Supporting Information

Polymeric vector-mediated targeted delivery of anti-PAK1 siRNA to macrophages for efficient atherosclerosis treatment

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Synthesis of copolymer. Firstly, poly(ethylene glycol)-block-poly(β-benzyl L-aspartate), i.e. *m*PEG-PBLA, was synthesized by ring-opening polymerization of BLA-NCA with *m*PEG-NH_2 as a macroinitiator.[1] In brief, PEG-NH_2 (1.2 g, 0.6 mmol) was vacuum-dried at 70 °C for 4 h in a 100 mL flask, and then dissolved in 40 mL of anhydrous dichloromethane. Subsequently, BLAsp-NCA (1.5 g, 6 mmol) dissolved in 4 mL of anhydrous DMF was added into the above solution under the protection of argon. After the reaction was kept stirring for 72 h at 35 °C, the mixture was precipitated into excessive cool diethyl ether. Then the precipitate was filtered, washed with diethyl ether, and vacuum-dried until a constant weight was attained (*m*PEG-PBLA: *M*n = 4.0 kDa, calculated from ^{1}H NMR spectrum; yield: 98%).

Then, linear polyethylenimine (PEI) was synthesized as previously reported.[2,3] Briefly, MPTS (0.37 g, 2 mmol), EtOx (2.38 g, 24 mmol), and CH_3CN (15 mL) were added into a 50 mL reaction flask and thermostated at 85 °C. After refluxing for 72 h under the protection of argon, the mixture was cooled at 0 °C whereas dry ammonia (NH_3) was slowly blown into the vial for 1 h to terminate the reaction. Subsequently, the solvent was removed under reduced pressure and the residue was dissolved in $CHCl_3$ and then precipitated into excessive diethyl ether. MeO-PEtOx-NH_2 was separated by filtration and dried in vacuum. Then, MeO-PEtOx-NH_2 (2.04 g, 2.0 mmol) was dissolved in 10 mL of aqueous HCl (10 wt%) and refluxed for 12 h under a nitrogen atmosphere at 100 °C. After reaction, the pH of the mixture was adjusted to 12 with 1 M NaOH, leading to precipitation of the product. The product was separated by centrifugation, washed twice with deionized water and then freeze-dried to obtain PEI-NH_2 (*M*n = 430 Da, calculated from the ^{1}H NMR spectrum; yield: 81%).

*m*PEG-PAsp-(*g*-PEI) was obtained by aminolysis of *m*PEG-PBLA with PEI.[4] Briefly, *m*PEG-PBLA (0.8 g, 0.20 mmol) and PEI (4.3g, 10 mmol) were dissolved in 30 mL of anhydrous DMSO, then the solution was stirred for 24 h at 35 °C. Then the reaction mixture was dialyzed (MWCO: 3.5 kDa) against deionized (DI) water for 3 days to remove excess PEI, and finally vacuum-dried to get the *m*PEG-PAsp-(*g*-PEI)

(Mn = 7.3 kDa, calculated from ^{1}H NMR spectrum; yield: 84%).

Finally, the maleimide-terminated polymer was synthesized by reaction of *m*PEG-PAsp-(*g*-PEI) with excess N-methoxycarbonylmaleimide according to our previous study.[5] Briefly, *m*PEG-PAsp-(*g*-PEI) (1.46g, 0.2 mmol) was dissolved in 10 mL of saturated aqueous solution of $NaHCO_3$ and put into an ice/salt bath. After the mixture was cooled to 0 °C, excess of N-methoxycarbonylmaleimide (0.31 g, 2 mmol) was added into the solution and the mixture was stirred for 30 minutes. Subsequently, 20 mL of deionized water was added. After the solution was stirred for another 1 h, the pH value was adjusted to 3.0 with 0.5 M sulfuric acid, then the mixture was extracted with CH_3Cl, dried with Na_2SO_4 and finally added into large amount of cold diethyl ether. The precipitate was collected by filtration and vacuum-dried to a constant weight at room temperature to get the *m*PEG-PAsp(-*g*-PEI)-Mal (yield: 80%).

