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Transiently responsive protein-polymer conjugates via a grafting-from RAFT approach: for intracellular co-delivery of proteins and immune-modulators

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Materials. Unless otherwise noted all chemicals were purchased from Sigma-Aldrich. 2.2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was purchased from Wako Chemicals. The pentafluorophenyl modified 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (PFP-CETPA) RAFT agent was synthesized as reported earlier.[1] The [(2,2-dimethyl-1,3-dioxolane)methyl] acrylamide (DMDOMA) was synthesized according to literature.[2] Cyanine5-NHS (Cy5-NHS) and cyanine3-alkyne (Cy3-alkyne) were purchased from Lumiprobe. CL075 was purchased from Invivogen. The immortalized dendritic cell line DC2.4 was a kind gift from Dr. Kenneth Rock.[3] Bone marrow derived dendritic cells were obtained as previously reported.[4] Cell culture medium and supplements, Hoechst and Cholera Toxin Subunit B, Alexa Fluor® 488 Conjugate (CTB-AF488) were purchased from Life Technologies. FC block, MHCII-FITC, CD11c-APC and CD86-PE were obtained from BD Pharmingen.

Conjugation of BSA with pentafluorophenyl CTA to obtain macroCTA. BSA (3.2 g; 48.4 µmol; 1 equiv.) was dissolved in phosphate buffered saline (PBS; 413 mL; pH 7.4) in a 1 L round bottom flask, equipped with a magnetic stir bar. The solution was purged with nitrogen for 40 min. A solution of the PFP-CETPA (415.6 mg, 968 µmol, 20 equiv.) in DMF (17 mL) was added dropwise, and the solution was stirred at room temperature overnight. The reaction mixture was centrifuged twice (4000 rpm, 10 min, 5 °C) to remove the excess of PFP CTA. Subsequently, the supernatant was dialyzed against deionized (DI) water (6 x 15 L) for 2 days, using a MWCO of 8000 Da, and then lyophilized to isolate the BSA macroCTA.

UV/VIS analysis BSA macroCTA. UV/VIS spectroscopy was carried out on a Shimadzu UV-1650PC UV/VIS double beam spectrophotometer. PFP-CETPA was dissolved in methanol to obtain a 3 mg/mL (7 mM) concentrated stock solution that was used to make a two-fold dilution series ranging from 6 µg/mL to 48 µg/mL. The absorbance of each solution was measured at 306 nm (λ_{max} of the PFP-

CETPA) to obtain a linear plot of CTA concentration *versus* absorbance. The CTA extinction coefficient was determined to be ε = 9875 M^{-1} cm^{-1}, and this value was used to calculate the concentration of the CTA content within the BSA macroRAFT agent. The molar ratio of CTA to BSA was 5.041, which indicates that on average 5 CTA molecules were attached to every BSA molecule. **Figure S1** shows the wave scans for native BSA, BSA macroCTA and PFP CTA respectively. In the wave scan of the BSA macroCTA a second peak appears around the λ_{max} value of the PFP CETPA, indicating successful modification of the BSA molecules with CETPA.

