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Supplementary information

Dual-Peptide Functionalized Acetalated Dextran-Based Nanoparticles for Sequential Targeting of Macrophages during Myocardial Infarction

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Experimental section

Synthesis of Putre-AcDEX. Putre-AcDEX polymer was synthetized from partially oxidated AcDEX, which was was synthetized as previously described.[1] First, partially oxidation of dextran (5 g, MW 9–11 kDa; Sigma-Aldrich, USA) was performed by adding sodium periodate (1.1 g; Sigma-Aldrich, USA) to an acqueous solution of the polymer (20 mL of Milli-Q water) and letting the mixture stirring for 5h at room temperature. Dialysis was used to purify the partially oxidized dextran against distilled water, using a regenerated cellulose membrane (Spectra/Por RC tubin, MWCO 3.5 k, USA). Upon lyophilization, partially oxidized dextran (3 g) was then modified with 2-methoxypropene (10.6 mL, Sigma–Aldrich, USA) and pyridinium p-toluenesulfonate (46.8 mg; Sigma–Aldrich, USA) in anhydrous dimethyl sulfoxide (30 mL, Sigma–Aldrich, USA) during 1 h under a Argon atmosphere to obtain the partially oxidized AcDX, as described elsewhere. Triethylamine (TEA, 1 mL; Sigma–Aldrich, USA) was used to quench the reaction. The resulting product was precipitated in Milli-Q water and centrifuged (10 min, 68320g). The pellet was washed twice with Milli-Q water and dried in vacuum oven at 40 °C for 48 h. Partially oxidized AcDEX (2.0 g) was then dissolved in 10 mL of methanol and putrescine (4.0 g, 19.8 mmol; Sigma-Aldrich, USA), dissolved in other 10 mL of methanol, was added to the solution, which was kept stirring for 24 h at room temperature. Sodium borohydride ($NaBH_4$, 1.0 g; Sigma-Aldrich, USA) was added to reduce the imine to amine. Reduction reaction was allowed to occur at room temperature during at least 24 h. Methanol (10 mL) and Milli-Q water (80 mL) were added to the flask to precipitate the polymer and to dissolve the excess $NaBH_4$. The polymer was washed with Milli-Q water pH 8 for five times. Residual moisture was removed by lyophilization, yielding white Putre-AcDEX powder.

The degree of functionalization was determined by ^{1}H NMR spectroscopy in deuterated dimethyl sulfoxide (DMSO), according to the method.[2] ^{1}H NMR (400 MHz, DMSO-*d6*): δ 1.40 (s, br, acetal), 3.25 (br, acetal), 3.45, 3.50−4.10, 4.90, 5.10 (br, dextran).

Preparation of Putre-AcDEX Nanoparticles. Putre-AcDEX nanoparticles were prepared by an oil–in–water (o/w) single emulsion technique, similarly as described elsewhere.[1] Briefly, putre-AcDEX polymer (6.5 mg) was dissolved in CH_2Cl_2 (0.125 mL). For the drug loaded particles, the two drugs, CHIR99021 (380 µg, Tocris, UK) and SB203580 (500 µg, ab120162, Abcam) were added to the polymer solution. After that, an aqueous solution of polyvinyl alcohol (PVA) (MW: 31 000–50 000 g/mol, Sigma-Aldrich, USA) (0.25 mL, 2.5%, w/v for empty particles and 2.0% for loaded ones) was added, mixed thoroughly and emulsified by sonication for 40 sec with an output setting of 5 and a duty cycle of 40%, using a probe sonicator (Sonics VCX 750, USA). The resulting o/w emulsion was then transferred to another solution of PVA (0.75 mL, 0.2% w/v for empty nanoparticles and 0.05% for loaded ones) and stirred for 3 h at room temperature to allow evaporation of the CH_2Cl_2. After 3h, the nanoparticles were pelleted by centrifugation (16 100g, 5 min), washed twice with a solution of Lutrol F127 0.5% pH 8 (BASF) and once with 2% w/v of sucrose (Sigma-Aldrich, USA). All supernatants were kept for detection of the drugs, CHIR99021 and SB203580, using high performance liquid chromatography (HPLC), as described below.

Surface Modification of Putre-AcDEX Nanoparticles. The surface of Putre-AcDEX nanoparticles was functionalized with a branched PEG (N-Mal-N-bis(PEG2-acid); MW 488.5 g mol^{-1}, BroadPharm, USA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxysulfosuccinimide (EDC/NHS, Sigma-Aldrich, USA) crosslinking chemistry. Briefly, a EDC/NHS solution was prepared by adding EDC (8 µL) and 2 mg of NHS to 2 mL of 10×10^{-3} M of 2-(N-morpholino)ethanesulfonic acid (MES, Sigma-Aldrich, USA). The pH was adjusted to 7.4, and the branched PEG and Putre-AcDEX nanoparticles were, respectively, dissolved and resuspended in the EDC/NHS solution. The EDC/NHS solution:nanoparticles ratio was 1:3, while the ratio branched PEG:Putre-AcDEX nanoparticles was 1:4. First, the branched PEG was dissolved in EDC/NHS solution and put to stir, and immediately after, the nanoparticles' suspension was added

