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Supplementary Information for

Functional Muscle Recovery with Nanoparticle-Directed M2 Macrophage Polarization in Mice

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Supplementary Information Text

SI Materials and Methods

Macrophage Depletion. Clodronate and PBS liposomes were purchased from Liposoma (SKU: LIP-01). Liposomes (70uL) were administered retro-orbitally 2 days before surgery, and again on days 1 and 5 after surgery (Fig. S1A); 10uL were also administered intramuscularly into the ischemic TA 1 day before surgery, and again on days 2 and 7 after surgery.

Blood Perfusion. Measurements of blood perfusion were performed using a Laser Doppler Perfusion Imaging (LDPI) analyzer (PeriScan PIM II; Perimed) on anesthetized mice.

Flow Cytometry. To assess cell recruitment and phenotype in the TAs, a single cell suspension was prepared using the skeletal muscle dissociation kit (MACS Miltenyl Biotec, # 130-098-305) with the gentleMACS^{TM} dissociator. Murine cells were blocked with CD16/CD32 monoclonal antibody (eBioscience # 14-0161-82) and then stained to assess immune cell presence, with the following antibodies CD45.2-FITC or CD45.2-PerCP/Cy5.5 (BioLegend #109828), CD3-PE/Cy5, CD4-PacBlue, CD11b-APC/Cy7 or CD11b-Pacific Blue (BioLegend #101224), Ly6C-PE/Cy7 or Ly6C-PE (BioLegend #128008), Gr-1-APC, F4/80-PE or F4/80-APC (BioLegend #123116), Ly6G-AlexaFluor 488 (BioLegend #127626), CD11c-APC/Cy7 (BioLegend #117324) at the concentrations recommended by the manufacturer to assess cell recruitment. A separate panel was used to assess macrophage polarization: CD86-FITC (eBioscience #11-0862-82 or BioLegend #105006), CD206-APC (BioLegend #141708), F4/80-PE (eBioscience #12-4801-82) or F4/80-PerCP/Cy5.5 (BioLegend #123128), CD11b-APC/Cy7 (BioLegend #101226), CD80-Pacific Blue (BioLegend #104724), and CD163-Cy3 (Bioss Antibodies #bs-2527R-Cy3) or CD163 (Santa Cruz #18796) + PerCP/Cy5.5 secondary (Santa Cruz #45102) at the concentrations recommended by the manufacturer. Unstained cells, fluorescent minus one controls, and the appropriate isotype controls recommended by the manufactures were used as controls in the gating. To identify recruited immune cells the following definitions were used: myeloid cells (CD11b+/CD11c-), macrophages (CD45+/CD11c-/CD11b+/Ly6G-/Ly6C-/ F4/80+) or (CD45+/CD11b+/CD3-/F4/80+) depending on the staining panel, monocytes (CD45+/CD11c-/CD11b+/Ly6G-/Ly6C+/ F4/80-) or (CD45+/CD11b+/CD3-/F4/80-/Gr1 lo or -) depending on the staining panel, monocyte/macrophages (CD45+/CD11c-/CD11b+/Ly6G-/Ly6C+/ F4/80+), neutrophils (CD45+/CD11b+/CD3-/F4/80-/Gr1^{hi}/Ly6C^{lo}), T cells (CD45+/CD11b-/CD3+), patrolling monocytes (CD45+/CD11b+/CD3-/F4/80-/Gr1-/Ly6C^{lo} or -). CD206 expression on macrophages was used as an indicator of M2a polarization, CD163 as an indicator of M2c polarization, and CD80 and CD86 as indicators of M1 polarization. Cells were fixed and stored in 0.4% PFA at 4^{o}C until they were run on an LSR II flow cytometer, within 3 days following staining.

For in vitro polarization studies, human THP-1 derived macrophages were stained with near-IR fixable dead cell stain (Invitrogen #L10119). Then they were blocked with Fc receptor blocking solution (Human TruStain FcX^{TM}, BioLegend #422302) and stained with the following antibodies: CD206-PE (BioLegend #321106), CCR7-APC (BioLegend #353214), and CD163-FITC (BioLegend #333618) at the concentrations recommended by the manufacturer. CD206 expression was used as an indicator of M2a polarization, CD163 as an indicator of M2c polarization, and CCR7 as an indicator of M1 polarization on live cells. Unstained cells, and the isotype controls recommended by the manufacture were used as

controls in the gating. Cells were fixed and stored in 0.4% PFA at 4^{o}C until they were run on an LSR II flow cytometer, within 3 days following staining.

Muscle Force Measurements. All animal work was performed in compliance with NIH and institutional guidelines. Intact TA muscles were dissected, and immediately mounted vertically midway between two cylindrical parallel steel wire electrodes (1.6 mm diameter, 21 mm long) by attaching their tendons to microclips connected to a force transducer (FORT 25, WPII) [1]. Muscles were bathed in physiologic saline solution in a chamber with continuously bubbled oxygen at 37^{o}C, and the muscle was adjusted to a physiologically relevant length. A wave pulse was initiated using a custom-written LabVIEW program and delivered to the stimulation electrodes via a purpose built power amplifier (QSC USA 1310). Three tetanic contractions were evoked at 250, 270, 300 Hz and 25, 27, 30 V, respectively, with a constant pulse width of 2ms and a train duration of 1s. The muscle was allowed to rest for 5min between each stimulation. Contraction force was determined as the difference between the maximum force during contraction and the baseline level (Fig. S2). Following ex vivo stimulation muscles were weighed and forces were then normalized to muscle wet weight. Contraction velocity was determined as the slope of the force curve at the time electrical stimulation was initiated (Fig. S2).

AuNP Synthesis. AuNPs were synthesized by the hydroquinone reduction of gold onto citrate stabilized seed particles [2]. Briefly, to make 15nm seed particles, 1mL of 1%w/v gold chloride (Sigma #254169) solution was added to 100mL of milliQ water in a clean Erlenmeyer flask, stirred slowly and heated on a 400^{o}C plate. As soon as the solution came to a boil, the stirring was set as fast as possible without splashing, and 3mL of 1% w/v sodium citrate (Sigma # 4641) was rapidly added. The solution color changed from clear to purple to red over the course of ~5min, a few minutes after the color changes completed, the solution was cooled at room temperature.

The 30, 60, and 100nm-core AuNPs were then synthesized by the reduction of ionic gold onto the 15nm seed particles by hydroquinone. MilliQ water was added to an Erlenmeyer flask followed by 25mM gold chloride solution, and 15nm seed particles. The solution was spun as fast as possible without splashing and then 15mM sodium citrate and 25mM hydroquinone were added simultaneously. The volume of each solution added to the hydroquinone reduction reaction depended on the desired nanoparticle size, and have been published previously [3]. The solution color changed from red to black and then to a darker red. The reaction was completed in a few minutes; then particles were stored at room temperature.