Polymer and polyplex characterizations. ^{1}H NMR spectra were carried out on a Varian Unity 400 MHz spectrometer using DMSO-d_6 or D_2O-d_2 as solvent according to the solubilities. FTIR spectral measurements were recorded using a Nicolet/Nexus 670 FTIR spectrometer with a resolution of 2 cm^{-1} and the powder samples were compressed into KBr pellets. The molecular weight distribution of copolymer was analyzed using a gel permeation chromatography (GPC) system consisting of a Waters 1515 pump, an Ultrahydrogel TM 500 column, an Ultrahydrogel TM 250 column, and a Waters 2417 differential refractive index detector with PEG as a calibration standard. DMF was used as an eluent at a flow rate of 1.0 mL/min.

The sizes and zeta potentials of polyplexes were determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C on a 90 Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA). For each sample, the data from five measurements were averaged to obtain the mean ± standard deviation (SD).

Transmission electron microscopy (TEM) was performed using a Hitachi model JEM-1400 TEM operated at 120 kV. The samples were prepared by drying a drop (10

μL, 0.5 mg/mL) of sample solution on a copper grid coated with amorphous carbon. For the negative staining of samples, a small drop of uranyl acetate solution (2 wt% in water) was added to the copper grid, which was then blotted with a filter paper after 1 min. The grid was finally dried overnight in a desiccator before TEM observation.

MTT assay. The murine macrophage cell line RAW 264.7 was provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). RAW 264.7 cells were cultured at 37 °C in a 5% CO_2 incubator with Dulbecco's modifier Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). RAW 264.7 cells were seeded at 2×10^3 cells per well in 96-well plate. After starvation for 3 h when reached 60%, the cells were incubated with polyplexes at different polymer concentration or at different N/P ratios with 100 nM SCR in DMEM for 48 h. Then 10 μL of 1% MTT (Promega, USA) was added into each well and kept incubation for another 4 h. Subsequently, the medium was removed carefully and 100 μL of DMSO（sangon，China） was added into each well, then the absorbance of solubilized blue formazan was read at the wavelength of 570 nm by using a Tecan Sunrise™ microplate reader (Tecan, Switzerland).

Preparation of SPIO labelled polyplexes. Superparamagnetic iron oxide nanoparticles (SPIO) were introduced to make polyplexes detectable *in vivo* using magnetic resonance imaging system by amino exchange reaction as previously reported.[6] Briefly, 10 mg of polymer and 1 mg of SPIO was co-dissolved in 2 mL of chloroform, after the mixed solution was kept stirring using glass magneton for 24 h, 10 mL of deionized water was added into the solution and another additional 6 h' stirring was carried out on an ice-salt bath. Subsequently, chloroform was removed by rotary evaporation, the solution was then filtered (0.45 μm), washed three times with deionized water using ultrafiltration (MWCO: 100 kDa) at 4 °C and finally freeze dried to get SPIO-labelled vector. Iron content of the nanoparticle was detected the absorbance at 284.3 nm on an atomic absorption spectrometer (ASS, Z-200, Hitachi,

Japan). To obtain a similar zeta potential compared with the SPIO-free polyplexes, mass ratio of 2.2:1 and 2.4:1 (SPIO-labeled nanoparticle to siRNA) were adopted for the *in vivo* MRI test for non-targeted and targeted group, respectively.

Prussian blue staining. After *in vivo* MR imaging, mice were sacrificed and aortic roots were collected to produce frozen sections for Prussian blue staining. Briefly, the sections were immersed into staining solution of a fresh mixture of ferric ferrocyanide with 2 wt% hydrochloric acid for 30 min, then transferred into nuclear fast red staining solution for 5 min. The sections were washed with running water for 10 min to remove residual dye. The samples are observed under BX51 microscope (Olympus, Japan).

References:

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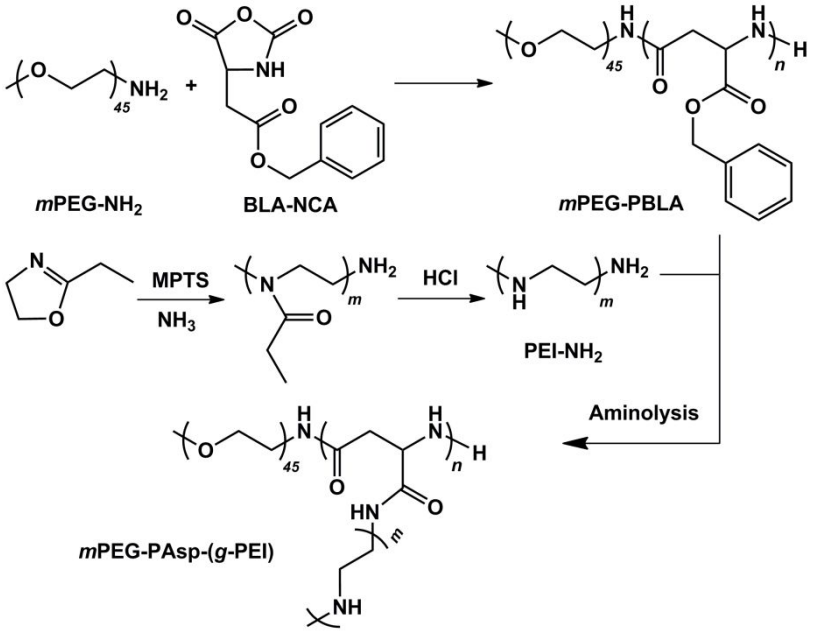

Scheme S1. Synthetic route of the non-targeted copolymer *m*PEG-PAsp-(*g*-PEI).