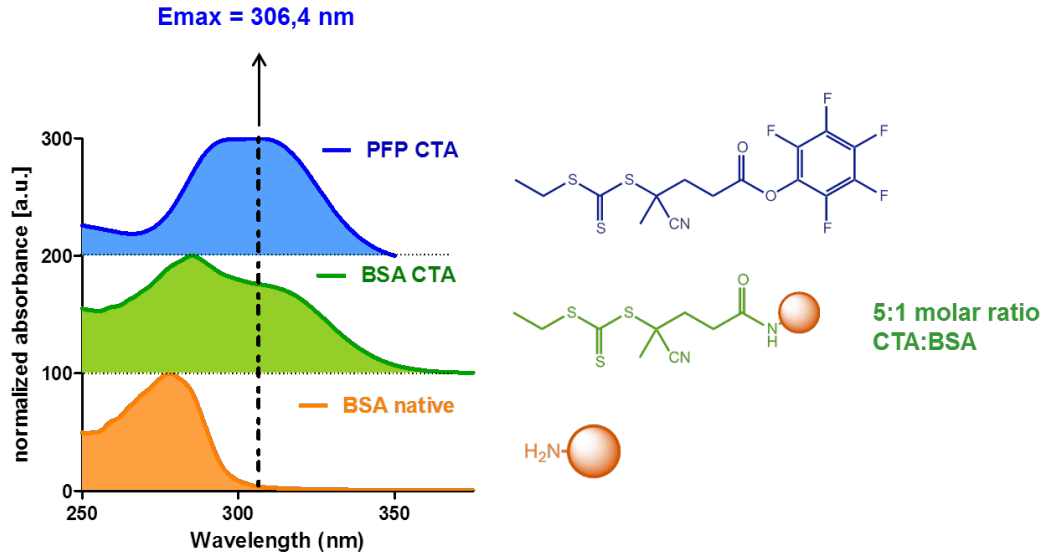

Figure S1. UV-Vis spectra of native BSA (orange), CTA-modified BSA (green) and PFP CTA (blue).

RAFT homopolymerization of DMDOMA. DMDOMA (15 mmol), PFP-CETPA (0.075 mmol for a DP 200) and AIBN (0.015 mmol) were transferred into a Schlenk tube and dissolved in anhydrous DMF (2M monomer concentration). After bubbling with nitrogen for 30 min, the solution was heated at 70°C in an oil bath for 4 h. The polymers were isolated by precipitation in hexane and dried under vacuum. Monomer conversion was measured by ^{1}H-NMR and calculated to be 92% (DP^{NMR} = 184, Mw^{NMR} = 34.4 kDa). The purified polymer was characterized by SEC analysis (Mn^{SEC} = 26,3 kDa, PDI = 1.15). ^{1}H-NMR spectra were recorded on a Bruker 300 MHz FT-NMR spectrometer using $CDCl_3$ as solvent. Size exclusion chromatography was carried out on Shimadzu Prominence GPC system equipped with a LC-20AD isocratic pump and a RID-20A refractive index detector. Measurement were done in DMA containing 50 mM LiCl at 50°C and with a flow rate of 0.593 mL/min. The two PL gel 5 µm mixed-D columns were calibrated with polymethylmethacrylate (PMMA) standards (Polymer standards service) in a molecular weight (Mn) range of 1980 Da to 372000 Da.

RAFT polymerization of DMDOMA with BSA macroCTA. A grafting-from RAFT polymerization of DMDOMA from the BSA macroCTA with an aimed degree of polymerization (DP) of 200 was conducted as follows. DMDOMA (185 mg, 1 mmol), BSA macroCTA (65 mg, 1 µmoL of protein, which contained 5 µmol of CTA functionality, as determined by UV/VIS), VA-044 (2 mg, 6.5 µmol) and phosphate buffer pH 6 (PB, 3.8 mL) were sealed in a Schlenk vial equipped with a magnetic stir bar. The solution was degassed by 5 cycles of freeze-vacuum-thaw, prior to immersing the Schlenk vial into a preheated oil bath at 25°C. For a DP of 100, the [DMDOMA]:[$CTA_{functionality}$] ratio was kept at 100:1 instead of 200:1. VA-044 was employed in a [VA-044]:[$CTA_{functionality}$] ratio of 1.3:1. After 16 h reaction the polymerization mixture had become turbid, indicative of polymerization-induced self-assembly (PISA). Time samples before (T_0) and after (T_e) polymerization were analyzed on SDS-PAGE and DLS. Half of the reaction mixture was diluted with DI water and dialyzed against DI water (6 x 5L; 4°C) for 2 days using a MWCO of 8000 Da. The other half was first diluted 3 times with a 0.1 M bicarbonate buffer pH 8.2 and fluorescently labeled with the Cy5-NHS (stock solution in DMSO, molar ratio BSA:Cy5-NHS = 1:10). After overnight reaction, the fluorescently labeled BSA-pDMDOMA conjugates were dialyzed against DI water (10 x 5 L, 4°C). The resulting solutions were lyophilized to obtain the unlabeled and Cy5-labeled protein-polymer conjugates respectively.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed with a 4-20 % polyacrylamide gradient gel using the Mini-PROTEAN Tetra Cell from Bio-Rad, at 175 V for 45 min. The polymerization time point samples were diluted 20 times with ice-cold phosphate buffer at pH 6 and were further diluted with 4x Laemmli sample buffer in a 3:1 ratio before loading on the SDS-PAGE gels. Staining was accomplished with Coomassie Blue. Integration of the protein bands was done by ImageJ software as reported earlier.[1]