dropwise to the PEG solution. The mixture was let to stir for 1 h at room temperature in dark. PEGylated nanoparticles were pelleted by centrifugation (16100*g*, 5 min), washed once with 2% w/v of sucrose (pH < 8) and then conjugated with carboxyfluorescein (FAM)–labelled Lin–TT1 peptide (AKRGARSTA), through a thioether bond between the thiol group of a cysteine residue of the peptide and the maleimide group on the functionalized particles. The reaction occurred in a solution of 10×10^{-3} M of MES (pH 7.8). The nanoparticles:peptide ratio was 60:1 (w/w), whereas the nanoparticles:MES ratio was 3:1 (w/w),. The peptide was dissolved in 1:10 of MES and added dropwise to the nanoparticles suspension. The mixture was left to stir for 2h at room temperature in dark. Particles were collected by centrifugation (16100*g*, 5 min), washed once with 2% w/v of sucrose (pH < 8) and then resuspended in a solution of ANP (MW 3080 g mol^{-1}, United BioSystems Inc, USA) in EDC/NHS pH 7.4 (3.6 mg mL^{-1}), and kept under stirring during 2 h at room temperature in dark.

Physicochemical Characterization. The hydrodynamic diameter and polydispersity of the nanoparticles were measured in a disposable polystyrene cuvette (SARSTEDT AG & Co., Germany) by DLS, while the ζ-potential was measured in a disposable folded capillary cell (DTS1070, Malvern, UK), using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK).

The surface chemical composition of the dry nanoparticles was evaluated by KBr–FTIR, using a Bruker VERTEX 70 series FTIR spectrometer (Bruker Optics, Germany). The FTIR spectra were recorded in the range of 4000–650 cm^{-1} with a resolution of 4 cm^{-1}, using an OPUS 8.1 software.

The amount of ANP covalently conjugated onto the nanoparticles was determined by elemental analysis using a vario MICRO cube CHNS analyzer (Elementar AnalysenSystem) using dry samples. The percentages of carbon, hydrogen, and nitrogen were recorded. The amount of ANP conjugated onto the nanoparticles' surface was calculated based on the percentage of N content and the chemical structure of the peptide.

The nanoparticles' morphology was evaluated by transmission electron microscopy (TEM, Jeol JEM-1400, Jeol Ltd., Japan). The samples were prepared by incubation of the carbon-coated copper grids (300 mesh; Electron Microscopy Sciences, USA) with a drop of 0.1 mg mL^{-1} nanocarrier suspensions during *ca.* 1 min, blotted away and left to dry at room temperature overnight.

TT1 Binding Assay. A fluorescent-based binding assay was performed to determine the effective functionalization of Putre-AcDEX nanoparticles with TT1 peptide, as described.[3] Briefly, Ni-NTA magnetic agarose beads (Qiagen, Germany) in binding buffer (50 mM of Tris (pH 7.4), 150 mM of NaCl, 0.05% of NP40, and 5 mM of imidazole) were coated with hexahistidine tagged p32 protein (at 15 μg of protein/10 μL beads). Fluorescently labelled Putre-AcDEX nanoparticles were incubated with the p32-coated beads in binding buffer containing 1% of bovine serum albumin (BSA) at room temperature for 1 h. Incubation was followed by washes and elution with 400 mM of imidazole containing binding buffer. The fluorescence of eluted samples was quantified using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific) with a λ_{Ex} of 488 nm and a λ_{Em} of 519 nm.

High-Performance Liquid Chromatography (HPLC), Encapsulation Efficiency (EE), Loading Degree (LD) and Drug Release Tests. The compounds encapsulated in the nanoparticles were quantified by using an Agilent 1100 series HPLC system (Agilent Technologies, Germany). A Discovery 5 μm C18 reversed phase column (100 × 4.6 mm, Supelco, USA) was used. The mobile phase was Na_2HPO_4:citric acid (2:1) (pH 6.0) and acetonitrile (ACN) (50:50, v/v). The flow rate used was 1.4 mL/min, the injection volume was 5 μL, and the wavelengths were set at 274 ± 20 nm and 304 ± 20 nm for CHIR99021 and SB203580, respectively.