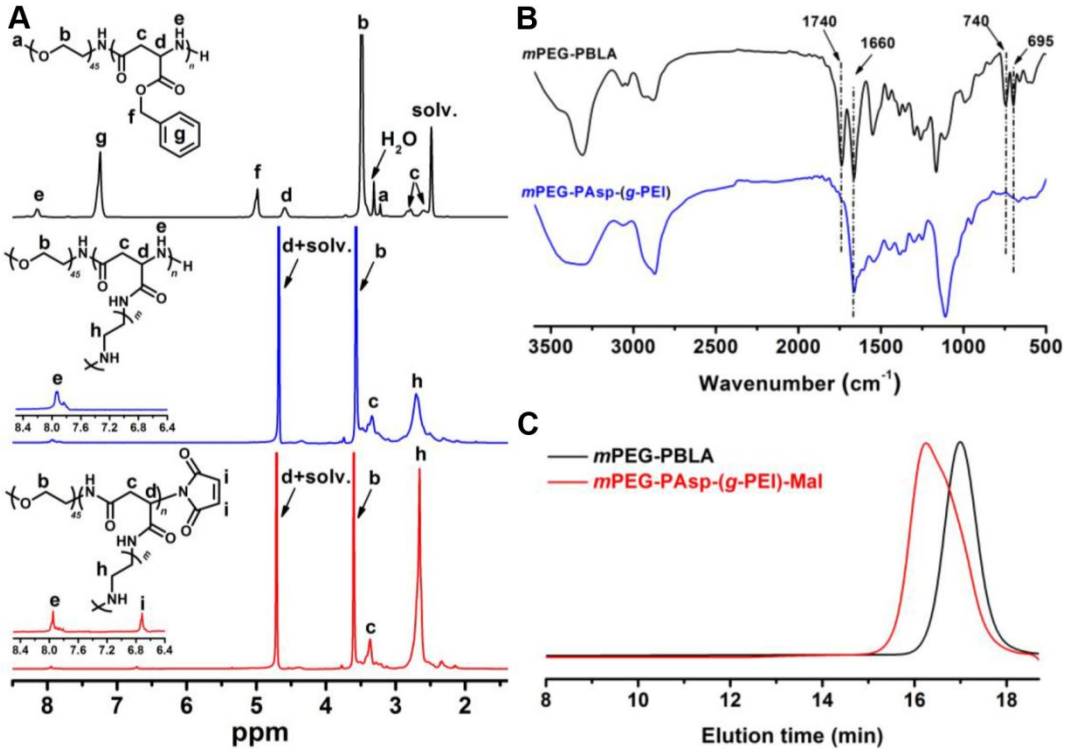

Figure S1. (A) ^{1}H NMR spectrum of *m*PEG-PBLA (black line) in DMSO-d_6, *m*PEG-PAsp-(*g*-PEI) (blue line) and *m*PEG-PAsp-(*g*-PEI)-Mal (red line) in D_2O-d_2. The major resonance peaks of the copolymer in the ^{1}H spectrum well fit into the expected chemical structure: 3.25 ppm (s, -OC***H***$_3$ of PEG, a), 3.38-3.60 ppm (m, -OC***H***$_2$C***H***$_2$- of PEG, b), 2.52-2.88 ppm (m, - C***H***$_2$CHCON- of PBLA, c), 4.53-4.66 ppm (m, - CH$_2$C***H***CON- of PBLA, d), 8.11-8.23 ppm (m, - CH$_2$CHCON***H***- of PBLA, e), 4.93-5.10 ppm (m, -C***H***$_2$C$_6$H$_5$ of PBLA, f), 7.16-7.38 ppm (m, -CH$_2$C$_6$***H***$_5$ of PBLA, g). The polymerization degree of PBLA was 10 according to the integral area ratio of proton from methylene in PEG chain and benzyl group in PBLA chain. After ammonolysis, the disappeared resonance peaks of benzyl group (4.93-5.10 ppm and 7.16-7.38 ppm) in ^{1}H spectrum of *m*PEG-PAsp-(*g*-PEI) showing a complete ammonolysis reaction taking place. In addition, ^{1}H NMR spectrum of *m*PEG-PAsp-(*g*-PEI)-Mal appeared the characteristic shift of maleimide at 6.71 ppm, indicating the successfully conjugation of Mal. (B) FT-IR spectra of *m*PEG-PBLA and *m*PEG-PAsp-(*g*-PEI). After ammonolysis reaction, the absorption peak of 1740 which shows the stretching vibration of carbonyl group (C=O) in ester bond disappeared, indicating the remove of benzyl group. (C) GPC curves of *m*PEG-PBLA and *m*PEG-PAsp-(*g*-PEI)-Mal in DMF at a flow rate of 1 mL/min. The prepolymer and the final polymer all showed a unimodal molecular weight distribution in their GPC chromatograms, and *m*PEG-PAsp-(*g*-PEI)-Mal showed an obviously higher molecular weight than *m*PEG-PBLA.

Table S1. The molecular weight of *m*PEG-PBLA and *m*PEG-PAsp-(*g*-PEI)-Mal

Polymer	M_n[a] (Da)	M_n[b] (Da)	M_w/M_n[b]
*m*PEG-PBLA	4,000	5,400	1.10
*m*PEG-PAsp-(*g*-PEI)-Mal	7,300	8,700	1.17

[a] calculated by ^{1}H NMR; [b] calculated by GPC.

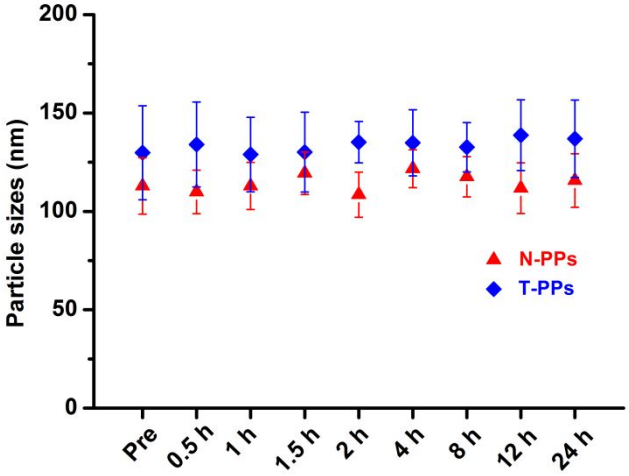

Figure S2. Stability of N-PPs and T-PPs in PBS (pH 7.4) containing 10% fetal bovine serum (FBS) measured by dynamic light scattering. "Pre" means before FBS addition. Data are mean ± SE (n = 3).

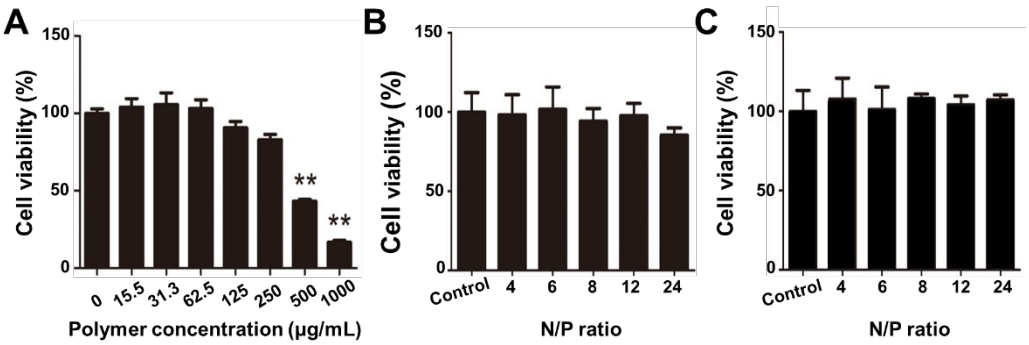

Figure S3. MTT assay were detected the cell toxicity of *m*PEG-PAsp-(*g*-PEI) (A) and at different concentration for 48 h treated. MTT assay were detected the cell toxicity of PEG-PAsp-(*g*-PEI) loading SCR (C) and CD36-mPEG-PAsp-(*g*-PEI) loading SCR (D) at different N/P ratios for 48 h treatment. $^{**}P < 0.01$ indicated significant difference.