Dynamic Light Scattering (DLS). DLS measurements of polymerization time point samples were conducted with a Malvern Zetasizer Nano-S. Both T_0 and T_e were measured below (3°C) and above (30°C) the T_{cp} of the protein-polymer conjugates.

Cloud Point Temperature (T_{cp}) measurements. The phase transition temperature of the protein-polymer conjugates was determined by DLS measurements at 1°C intervals ranging from 5 to 40°C. The purified BSA-pDMDOMA conjugates were dissolved in ice-cold PBS (2.5 mg/mL) and filtered through a 0.45 µm syringe filter prior to measurements. The T_{cp} was defined as the temperature where the mean volume and derived count rate abruptly shifted to higher values (**Figure S2**). BSA-$pDMDOMA^{DP100}$ and BSA-$pDMDOMA^{DP200}$ conjugates had a T_{cp} of 27°C and 19°C respectively.

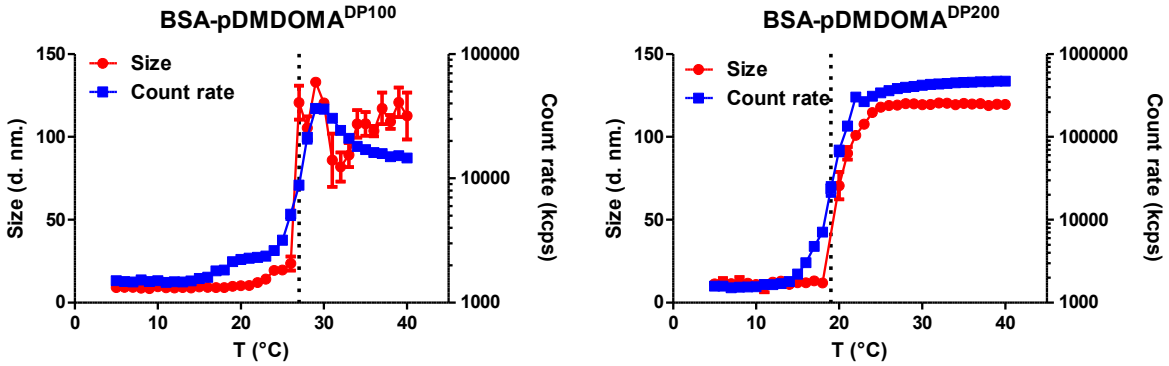

Figure S2. Volume mean diameter (red data points) and count rate (a measure for the light scattering intensity) (blue data points) of the BSA-pDMDOMA conjugates as function of temperature, measured by DLS.