PEGylation and IL-4 conjugation to the AuNPs. AuNPs were sterilized by filtration through a 0.2um filter. Then they were divided into 1mL aliquots in epindorff tubes and pelleted via centrifugation; an aliquot was resuspended at 1x concentration in milliQ water and the DLS size and absorbance were measured. The size and absorbance were used to calculate the AuNP concentration using the Beer-Lambert Law. The remaining AuNPs were then resuspended in milliQ water containing 5kDa methoxy PEG-SH (Laysan Bio, Inc) to a final concentration 15x more concentrated than the AuNP synthesis solution; 2.5 PEG ligands/nm^2 were provided, only enough to partially cover the AuNP core. PEGylation was allowed to occur overnight at room temperature, on a slow shaker.

Following overnight PEGylation, recombinant human or murine IL-4 (Peprotech) was added to the reaction along with trehalose (Sigma #T0167). Enough IL-4 was added to provide 0.5 ligands/nm^2, and the final concentration of trehalose was 10% w/v. IL-4 conjugation was allowed to occur overnight at

room temperature, on a slow shaker. The unbound IL-4 and trehalose were removed by centrifugation, 4 washes in total. The supernatants were collected and the unbound IL-4 in the supernatants was quantified with LavaPep (Gel Company #LP-022010); subtractive analysis was used to quantify the bound IL-4. Following PEGylation and IL-4 conjugation, PA4s were resuspended in milliQ water and the DLS size and zeta-potential were measured.

In vitro IL-4 release. PA4s were incubated in 1% BSA in PBS, or RPMI media (10% HI-FBS, 1% pen/strep, HEPES, sodium bicarb, sodium pyruvate) at 37^{o}C, 5% CO_2. Supernatants were collected and released IL-4 was quantified with ELISAs. After 1 day, size distribution was measured by DLS (Zsizer, Marvern Instruments).

In vitro PA4 bioactivity assay with human THP-1 cells. THP-1 cells (ATCC® TIB-202^{TM}) were cultured in RPMI-1640 media (containing 10% HI-FBS) as recommended by ATCC, except 2-mercaptoethanol was excluded. To differentiate the THP-1 monocytes to macrophages, cells were seeded at 10^6cells/well in non-TC treated 6-well plates with 2mL media/well containing 100ng/mL phorbol 12-myristate 13-acetate (PMA). After 3 days, the PMA-media was removed and the following conditions were used to polarize the macrophages: 20ng/mL soluble IL-4 or 20ng/mL IL-4 delivered as PA4 for M2a polarization, complete RPMI media without additives or AuNP-PEG (dose matched to the AuNP dose in the PA4 condition) for M0 polarization. Polarization media was changed daily.

In vitro THP-1 macrophage polarization switching. After PMA differentiation, the following M1 polarization media was used: 20ng/mL IFN-γ + 2400ng/mL LPS for 2 days. After M1 polarization, the media was replaced with media containing 40ng/mL soluble IL-4, 40ng/mL IL-10, 40ng/mL IL-4 + 40ng/mL IL-10, or RPMI media without additives. Flow cytometry was used to assess polarization after the 2 day M1 polarization and at various timepoints after exposure to IL-4 and/or IL-10. Media was changed daily.

In vitro macrophage polarization in response to varying degrees of IL-4 valency on PA4. Following PEGylation of 30nm-core AuNPs as described above, varying amounts of IL-4 were added to the conjugation reaction to give 1x, 0.5x, 0.1x or 0.01x the amount of IL-4 typically provided per Au surface area, as described above in "PEGylation and IL-4 conjugation to the AuNPs." Conjugation of IL-4 to the AuNP was again quantified by subtractive analysis using LavaPep, also as described above.

After PMA differentiation of THP-1 cells, they were polarized with 20ng/mL IL-4 presented as soluble IL-4 or as the 1x, 0.5x, 0.1x or 0.01x PA4s described above. Basal medial lacking any IL-4 was used as a negative control. Polarization media was changed daily, for 3 days, then macrophage polarization was assessed using flow cytometry.

Histological Assessment of Skeletal Muscle. Following ex vivo muscle force measurements, the TA muscles were fixed in 4% PFA, washed, paraffin embedded and stained with H&E. H&E stained cross sections were imaged with a 10x lens on an Olympus-IX81 light microscope connected to a Olympus DP70 digital image capture system, and images were tiled across the entire muscle cross section. To assess the percentage of the muscle cross sectional area that was composed of muscle fibers and the percentage that consisted of cell nuclei, color deconvolution was performed with ImageJ. A threashold was then applied to the pink (eosin) color channel such that only muscle fibers were included in the threashold and background was excluded. The "analyze particles" function in ImageJ was then used to

quantify the area within the threashold. To assess the nuclear area, the blue (hematoxylin) channel was processed by first increasing the contrast, then applying a threashold to include nuclei and exclude background. The threasholded image was then made binary, and finally the "watershed" function in ImageJ was used to separate distinct cell nuclei. Again the "analyze particles" function was used to quantify the area within the blue (hematoxylin) threashold. The total cross sectional area of the muscle was measured in ImageJ by using freehand selection and the percentage of that cross section falling within the eosin and hematoxylin channels was calculated. The empty area was calculated by subtracting the muscle fiber area and the nuclear area from the total muscle cross sectional area. The investigator was blinded to treatment before performing the ImageJ analysis.