For determination of the EE (ratio between the amount of drugs encapsulated and the total amount of drugs) and LD (ratio between mass of loaded drugs and total mass of drug encapsulated nanoparticles), a known amount of loaded Putre-AcDEX, Putre-AcDEX-PEG, Putre-AcDEX-PEG-

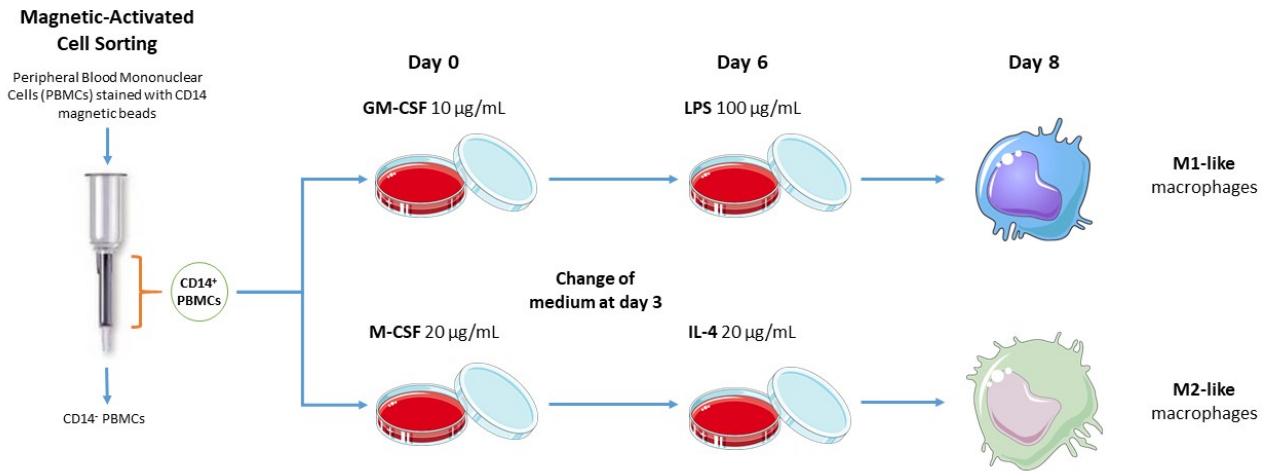
TT1, and Putre-AcDEX-PEG-TT1-ANP nanoparticles was dissolved in ACN. The CHIR99021 and SB230580 contents were measured by HPLC from the resulting solution, and all the supernatants resulting from the nanoparticle preparation. *In vitro* release studies were performed in sink conditions, in PBS (pH 7.4) and acetate buffer (pH 5), to simulate both the extracellular (pH 7.4) and intracellular (pH 5) microenvironments. The drug-loaded nanoparticles were immersed in the appropriate release medium, stirring at 150 rpm and 37 ± 1 °C. Free drugs were used as controls. Aliquots of 200 µL of samples were taken at specific time points, replaced with the same volume of fresh preheated medium, and analyzed by HPLC.

Macrophages Cell Lines and Cell Culture. RAW 264.7 and KG-1 macrophages (ATCC® TIB-71™ and ATCC® CCL-246™, respectively) were used for biocompatibility and uptake studies. RAW 264.7 and KG-1 macrophages were cultured with Dulbecco's Modified Eagle's Medium (DMEM) and Iscove's Modified Dulbecco's Medium (IMDM), respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 1% L-glutamine and 1% penicillin-streptomycin. Cells were kept in an incubator (BB 16 gas incubator, Heraeus Instruments GmbH) at 37 ºC, 5% CO_2 and 95% relative humidity.

Isolation of Monocytes from human Peripheral Blood Mononuclear Cells (PBMCs) and Polarization in M1– / M2–like Macrophages. Isolation of monocytes was performed, as described elsewhere with some modifications.[4] Briefly, PBMCs were isolated on Ficoll-Paque (1.077 g mL^{-1} density) gradients according to the manufacturer's instructions (GE Healthcare Bio-sciences, Piscataway, NJ) from buffy coats obtained from unknown healthy anonymous donors from the Finnish Red Cross. Once counted, $CD14^+$ monocytes were isolated from PBMCs by magnetic labelling using MAb CD14 conjugated microbeads (Miltenyi, Biotech, GmBH, USA), according to the manufacturer's instructions and then growth in Petri dishes at a density of 2×10^5 cells per mL in