Determination of the Critical Micellar Concentration (CMC). Similar to a previously reported protocol, the CMCs of the BSA-pDMDOMA conjugates were determined by fluorescence microscopy using pyrene as a fluorescent probe.[5] First, 5 mL of protein-polymer conjugate solutions were prepared in ice-cold PBS with concentrations ranging from 0.001 to 1 mg/mL. The samples were kept on ice and a pyrene working solution in acetone (16.67 µL, 36 µg/mL) was added under continuous stirring and heating above the T_{cp}. After overnight evaporation of the acetone, fluorescence excitation spectra were collected at 25°C on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) equipped with a Varian Cary Temperature Controller. The CMC was quantified based on the change in excitation intensity ratio at 338 and 333 nm upon dilution (**Figure S3**). The CMC was similar for BSA-pDMDOMA conjugates with different polymer chain lengths: 48 µg/mL for DP100; 51 µg/mL for DP200.

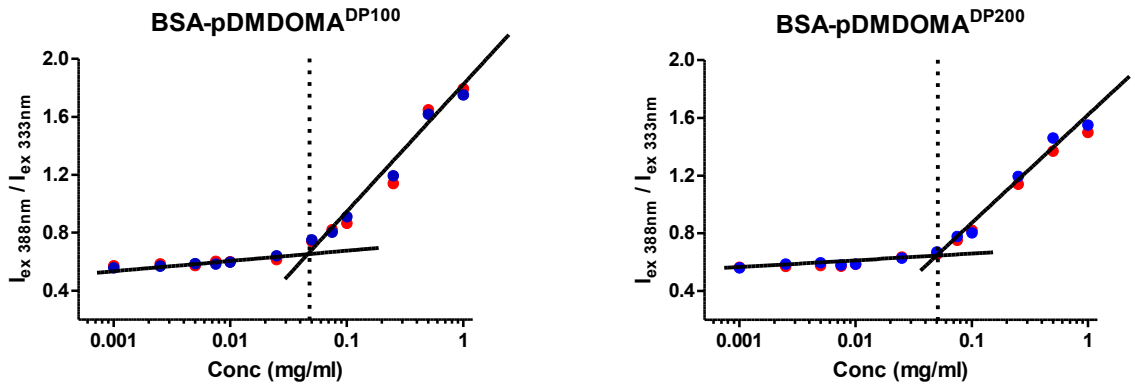

Figure S3. Intensity ratio of pyrene at 338 nm (I_3) and 333 nm (I_1) as function of the BSA-pDMDOMA concentration determined by fluorescence spectroscopy at 25°C.

Hydrolysis of dioxolane side groups. A solution of the BSA-pDMDOMA conjugates (2.5 mg/mL in ice-cold PBS) was filtered through a 0.45 µm syringe filter and 475 µL was transferred into a DLS cuvette. The sample was measured on DLS at 37°C, followed by addition of an HCl stock solution (25 µL, 1M) to obtain an 50 mM HCl final concentration. The evolution of particle size and light scattering intensity was followed in function of time by DLS at 37°C. To confirm that the evolution in particle size is due to hydrolysis of the pDMDOMA dioxolane side groups rather that degradation of the BSA itself, several control experiments were included. Both soluble BSA and the BSA-pDMDOMA conjugates (2.5 mg/mL) treated with 50 mM HCl were compared with untreated samples by SDS-PAGE. **Figure S4** clearly demonstrates no significant changes in gel retardation, indicating no significant changes in molecular weight occurred. Importantly also no lower molecular weight protein fractions were observed that could result from protein hydrolysis. By contrast, the ^{1}H-NMR analysis of the pDMDOMA homopolymer clearly revealed removal of the dimethyl groups on the dioxolane moieties after treatment with 50 mM HCl (**Figure S5**).