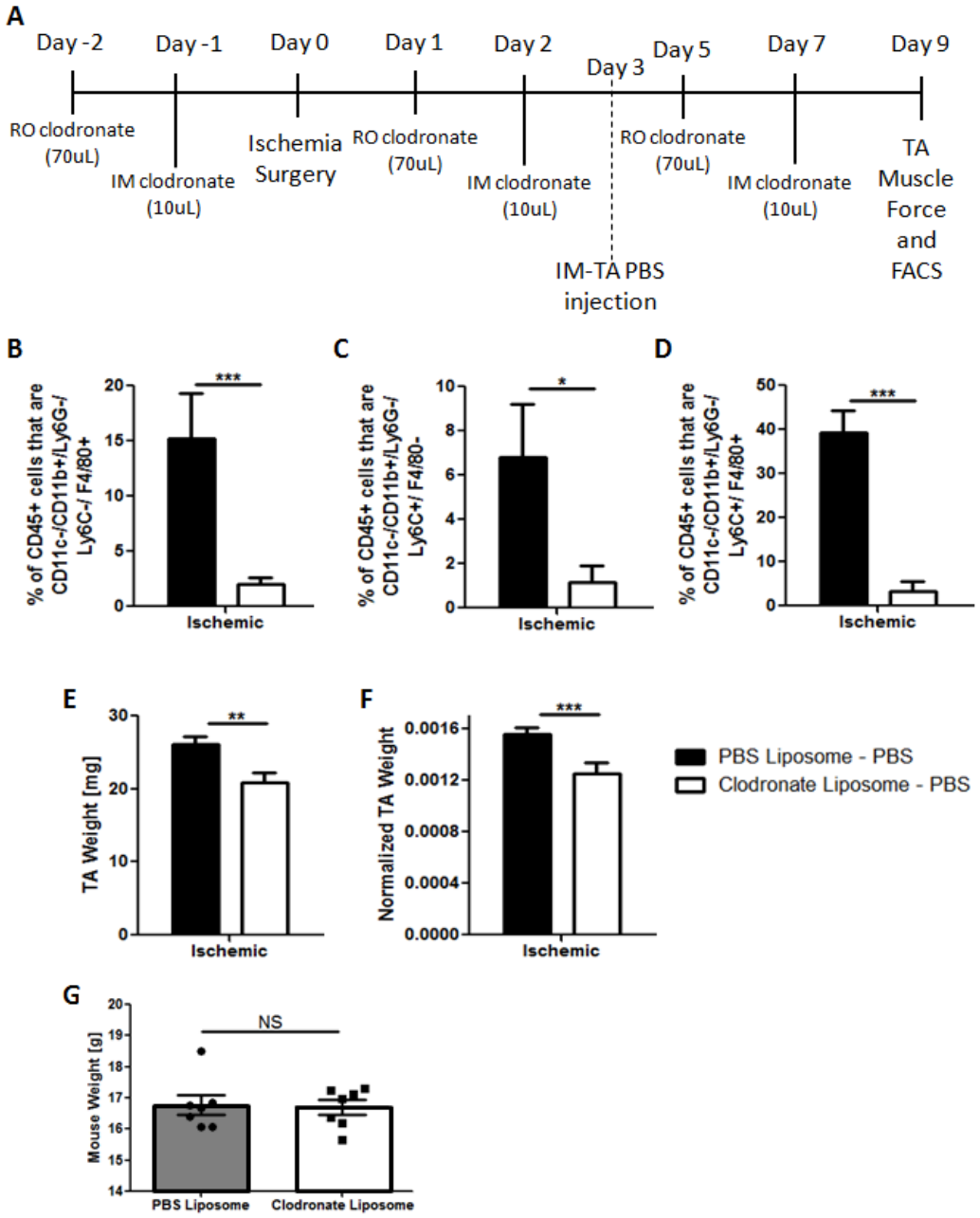
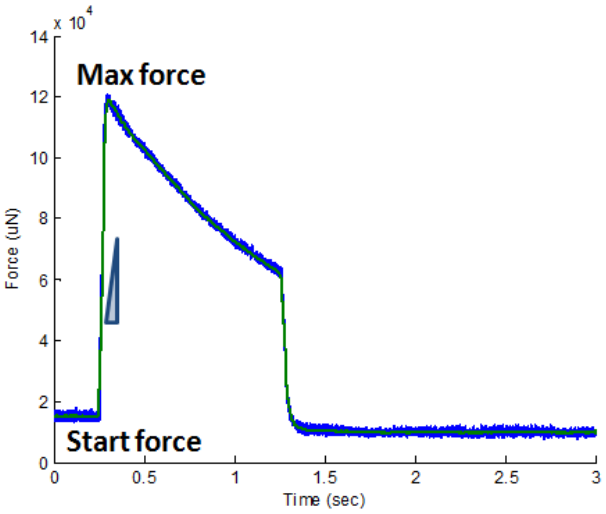


Fig. S1. Clodronate depletes monocyte/macrophages and inhibits muscle regeneration. (A) Experimental design showing clodronate and PBS-treatment schedule in relation to the surgical induction of ischemia and muscle assessment. (B-D) The percentage of CD45+ immune cells in the TAs that were macrophages (CD11c-/CD11b+/Ly6G-/Ly6C-/ F4/80+) (B), monocytes (CD11c-/CD11b+/Ly6G-/Ly6C+/ F4/80-) (C), and monocyte/macrophages (CD11c-/CD11b+/Ly6G-/Ly6C+/F4/80+) (D), quantified by flow cytometry on day 9 after surgery. (E) The weight of the TA muscle isolated from the ischemic limb 9 days after surgery, following treatment with PBS-liposomes (control, black bar), or clodronate-liposomes (white bar). (F) The weight of the TA was normalized to the weight of the mouse. (G) Mouse weight on day 9 after surgical induction of ischemia. All data are means ±SEM of 7 mice per group. *P < 0.05, * P < 0.01, and ***P < 0.001

A



B

$$\text{Maximum Force [uN/mg]} = \frac{\text{Max force – Start force [uN]}}{\text{TA weight [mg]}}$$

C

Contraction Velocity = slope at 0.26s

Fig. S2. Contraction force curve data from ex vivo tetanic stimulation of TA muscles. (A) Intact TA muscles were mounted between 2 parallel steel wire electrodes, and connected by microclips to a force transducer. A pulse wave was used to stimulate the muscle at 25V, 250Hz; 27V, 270Hz; and 30V, 300Hz for 1s. Each muscle was tested at all 3 stimulations with 5min rest between each test. The contraction force curves (blue) were smoothed in MATLAB (green line). (B) The maximum force was determined to be the difference between the maximum force recorded and the starting baseline, and was normalized by the TA mass. (C) The contraction velocity was calculated as the slope of the force curve at the time of stimulation, 0.26s.

