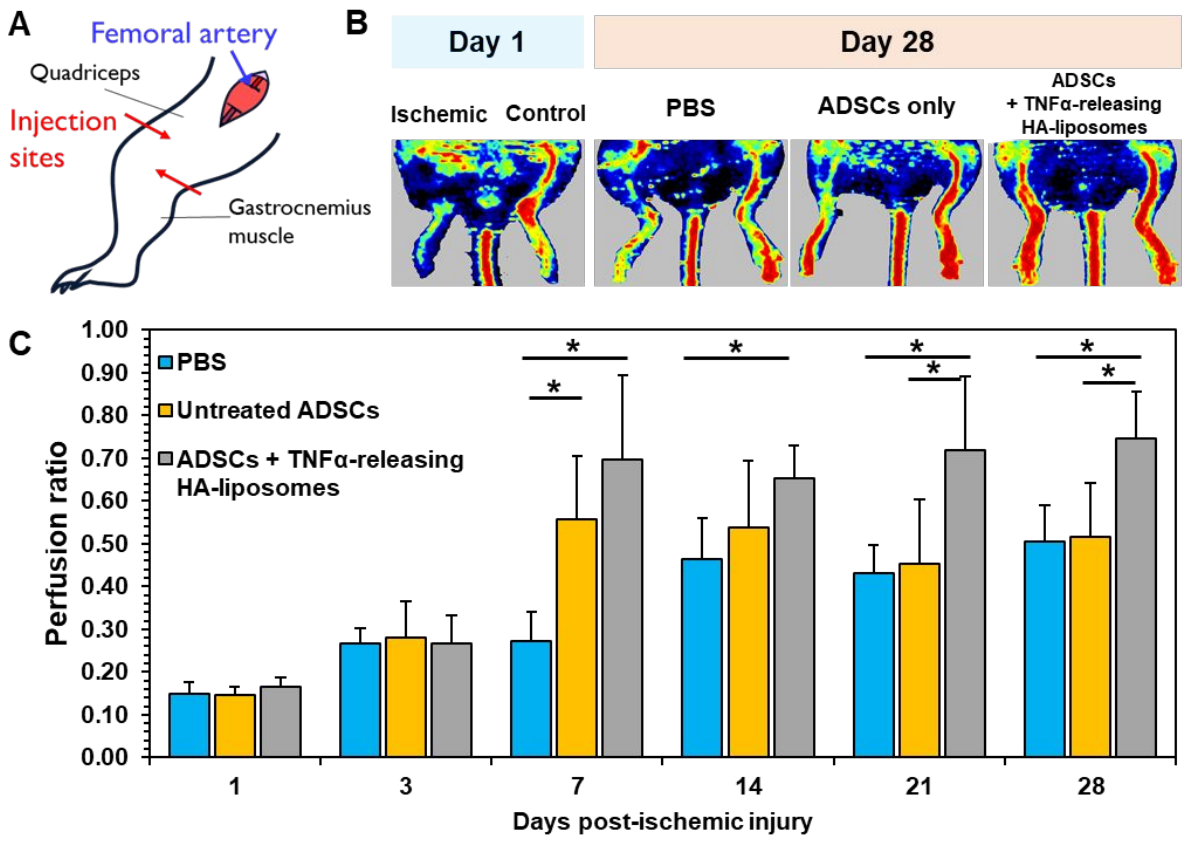

Figure S10. Laser Doppler perfusion imaging of mice induced with ischemic hindlimb injury. (A) LDPI images of mice after the ischemic hindlimb surgery. Ischemia was introduced by ligating the femoral artery in the right leg. The red color intensity represents the relative intensity of perfusion. The ischemic leg imaged after 24 h and after it was treated for 28 days with 1 million ADSCs, 1 million untreated ADSCs, and 1 million ADSCs tethered with TNFα-releasing HA liposomes. (A) Quantification of the mean perfusion ratio defined as the perfusion in the ischemic limb divided by the perfusion in the non-ischemic limb. (N = 7-8 mice, $p < 0.05$).

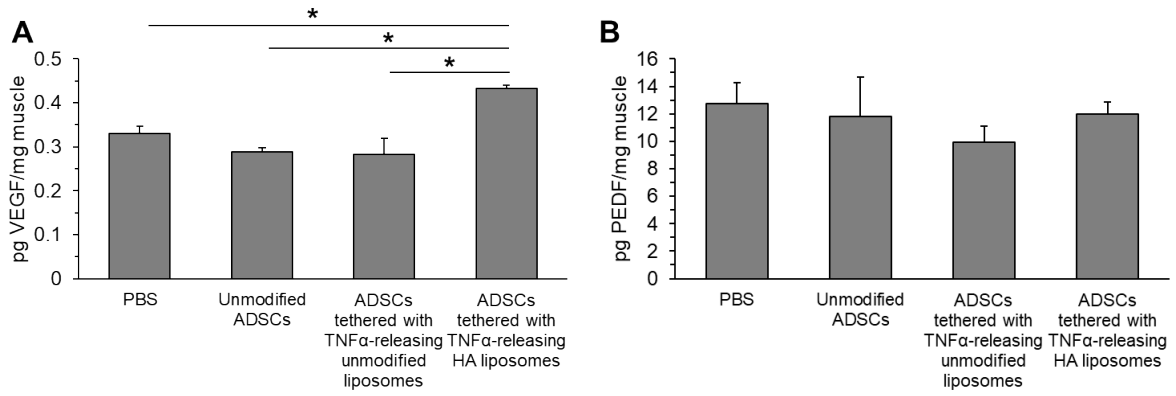

Figure S11. Quantification of (A) pro-angiogenic VEGF and (B) anti-angiogenic PEDF levels in the muscle after 14 days of vascular ligation. Data points represent the mean and error bars represent standard deviations. N=3, * represents the statistical significance between the conditions indicated. *$p < 0.05$.