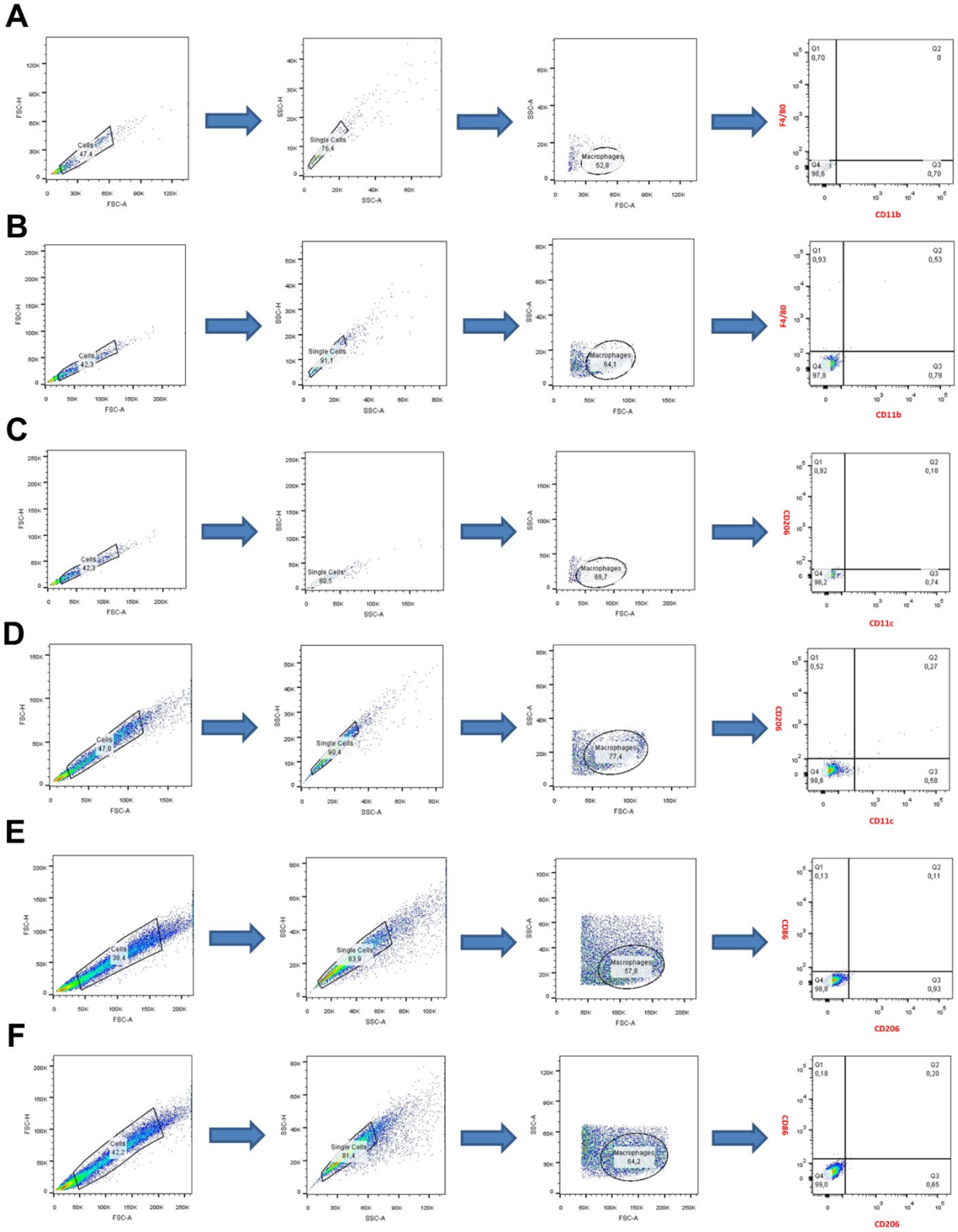
Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% FBS, 1% NEAA, 1% L-glutamine and 1% penicillin-streptomycin. Once isolated, PBMCs were cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium further supplemented with GM–CSF (10 ng mL^{-1}) or M–CSF (20 ng mL^{-1}), according to **Scheme S1** depending on whether monocytes were destined to M1 or M2 polarization. After three days, the medium was changed and monocytes were left to maturate until day six. Then, cells were kept for 48h in medium supplemented with 100 ng mL^{-1} of LPS or 20 ng mL^{-1} IL-4 to obtain, respectively, M1 and M2–like macrophages.

Isolation of murine Bone Marrow Derived Macrophages (BMDMs) and Polarization in M1– / M2–like Macrophages. Female 6-8 week old BALB/c mice were sacrificed with CO_2 and cervical dislocation. The femur and tibia bones were collected, cleaned from extra tissues and rinsed in sterile PBS (pH 7.4) at 4 ºC. The bones were then cut open, without losing the bone marrow, which was then flushed with a syringe, equipped with a 21G needle, in a Petri dish containing cold PBS. Clumps were broken by pipetting, and the cell suspension was transferred to a 15 mL Falcon and centrifuged at 500*g* for 5 min. The pellet was then gently dispersed into 1 mL of Ammonium-Chloride-Potassium (ACK) lysing buffer (150×10^{-3} M of NH_4Cl, 10×10^{-3} M of $KHCO_3$, and 0.1×10^{-3} M of Na_2EDTA) and incubated for 5 min to remove red blood cells. After this passage, 9 mL of complete RPMI medium were added and the cell suspension centrifuged again at 500*g* for 5 min. Cell were counted, dispersed in complete RPMI medium at density of 1×10^7 cells per mL and 200 μL of cell suspension were added in Petri dishes, containing 10 mL of complete RPMI medium supplemented with GM–CSF or M–CSF, depending on whether cells were destined for M1– / M2–like polarization. Maturation and polarization steps are the same used for human monocytes **(Scheme S1)**.



Scheme S1. M1- and M2-like macrophages polarization. Scheme of the protocol used to obtain the macrophages. Scheme was partially constructed by using Servier Medical art.