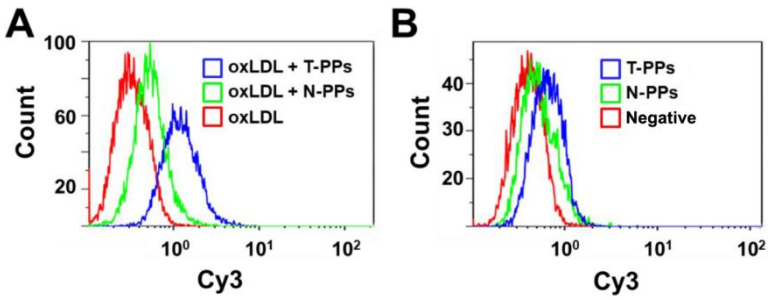

Figure S4. Cytometry detected the *in vitro* nanomedicine-mediated delivery efficiency of Cy3-labeled siRNA in RAW 264.7 cells with (A) and without (B) the pre-treatment of oxLDL. The co-incubation time was 6 h.

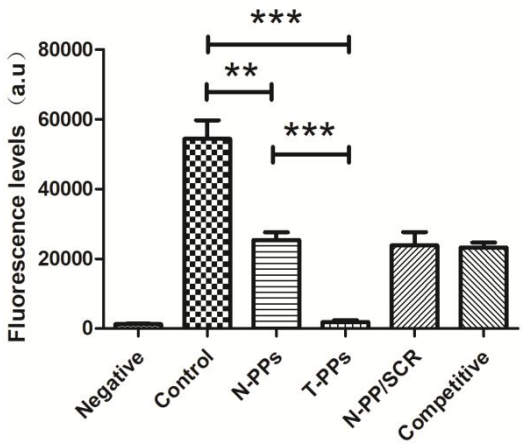

Figure S5. The statistics for sum intensity levels of Dil-oxLDL in RAW 264.7 cells after receiving the treatment of different samples in Figure 4D. $^{**}P<0.01$ and $^{***}P<0.01$.

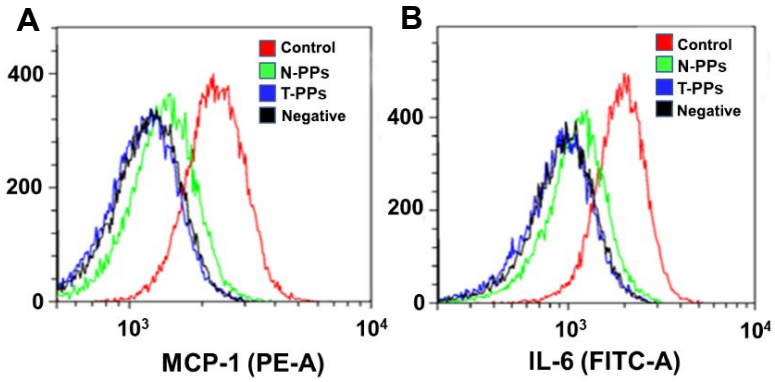

Figure S6. Cytometric analysis evaluated the MCP-1 (A) and IL-6 (B) translational levels in treated RAW 264.7 cells.