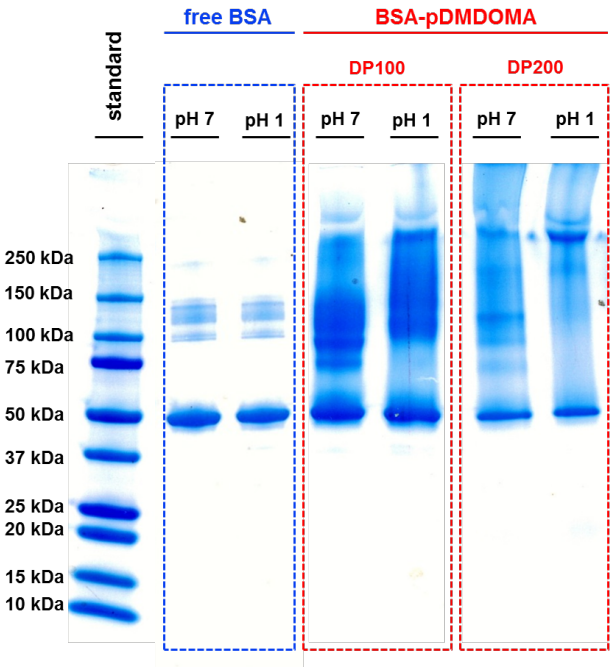

Figure S4. SDS-PAGE analysis of free BSA and BSA-pDMDOMA conjugates treated with 50 mM HCl (pH 1), compared with corresponding untreated samples (pH 7).