Flow Cytometry Study of the Expressed Markers. Human-derived M1 and M2–like macrophages were stained with allophycocyanin (APC)–CD86 and fluorescein isothiocyanate (FITC)–CD206 (Miltenyi, Biotech, GmBH, Germany) for 15 min at 4 ºC in the dark. Analogously, murine-derived matured and forty-eight hour stimulated MØ macrophages were treated instead with cocktails made, respectively, of APC–F4/80 and phycoerythrin–Cy7 (PE–Cy7)–CD11b, and APC–CD206 and FITC–CD11c. Fluorescence was detected by a LSR II flow cytometer (BD Biosciences, USA), and the data were analyzed with FlowJo software (Tree Star, Inc., USA). Cells were gated according to Scheme S2.



Scheme S2. Gatings for the Flow Cytometry Study of the Expressed Markers. Gatings adopted during the study of markers expressions on murine (**A–D**) and human (**E–F**) macrophages. Murine MØ macrophages matured after treatment with GM–CSF (**A**) and M–CSF (**B**), have been stained

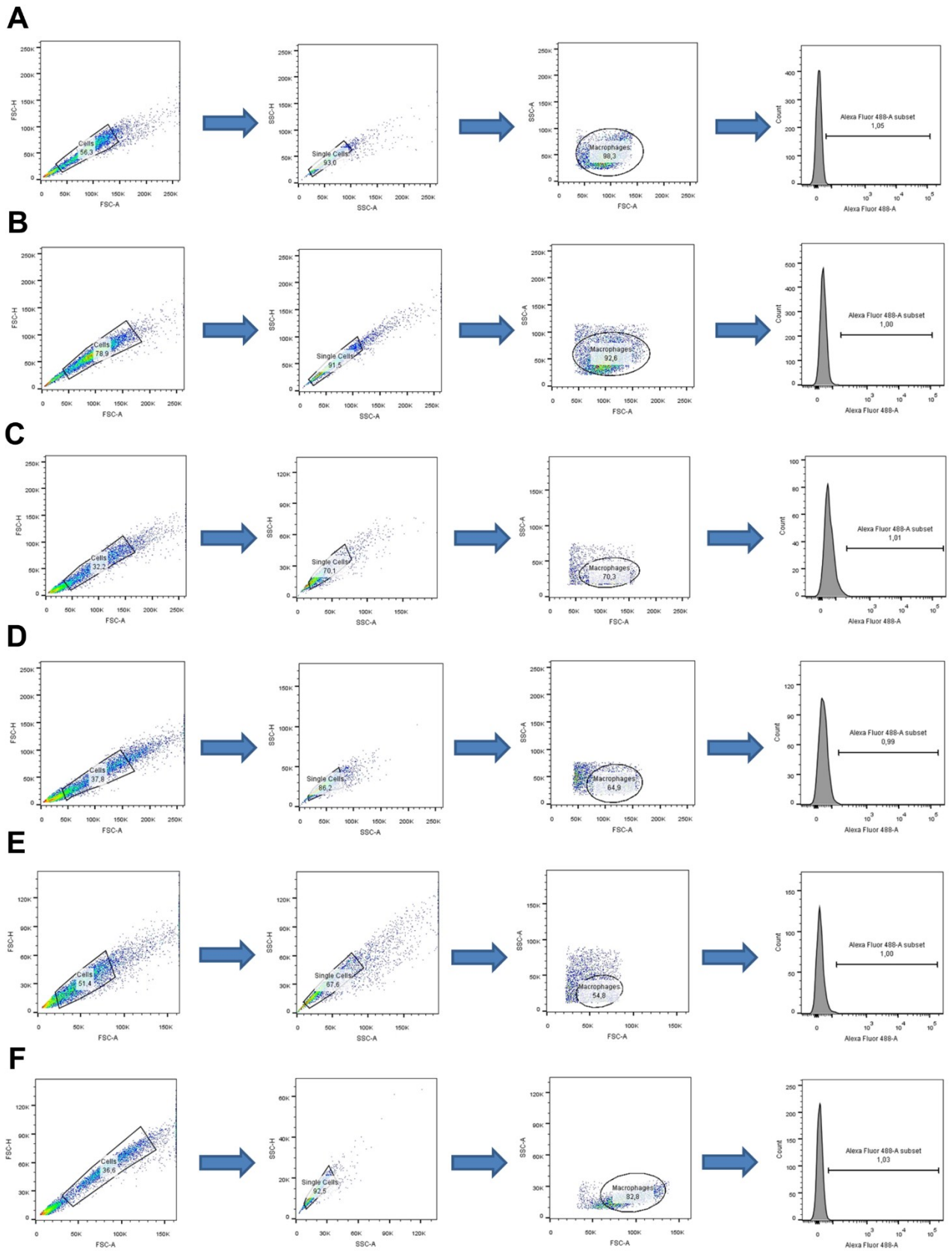
with APC–F4/80 and phycoerythrin–Cy7 (PE–Cy7)–CD11b, while macrophages stimulated with LPS (**C**) and IL-4 (**D**) have been screened for expression of APC–CD206 and FITC–CD11c. Human MØ macrophages stimulated with LPS (**E**) and IL-4 (**F**) have been stained with allophycocyanin (APC)–CD86 and fluorescein isothiocyanate (FITC)–CD206. Human MØ macrophages matured after treatment with GM–CSF and M–CSF were screened for the same markers and were gated in the same way (data not shown).