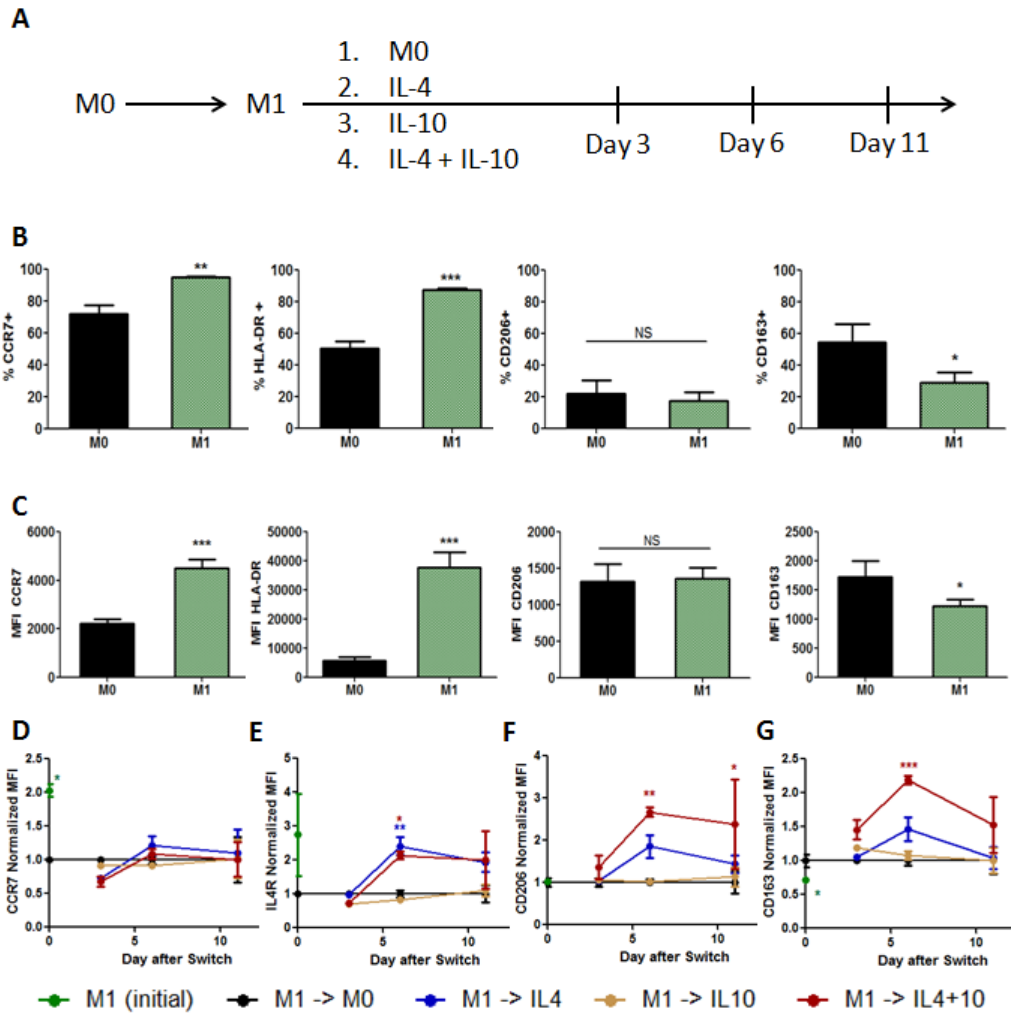
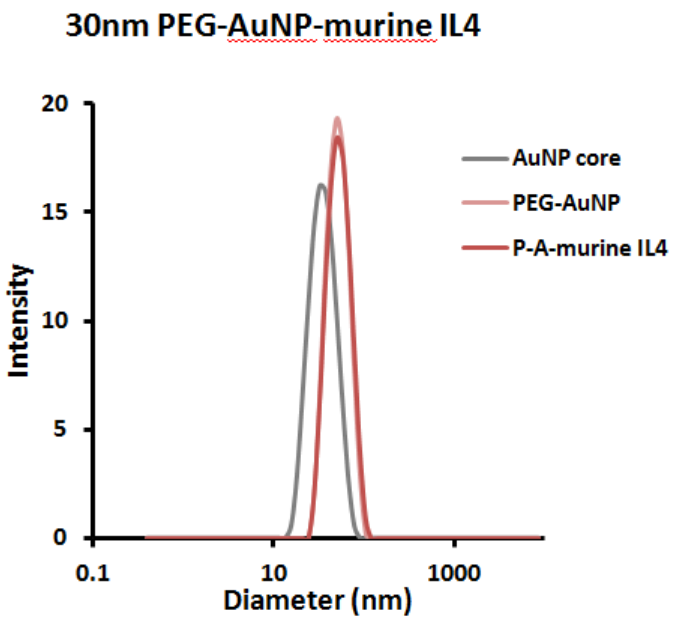


Fig. S3. IL-4 and IL-10 can shift an M1 macrophage population towards the M2 phenotype. (A) THP-1 monocytic cells were differentiated to M0 macrophages (3 days with 100ng/mL PMA) and then polarized towards the M1 phenotype (2 days with 2400ng/mL LPS, and 20ng/mL IFN-gamma). M1 macrophages were then exposed to 40ng/mL IL-4, 40ng/mL IL-10, 40ng/mL of each cytokine, or to complete media with no additives (M0) for 3, 6, or 11 days and then analyzed by flow cytometry. (B) The percentage of the initial M1 and M0 macrophage populations that were expressing markers of M1 polarization (CCR7, HLA-DR) and M2 polarization (CD206, CD163) were assessed with flow cytometry. (C) Median fluorescent intensity (MFI) per cell of the initial M1 and M0 populations. Data are means ±SD of n=3. *P < 0.05, **P < 0.01, ***P < 0.001 (D-G) Macrophage MFI data were normalized by the time matched M0 condition to allow for accurate comparison across different timepoints. The expression levels of CCR7 (D), IL-4R (E), CD206 (F), and CD163 (G) on the initial M1 macrophage population (green) in response to IL-4 (blue), IL-10 (yellow), or IL-4 + IL-10 (red) were assessed by flow cytometry. Data are means ±SD of n=3. CCR7: *P < 0.05; IL4R: *P < 0.05 IL-4 + IL-10 vs IL-10 and M0 conditions, **P < 0.01 IL-4 vs IL-10 and M0 conditions; CD206: *P < 0.05 IL-4 + IL-10 vs M0, **P < 0.01 IL-4 + IL-10 vs IL-10 and M0 conditions; CD163: ***P < 0.001 IL-4 + IL-10 vs IL-10 and M0 conditions, also P < 0.05 vs IL-4, *P < 0.05 IL-4 + IL-10 vs M0 condition.



	Z-average (d.nm)
AuNP Core	29.9 ± 0.2
AuNP-PEG	49.2 ± 0.5
PEG-AuNP-murineIL4	50.1 ± 0.1

Fig. S4. Representative DLS size distribution for 30nm AuNP core particles (gray) following PEGylation (light pink), and murine IL-4 conjugation (dark pink), measured in MilliQ water.

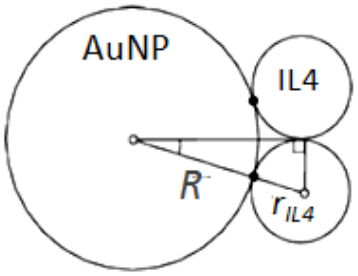


Fig. S5. Schematic used to determine the theoretical maximum loading of IL-4 onto AuNPs based on sphere packing.