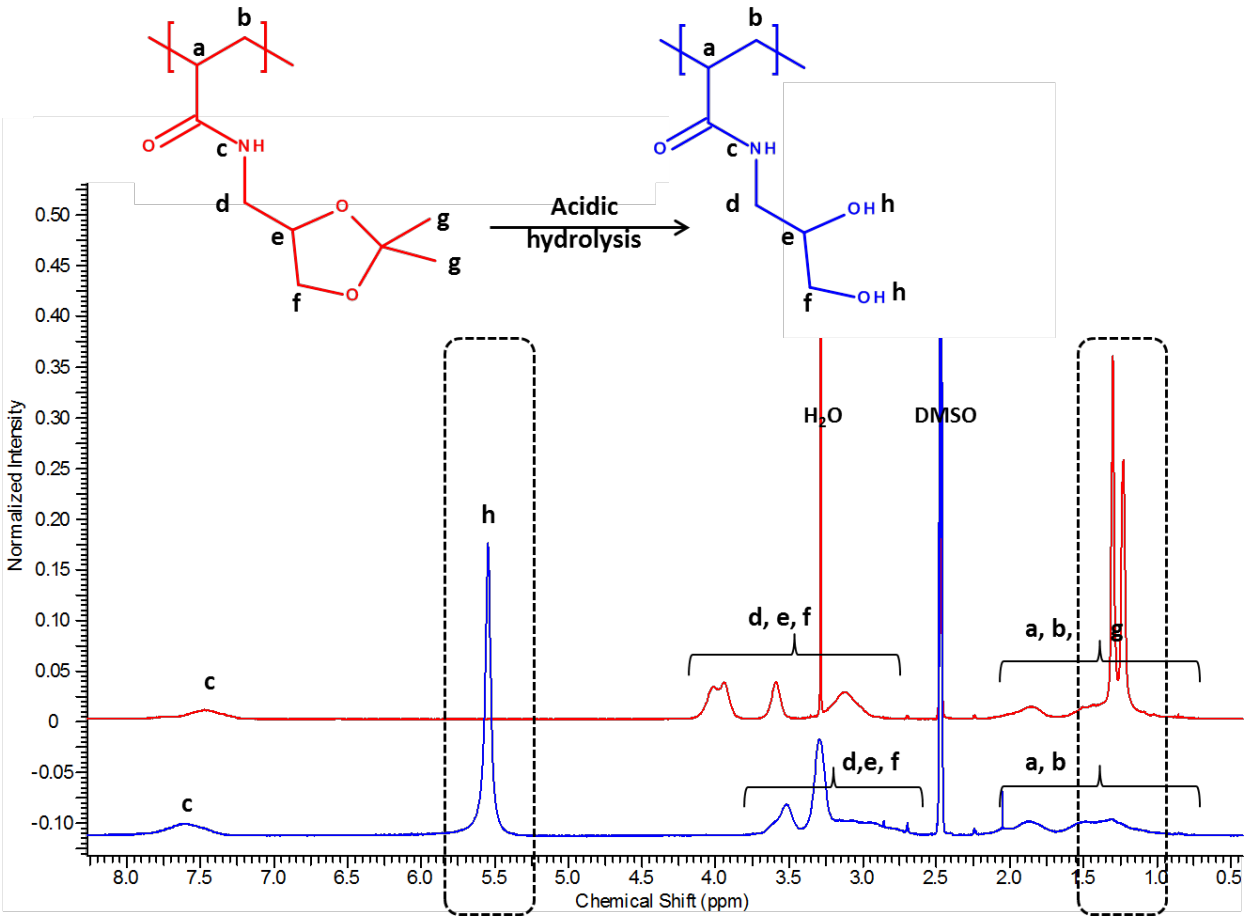

Figure S5. ^{1}H-NMR spectra in $(CD_3)_2SO$ of pDMDOMA before (red curve) and after (blue curve) treatment with 50 mM HCl. The spectra clearly show the removal of the dimethyl groups (g) on the dioxolane moieties by acidic hydrolysis, yielding hydroxyl groups (h).

Loading of BSA-pDMDOMA nanoparticles with hydrophobic molecules. A typical solvent displacement loading protocol for the BSA-pDMDOMA particles was as follows. The protein-polymer conjugates were dissolved in ice-cold PBS buffer (2.5 mg/mL) and a stock solution of the hydrophobic compound in ethanol was added under continuous stirring and heating above the T_{cp}. After overnight evaporation of ethanol at 37°C, the non-encapsulated hydrophobic compound was removed by filtration (0.45 µm syringe filter). In this way, Cy5-labeled BSA-pDMDOMA conjugates (1 mL, 2.5 mg/mL in PBS) were loaded with Cy3-alkyne (5 µL, 1 mg/mL in ethanol) and unlabeled BSA-pDMDOMA conjugates (0.5 mL, 2.5 mg/mL in PBS) were loaded with CL075 (15 µL, 5 mg/mL in ethanol). As a control, the same procedure was repeated for solutions of PBS buffer and (Cy5-)BSA macroCTA.

***In vitro* uptake experiment by DC2.4 cells.** DC2.4 cells (immortalized dendritic cell line) were cultured in RPMI-glutamax, supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES buffer, 0.05 mM 2-mercaptoethanol, MEM NEAM and antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin). Cells were incubated at 37 °C in an controlled, sterile environment of 95% relative humidity and 5% CO2. DC2.4 cells were seeded into 24-well titer plates (250 000 cells per well, suspended in 0.95 mL of culture medium) and incubated overnight to allow cell sedimentation and subsequent adhesion to the bottom of the wells. Next, 50 µL of the Cy3-alkyne loaded Cy5-BSA-pDMDOMA particles (2.5 mg/mL, *cfr. supra*) was added to the cells (conjugate concentration in wells of 0.125 mg/mL), followed by 24 h of incubation to allow cellular uptake. The same procedure was followed for Cy3-alkyne loaded PBS and Cy5-BSA macroCTA control samples. After overnight incubation, the wells were aspirated, washed with 1 mL of PBS and incubated with 500 µL of Cell Dissociation Buffer (15 min., 37°C). The cell suspensions were transferred into Eppendorf tubes and immediately centrifuged (350 g, 10 min., 5 °C). Finally, the supernatant was aspirated and the cell pellets were suspended in 200 µL of PBS and kept on ice to maintain cell integrity. FACS was performed on a BD Accuri C6 (BD Biosciences). The data were processed by FlowJo software.