Cell Viability Studies. The cellular viability of all macrophages was evaluated with a luminescence assay, in which luminescence is proportional to the amount of ATP produced, and thus, to the percentage of viable cells. Cells were seeded in white bottom 96-well plates (Corning, USA) at a density of 2×10^4 cells per well and left to attach overnight if adherent. Nanoparticles' suspensions were prepared at different concentrations (10, 25, 50, 100, 250 and 500 μg mL^{-1}) in the specific cell culture medium. After incubation with the nanoparticles at 37 ºC for 24 and 48h, plates were taken out from the incubator and kept at room temperature for 30 min. After that, adherent cells were washed twice with Hank's Balanced Salt Solution (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (HBSS−HEPES, pH 7.4). The assay reagent (CellTiter-Glo®, Promega, USA) was diluted 1:2 with HBSS–HEPES and added to the cells. Non-adherent cells instead, were directly treated with 100 μL of CellTiter-Glo®. Plates were shaken for 2 min and the luminescence was measured using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific). A solution of 9% of Triton X-100, cell medium, and CellTiter-Glo® reagent were used as negative, positive and blank controls, respectively.

Quantitative Cellular Uptake Studies. All the different types of macrophages were seeded in 6-well plates at a density of 5×10^5 cells per well, and left attaching overnight at 37 ºC. Cells were then incubated with 50 μg mL^{-1} of fluorescent-labelled nanoparticles for 1h: Putre-AcDEX nanoparticles

conjugated with the peptides were fluorescent due to the presence of FAM moiety attached to TT1 peptide; PEGylated nanoparticles were made fluorescent by further conjugation of AlexaFluor® 488 by EDC/NHS chemistry onto the nanoparticles' surface. The ratio of Putre-AcDEX-PEG NPs:AlexaFluor® 488 was 200:1 (w/w) and the fluorescent dye was used from aliquots of 500 µg mL^{-1}.

After incubation, RAW 264.7 were washed twice with PBS–ethylenediaminetetraacetic acid (EDTA), collected by trypsinization and then washed again. KG-1 were pelleted and washed twice with PBS–EDTA. Both human and murine macrophages were washed twice with PBS-EDTA and then kept for 15 min on ice with PBS–EDTA solution (2 mM of EDTA). All the cells were finally dispersed in PBS–EDTA and quantitative uptake was evaluated by an LSR II flow cytometer (BD Biosciences, USA). In order to quantify the cellular uptake, external fluorescence was quenched by incubation for 5 min with trypan blue (TB; 0.005% v/v). Cells were then pelleted by centrifuging, dispersed in fresh PBS–EDTA and samples were run again. All the data were analyzed with FlowJo software (Tree Star, Inc., USA) and results were reported as median fluorescence intensity (MFI) values. Cells were gated according to Scheme 3.



Scheme S3. Gatings for the Flow Cytometry Uptake Studies. Gatings adopted during the quantitative uptake studies on RAW 264.7 (**A**), KG-1 (B), human M1–like (C) and M2–like (D)

macrophages and murine M1–like (E) and M2–like (F) macrophages. Uptake was detected and quantified on the AlexaFluor® 488 channel according to nanoparticles fluorescent spectrum.

Qualitative Cellular Uptake Studies. The qualitative intracellular uptake of the nanoparticles was evaluated by confocal microscopy with a Leica TCS SP8 STED 3X CW 3D inverted microscope (Leica Microsystems, Germany). RAW 264.7 and primary macrophages were seeded at a cell density of 7×10^4 cells per well into 8-well chambers (Lab Tek™, Thermo Fisher Scientific, USA) and let attaching overnight. Cells were then incubated with nanoparticles at concentration of 50 $\mu g\ mL^{-1}$ for 1h. After incubation, macrophages were washed twice with PBS and then stained with CellMask Deep Red (Thermo Fisher, USA), followed by fixation with 4% paraformaldehyde (Sigma-Aldrich, USA), and nuclear staining with DAPI (Thermo Fisher, USA). After staining steps and fixation, cells were washed three times with PBS. Images were captured by using a 63× water objective and then processed with Leica AS software (Leica Microsystems, Germany).

Uptake Mechanism Studies. Before incubation with nanoparticles decorated with both peptides, primary human and murine M1– /M2–like macrophages were treated with the compounds listed in **Table S1**, in order to inhibit specific uptake pathways, and thus, evaluate the mechanism of internalization of the nanoparticles. Incubation with the compounds was performed for 30 min, followed by addition of Putre-AcDEX-PEG-TT1-ANP nanoparticles. After incubation, cells were washed, detached, dispersed in PBS–EDTA and analysed by flow cytometry, as described above. Also in this case, interaction was subdivided in association and uptake.

Table S1 Compounds used to inhibit the different mechanisms of endocytosis.