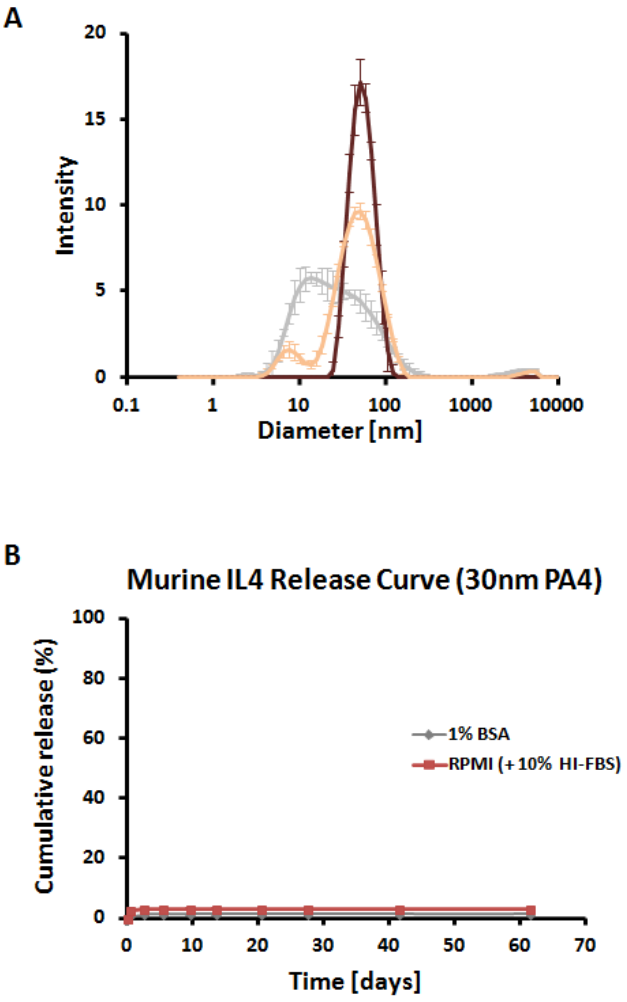


Fig. S6. AuNP-PEG particles and murine IL-4 conjugation to AuNPs is stable. (A) The stability of AuNP-PEG particles, lacking IL-4, in cell culture media was assessed by measuring the DLS size distribution of the 30nm-core particles immediately after PEGylation and washes in MilliQ water (brown) and comparing this to the DLS size distribution of the AuNP-PEG particles after 1 day incubation in cell culture media containing 10% HI-FBS at 37°C, 5% CO_2 (orange; 12ug/mL Au). The gray line shows the DLS distribution of cell culture media containing 10% HI-FBS without nanoparticles. Data are means ±SD of n=3. (B) The release of murine IL-4 from the surface of 30nm core PA4 into 1% BSA (gray) or RPMI containing 10% HI-FBS (red) at 37°C, 5% CO_2 was quantified over the course 61 days with ELISAs. Data are means ±SD of n=3.

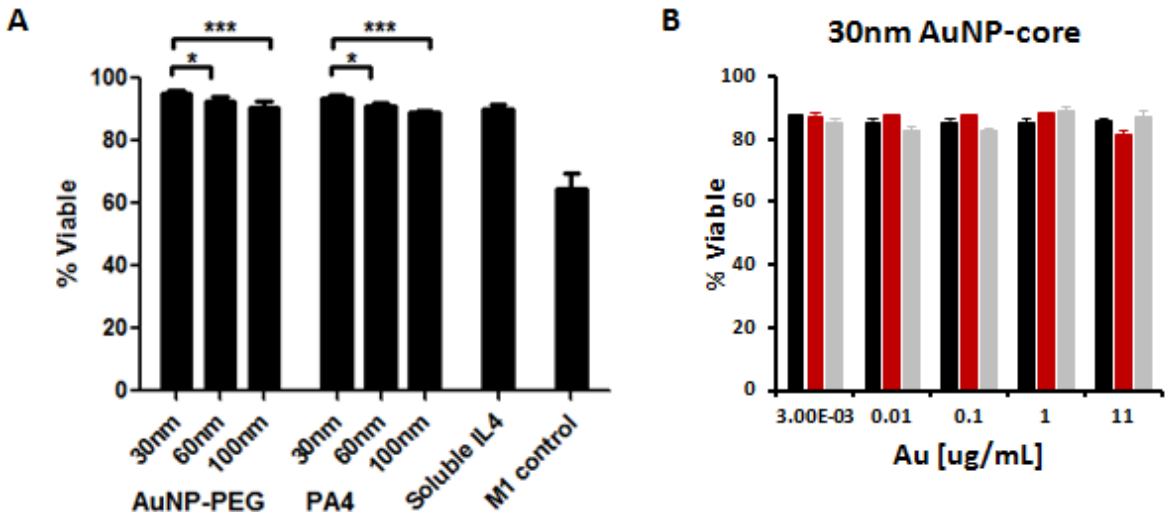


Fig. S7. PA4 do not reduce cell viability. (A) THP-1 derived macrophages were treated for 3 days with 30, 60, or 100nm core PA4 particles delivering 20ng/mL IL-4, 20ng/mL soluble IL-4, or the same concentration of AuNP-PEG particles lacking IL-4; stimulation with 2400ng/mL LPS, and 20ng/mL IFN-gamma was used as an inflammatory M1 control. Macrophage viability was assessed with flow cytometry by staining cells with an Invitrogen fixable dead cell stain. Data are means ±SD of n=4. *P < 0.05, ***P < 0.001 (B) A dose curve of PA4 (red), AuNP-PEG (gray), and soluble IL-4 extending through the equivalent of 200ng/mL IL-4 (11ug Au/mL) shows no significant reduction in viability, assessed by flow cytometry. Data are means ±SD of n=3.