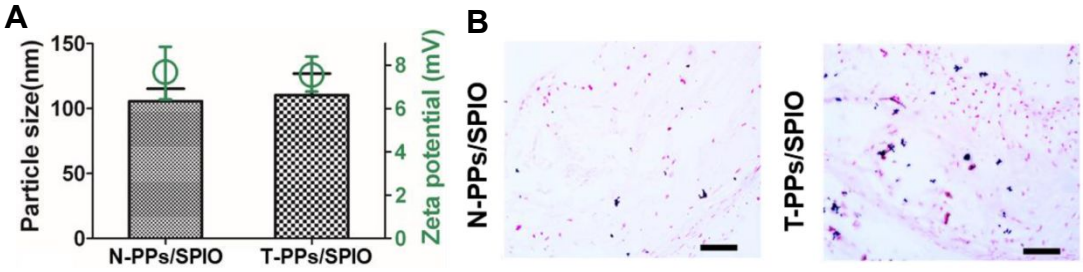

Figure S7. (A) Particle sizes and zeta potentials of SPIO-labeled N-PPs (N-PPs/SPIO) and T-PPs (T-PPs/SPIO) measured by dynamic light scattering (DLS). (B) Prussian blue staining of arotic root sections from the mice receiving N-PPs/SPIO and T-PPs/SPIO for 12 h. The scale bars represent 50 μm.

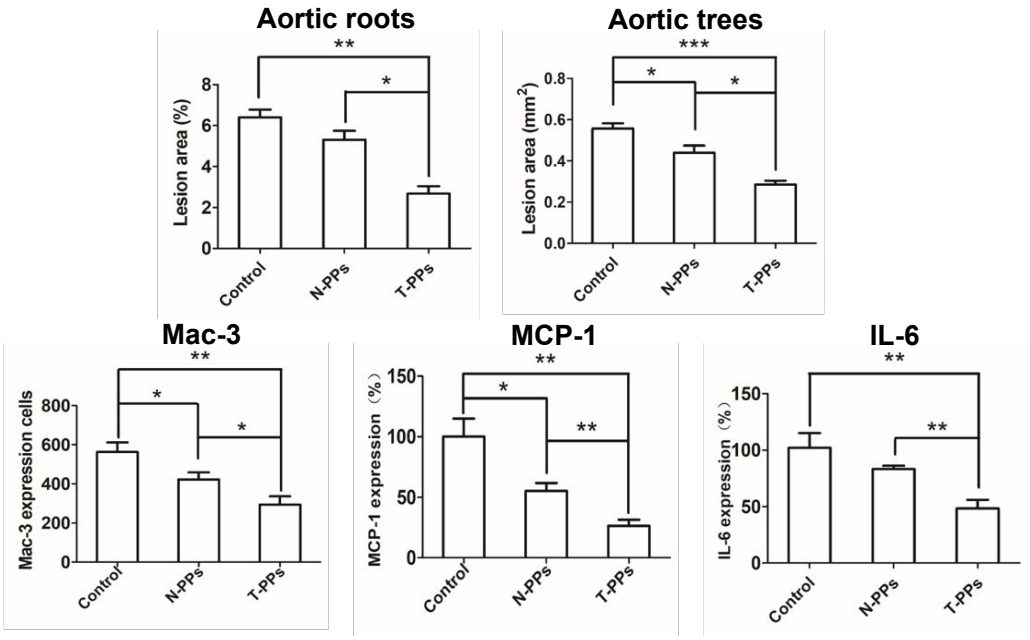

Figure S8. The statistics of positive areas of the aortic roots and aortic trees, the Mac-3 positive cells, MCP-1and IL-6 expression levels in Figure 7. **P*<0.05, ***P*<0.01 and ****P*<0.01.

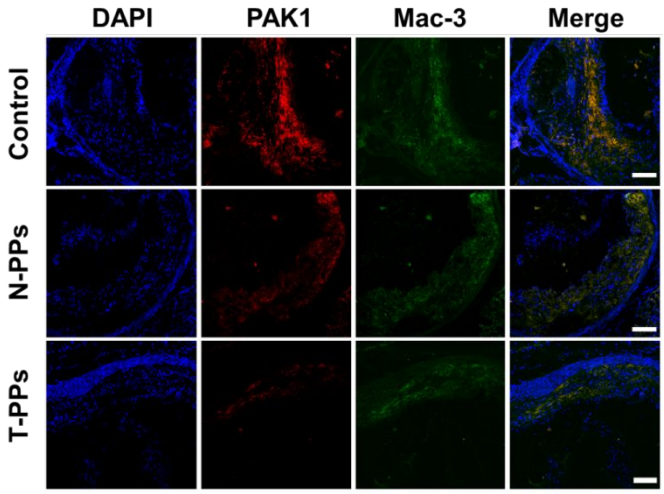

Figure S9. Images of immunofluorescence staining for PAK1 (red) and Mac-3 (green). Nucleus are stained by DAPI (blue). The scale bars represent 100 μm.