Compound	Uptake Mechanism	Concentration

Inhibited

Cytochalasin D (Sigma-Aldrich, USA)	Macropinocytosis (actin polarization/depolarization)	5-10 μM
Nocodazole (Sigma-Aldrich, USA)	Macropinocytosis (microtubule disruptor)	20 μM
Genistein (Sigma-Aldrich, USA)	Caveoline-mediated endocytosis	200 μM
Chlorpromazine (TCI, Japan)	Clathrin-mediated uptake	10 μg/mL
Sodium azide (Sigma-Aldrich, USA)	Active transport	100 mM
Protamine sulfate (TCI, Japan)	Adsorptive-mediated endocytosis	1 mM

Statistical Analysis. Statistical analysis was performed using a GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). The statistical methods used to analyze the data from each experiment are described in each figure caption. In general, one-way ANOVA followed by a Tukey–Kramer post hoc test was used for the statistical analyses during the different studies.

Results and discussion

Table S2. Elemental analysis of Putre-AcDEX nanoparticles.

Sample	Weight (mg)	N %	C %	H %	S %
Putre-AcDEX	1.84	0.24	52.25	7.60	0.47
Putre-AcDEX-PEG	1.74	0.48	51.57	7.50	0.14
Putre-AcDEX-PEG-TT1	1.84	0.55	53.76	7.54	0.19
Putre-AcDEX-PEG-TT1-ANP	1.80	0.84	52.05	7.32	0.14

Table S3. Loading degree and encapsulation efficiency values for the different types of Putre-AcDEX nanoparticles.

Formulation	LD% (CHIR99021)	LD% (SB203580)	EE% (CHIR99021)	EE% (SB203580)
Putrescine-AcDEX	1.62 ± 0.39	3.73 ± 1.01	88.70 ± 3.32	81.30 ± 1.61
Putrescine-AcDEX-PEG	1.35 ± 0.69	2.87 ± 1.72	72.34 ± 4.39	56.36 ± 11.21
Putrescine-AcDEX-PEG-TT1	1.04 ± 0.19	1.96 ± 0.36	48.25 ± 2.98	31.22 ± 1.33
Putrescine-AcDEX-PEG-TT1-ANP	0.97 ± 0.60	1.67 ± 1.14	33.08 ± 16.06	25.29 ± 9.46