Confocal microscopy on DC2.4 cells. DC2.4 cells were plated out on Willco-Dish glass bottom dishes (50 000 cells, suspended in 200 µL of culture medium) and incubated overnight. Next, 10 µL of the Cy3-alkyne loaded Cy5-BSA-pDMDOMA particles (2.5 mg/mL, *cfr. supra*) was added, followed by 24 h of incubation. Hoechst and CTB-AF488 staining was carried out simultaneously on fixed cells. In summary, culture medium was aspirated and cells were washed with PBS. Next, 200 µL of 4 % paraformaldehyde was added and allowed to fixate for 30 min. A staining solution was prepared by adding Hoechst (10 µL of a 1 mg/mL stock in DMSO) and CTB-AF488 (5 µL of a 1 mg/mL stock in PBS) to a PBS buffer containing 1% of BSA (2.5 mL). After aspiration and washing, 200 µL of this staining solution was added to the fixed cells and incubated for 40 minutes at room temperature. Finally, the samples were washed with 1% BSA PBS buffer. Confocal microscopy was carried out on a Leica DMI6000 B inverted microscope equipped with an oil immersion objective (Leica, 63x, NA 1.40) and attached to an Andor DSD2 confocal scanner. Images were processed with ImageJ software.

***In vitro* maturation experiment in murine bone marrow derived DCs.** Day 8 bone marrow derived DCs were incubated with 25 µL of the CL075 loaded BSA-pDMDOMA particles (2.5 mg/mL, *cfr. supra*). CL075 loaded PBS and BSA macroCTA were included as control samples. After overnight incubation, the cell suspensions were transferred into Eppendorf tubes and immediately centrifuged (350 g, 10 min., 5 °C). The supernatant was aspirated and the cell pellets were suspended in 50 µL of an antibody cocktail solution containing Fc block (diluted 200x), MHCII-FITC (diluted 500x), CD11c-APC

(diluted 200x) and CD86-PE (diluted 200x) in PBS buffer. After 30 min. of incubation on ice, 200 µL of PBS was added to the samples prior to centrifugation (350 g, 10 min., 5 °C). Finally, the supernatant was aspirated and the cell pellets were suspended in 200 µL of PBS and kept on ice to maintain cell integrity. FACS was performed on a BD Accuri C6 (BD Biosciences). The data were processed by FlowJo software. **Figure S6** illustrates the applied gating strategy, where the CD11c mouse DC surface marker is used to distinguish the dendritic cells from other bone morrow derived cell types.

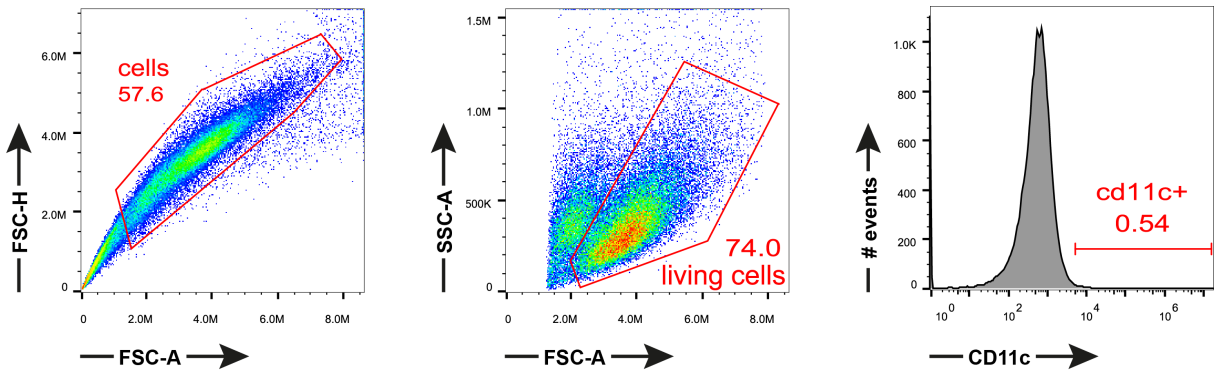

Figure S6. Flow cytometry gating strategy to select DCs from bone marrow cultures.

References.

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