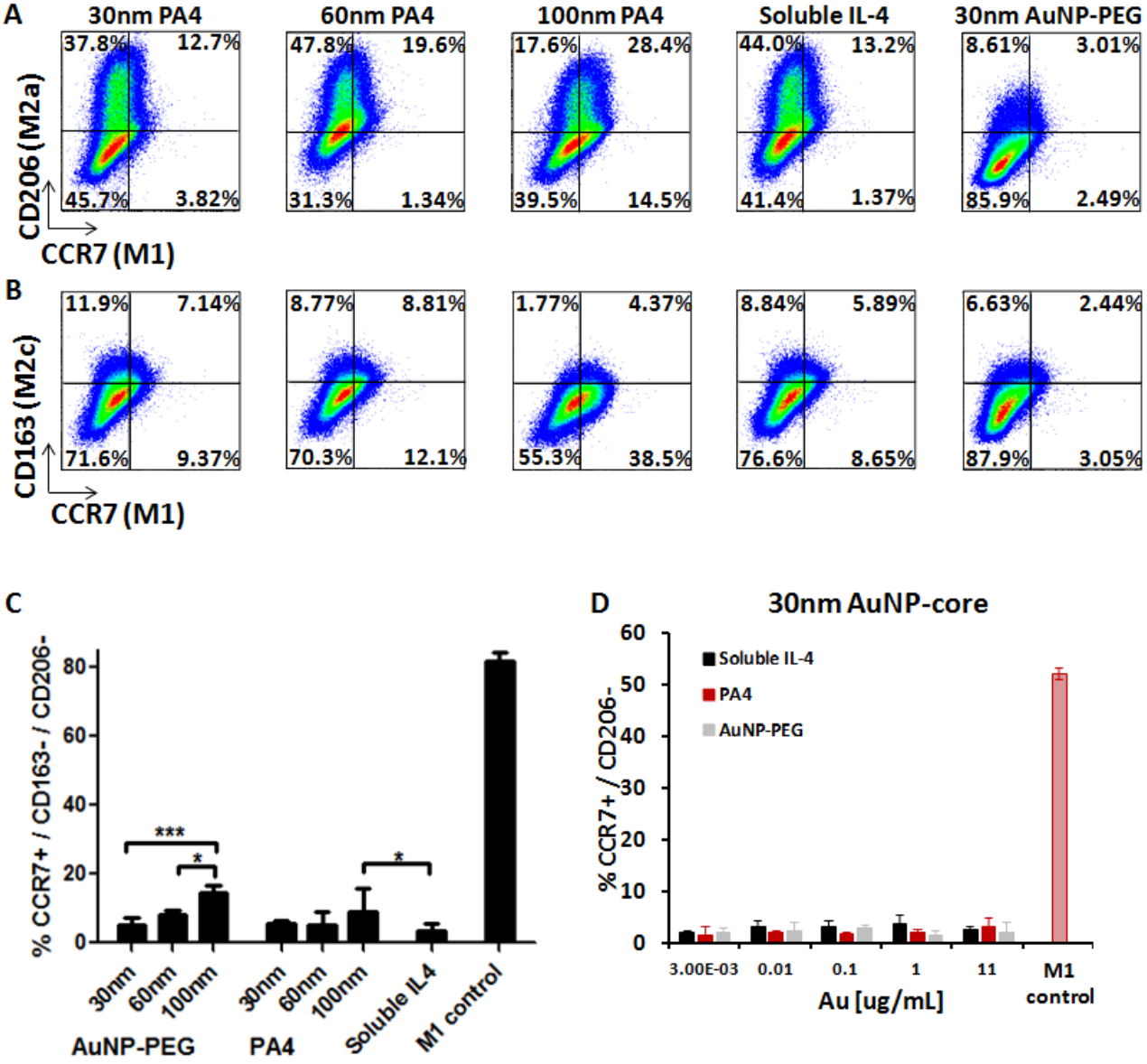


Fig. S8. PA4 direct M2a polarization and are not inflammatory. (A-B) THP-1 derived macrophages were treated for 3 days with 20ng/mL IL-4 as PA4 or soluble IL-4; or AuNP-PEG. Plots show expression of the M2a (CD206) or M2c (CD163) vs the M1 marker (CCR7). (C) Flow cytometry was also used to assess the percentage of macrophages expressing the inflammatory M1 phenotype (CCR7+/CD163-/CD206-) following exposure to the different size nanoparticles; stimulation with 2400ng/mL LPS, and 20ng/mL IFN-gamma was used as an inflammatory M1 control. Data are means ±SD of n=4. *P < 0.05, ***P < 0.001 (D) Inflammatory phenotype expression in response to increasing doses of nanoparticles and soluble IL-4. Data are means ±SD of n=3.

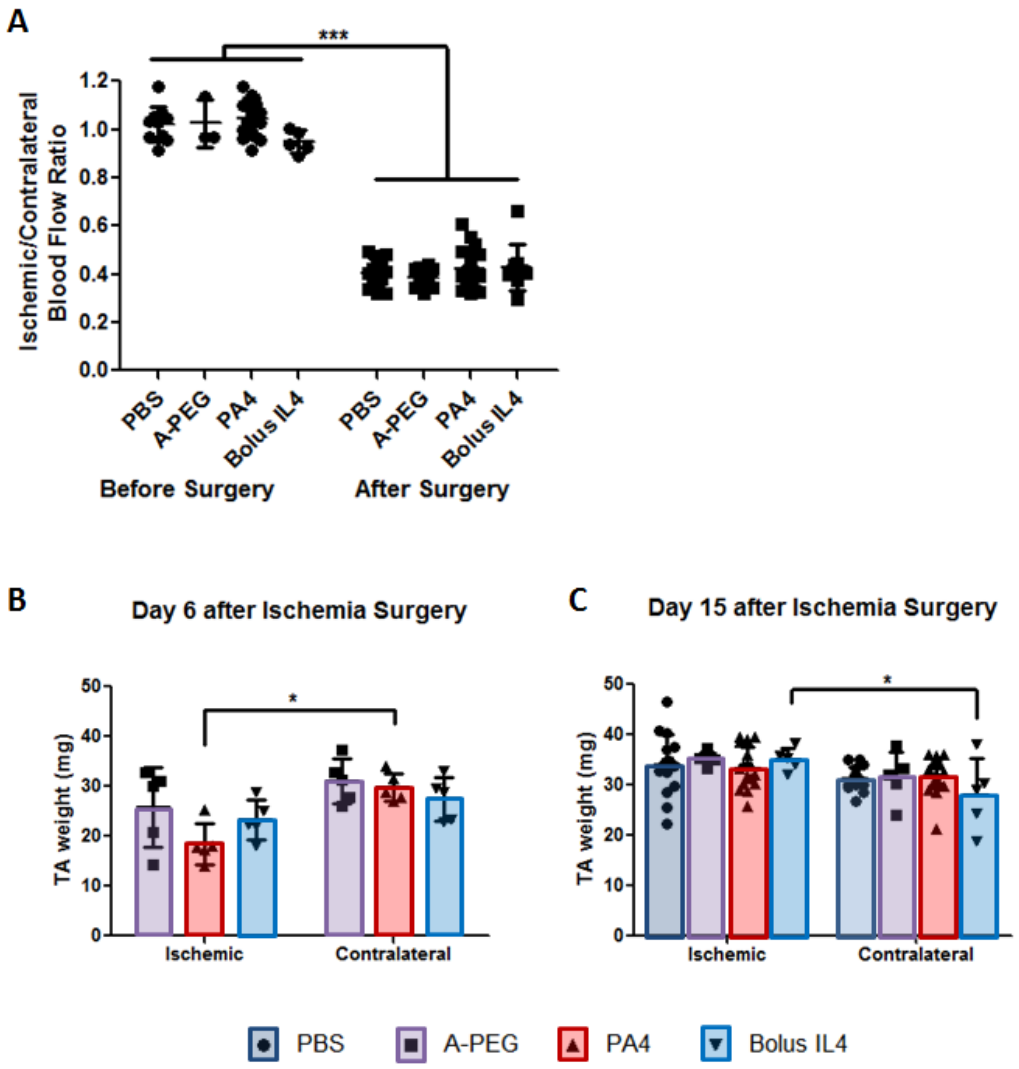


Fig. S9. Surgical induction of ischemia reduces blood flow and decreases muscle mass. (A) LDPI blood perfusion measured immediately before and after surgical induction of ischemia. LDPI readings were normalized to the contralateral (uninjured) limb. (B-C) TA muscle mass measured ex vivo, on day 6 (B), and day 15 (C) after surgery. Data are means ±SD of 3-16 mice per group.

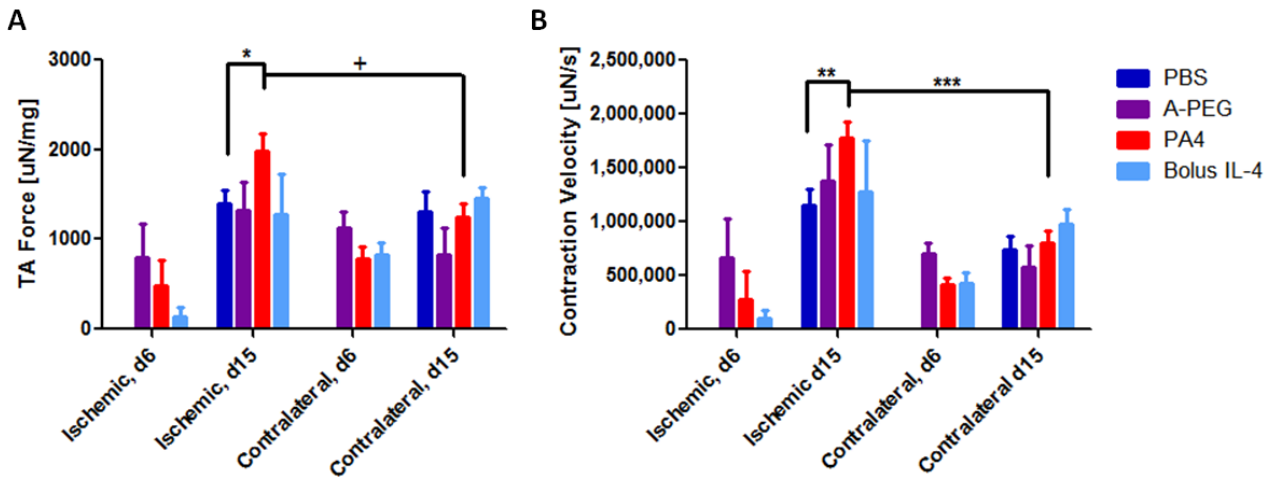


Fig. S10. PA4 improve muscle contraction force and velocity following unilateral ischemic injury. (A) Maximum contraction force of the TA muscle isolated from the ischemic limb in comparison to that from the uninjured contralateral limb on days 6 and 15 after surgery. Force was normalized to TA mass. (B) Contraction velocity of the TA muscles isolated from the ischemic and uninjured contralateral limbs on days 6 and 15 after surgery. Data are means±SEM. Day 6: n=5; day 15: n=16 for PA4 and PBS, n=5 for A-PEG and bolus IL-4 groups. *P<0.05, Bonferroni planned comparison; +P<0.05, 2-way ANOVA (considers each condition, ischemic vs. contralateral); **P<0.01, ***P<0.001, 2-way ANOVA

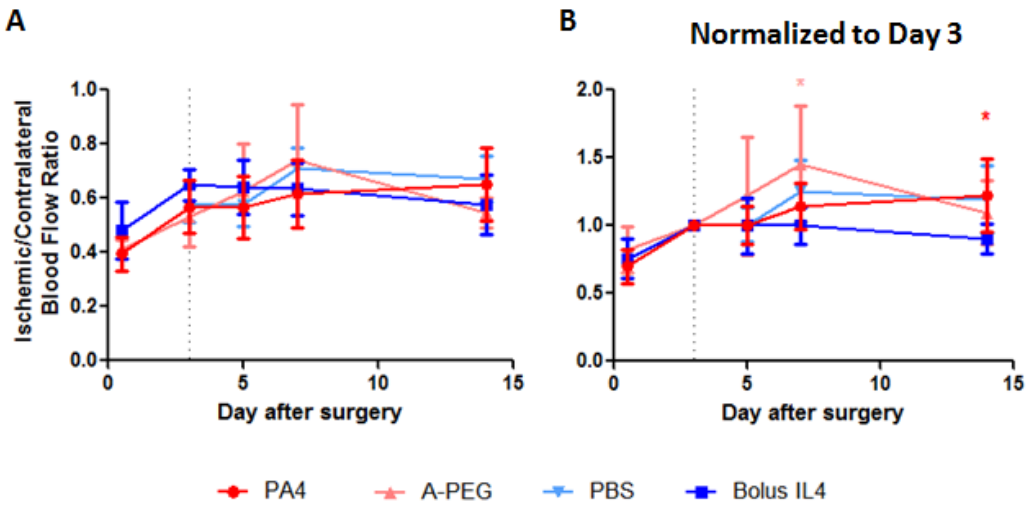


Fig. S11. PA4 treatment does not significantly impact blood perfusion. (A) LDPI blood perfusion measured at various timepoints after surgical induction of ischemia. LDPI readings were normalized to the contralateral (uninjured) limb. (B) The ischemic/contralateral ratio LDPI measurements were normalized to the day 3 reading, to more easily compare the effect of treatment (administered on day 3 after surgery). Data are means ±SD of 5-16 mice per group. *(pink) $P < 0.05$ AuNP-PEG vs bolus IL-4 and PA4; *(red) $P < 0.05$ PA4 vs bolus IL-4

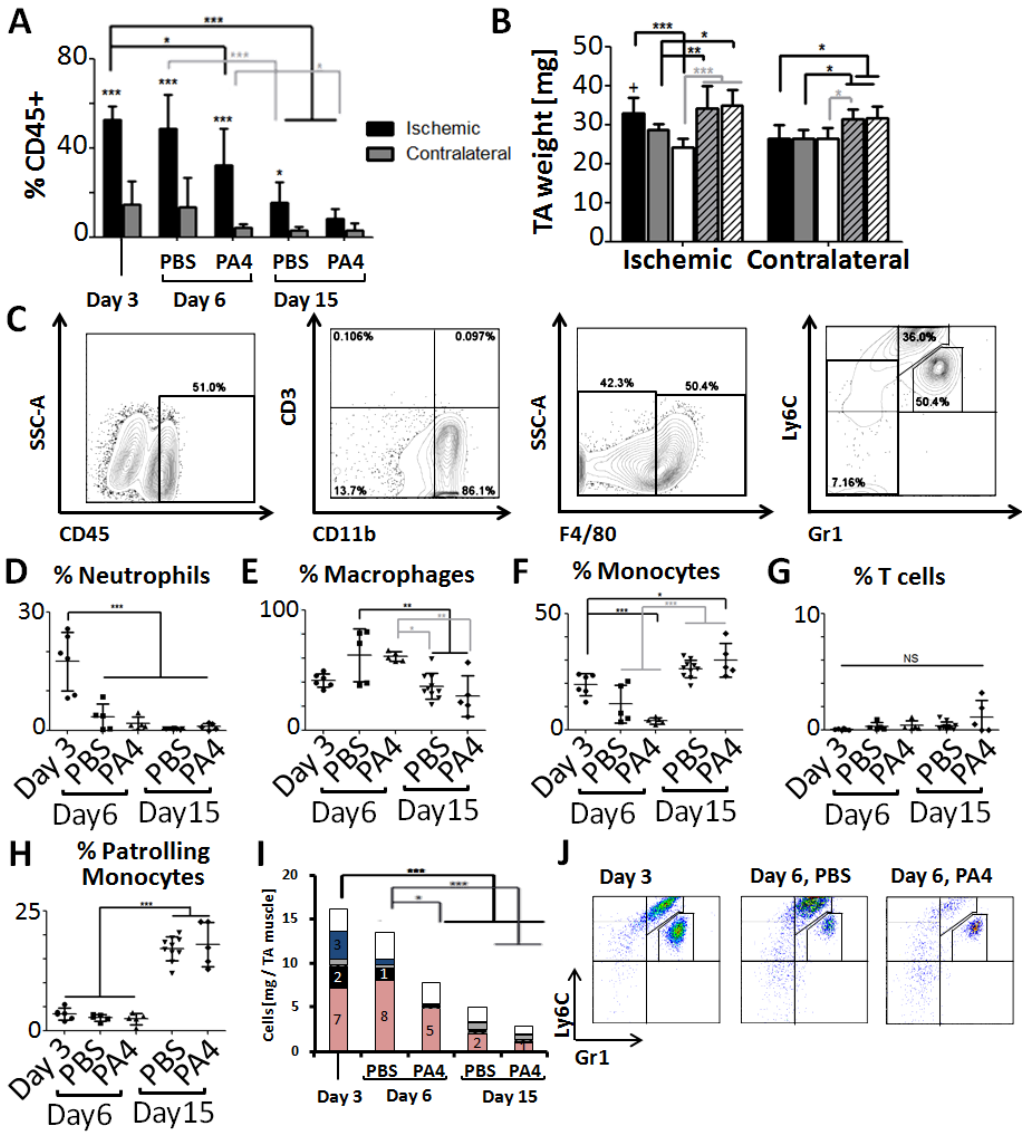


Fig. S12. Characterization of immune cell recruitment to the TA muscle following ischemic injury. (A) The percentage of cells isolated from the ischemic and contralateral (uninjured) TA muscles that were CD45+ immune cells were quantified with flow cytometry. (B) Weight of the isolated TA muscles at the time of treatment, day 3 (black bars), day 6 after surgery and PBS treatment (gray bars) or PA4 treatment (white bars), and day 15 after surgery and PBS treatment (gray stripped bars) or PA4 treatment (white stripped bars). (C) Gating strategy used to identify the immune cell populations. (D-H) The percentage of CD45+ immune cells collected from the TA muscles that were neutrophils (CD11b+/CD3-/ F4/80-/Gr1^{hi}/Ly6C^{lo}) (D), macrophages (CD11b+/CD3-/ F4/80+) (E), monocytes (CD11b+/CD3-/ F4/80-/Gr1$^{lo\ or\ -}$) (F), T cells (CD11b-/CD3+) (G), or patrolling monocytes (CD11b+/CD3-/ F4/80-/Gr1-/Ly6C$^{lo\ or\ -}$) (H). (I) The presence of each immune cell population was normalized to the TA mass; macrophages (pink; CD11b+/CD3-/F4/80+), inflammatory monocytes (black; Ly6C^{Hi}/Gr1^{Lo}), monocytes (black above gray border; Ly6C^{Hi}/Gr1-), patrolling monocytes (gray; Ly6C$^{Lo\ or\ -}$/Gr1-), neutrophils (blue; Ly6C^{Lo}/Gr1^{Hi}); T cells (dark gray above the blue box; CD11b-/CD3+), T cells (light gray above the blue box; CD11b+/CD3+), CD11b-/CD3- (white). (J) Gating of myeloid (CD45+/CD11b+/CD3-/ F4/80-) cells. Plots show diminishing inflammatory monocytes (Ly6C^{Hi} /Gr1^{Lo}) with PA4 treatment. All data are means ±SD of 5-10 mice per group. *P < 0.05, **P < 0.01, *** P < 0.001

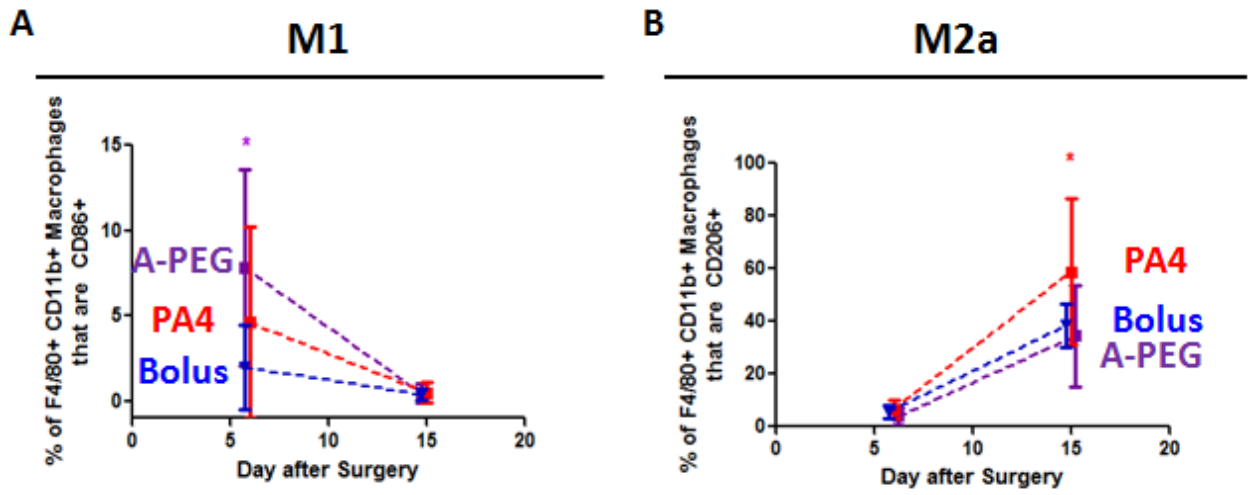


Fig. S13. Bolus IL-4 is not as effective as PA4 at shifting macrophage polarization in vivo at longer timepoints. Flow cytometry was used to characterize the polarization state of macrophages isolated from the injured TA muscles on days 6 and 15 after surgery. CD86 expression was used to identify M1 macrophages (A), and CD206, M2a macrophages (B). Data are means ±SD, n=5. For CD86 expression (A) *P < 0.05 for bolus IL-4 vs. AuNP-PEG; for CD206 expression (B) *P < 0.05 for PA4 vs. AuNP-PEG.

Table S1. DLS size and ζ-potential following PEGylation and human or murine IL-4 conjugation.

NP name	Z-average [nm]	PDI	ζ-Potential [mV]
100-nm AuNP	98.7	0.062	-38.7
100-nm AuNP-PEG	115.5	0.063	-27.8
100-nm human PA4	130.6	0.082	-15.5
60-nm AuNP	61.4	0.130	-37.9
60-nm AuNP-PEG	76.3	0.113	-29.6
60-nm human PA4	85.7	0.133	-15.7
30-nm AuNP	34.3	0.122	-44.1
30-nm AuNP-PEG	47.7	0.098	-24.0
30-nm human PA4	50.3	0.112	-10.5
30-nm murine PA4	50.3	0.092	-1.67

PDI, Polydispersity index.

Table S2. Murine IL-4 Conjugation to PA4.

NP name	IL4/nm^2	% of max. packing
30nm murine PA4	0.084	57

Max., maximum.

Table S3. PA4 concentration in cell culture.

Core (nm)	ug AuNP/mL	nm^2 Au core/mL ($x10^{13}$)	coated nm^2/mL ($x10^{15}$)	coated nm^3/uL ($x10^{13}$)	number of AuNPs/uL
34.3	1.2	1.1	5.7	4.8	7.17E+08
61.4	2.4	1.2	3.0	4.4	1.32E+08
98.7	4.2	1.3	1.2	2.6	2.22E+07

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