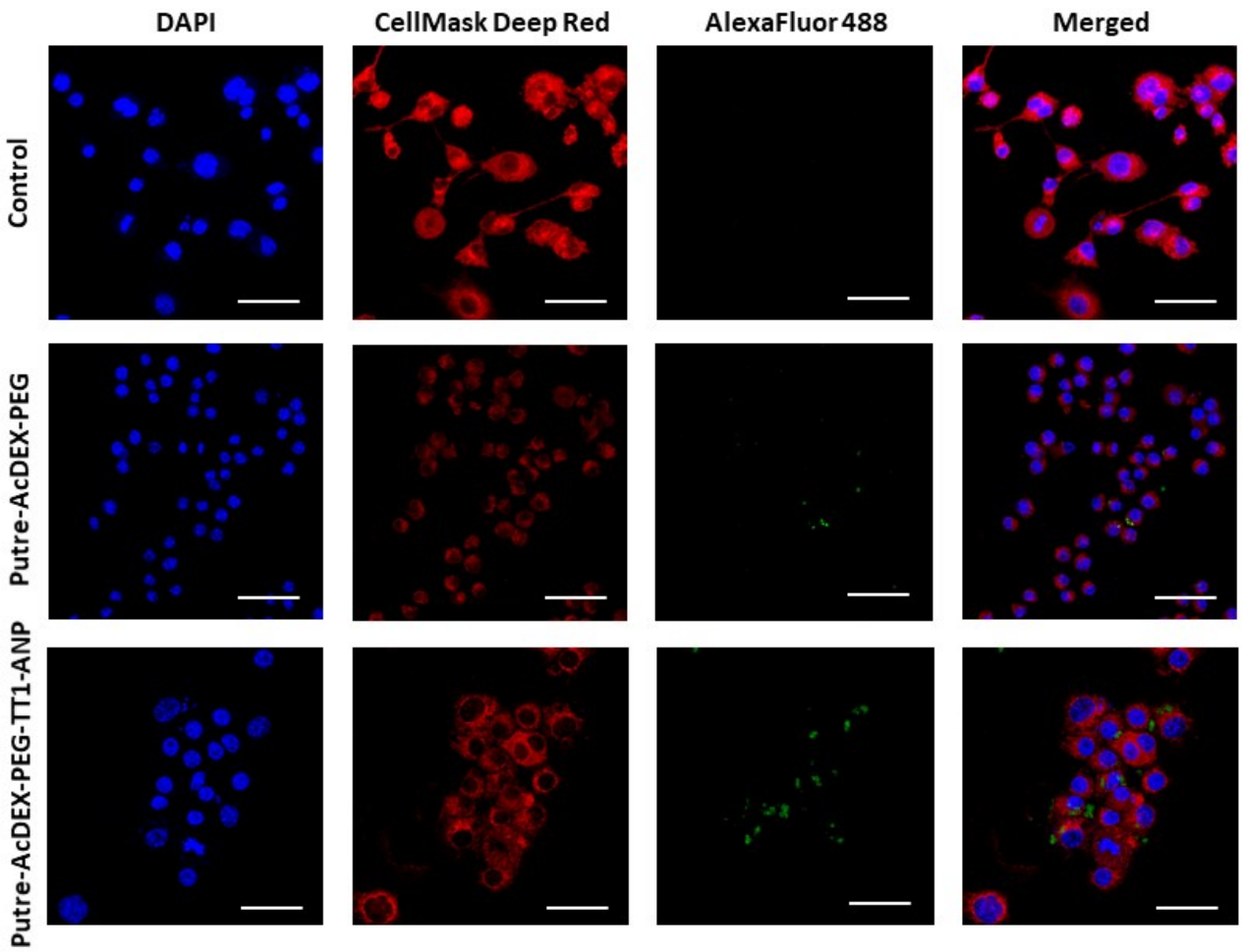


Figure S1. Qualitative uptake studies of RAW 264.7 macrophages. Uptake was evaluated by confocal fluorescence microscopy after incubation with the nanoparticles for 1 hour. Particles were either conjugated with AlexaFluor488 (Putre-AcDEX-PEG) or with FAM–conjugated TT1 peptide (Putre-AcDEX-PEG-TT1-ANP). In both cases, the nanoparticles were detected using the AlexaFluor 488 channel. Cells were stained with DAPI (nuclei) and CellMask Deep Red (membranes). Scale bars are equal to 21,8 μm.

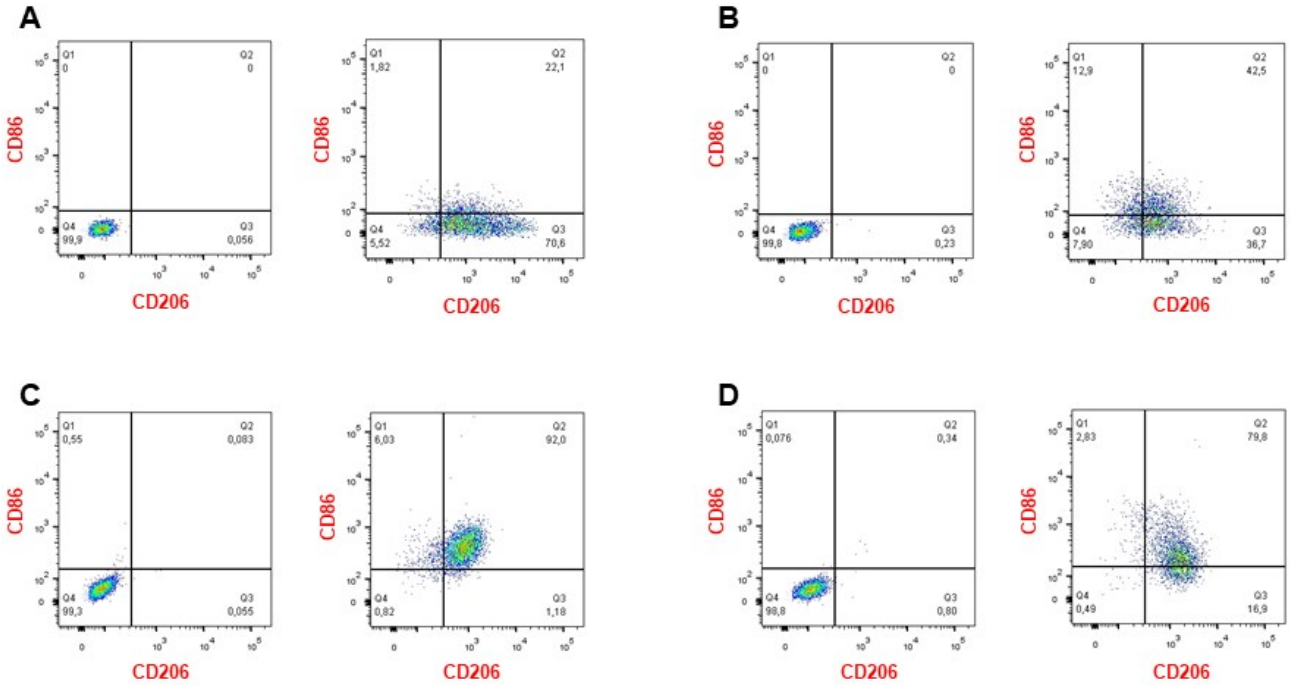


Figure S2. Markers expression of M1– / M2–like macrophages of human origin. CD14^{+} monocytes were magnetically isolated from human PBMCs and matured either with GM–CSF (**A**) or M–CSF (**C**) for six days. After that, cells were stimulated with LPS (**B**) and IL–4 (**D**) for two days to obtain M1– / M2–like macrophages. Both MØ and stimulated macrophages were then characterized according to the different expression of CD86 and CD206.

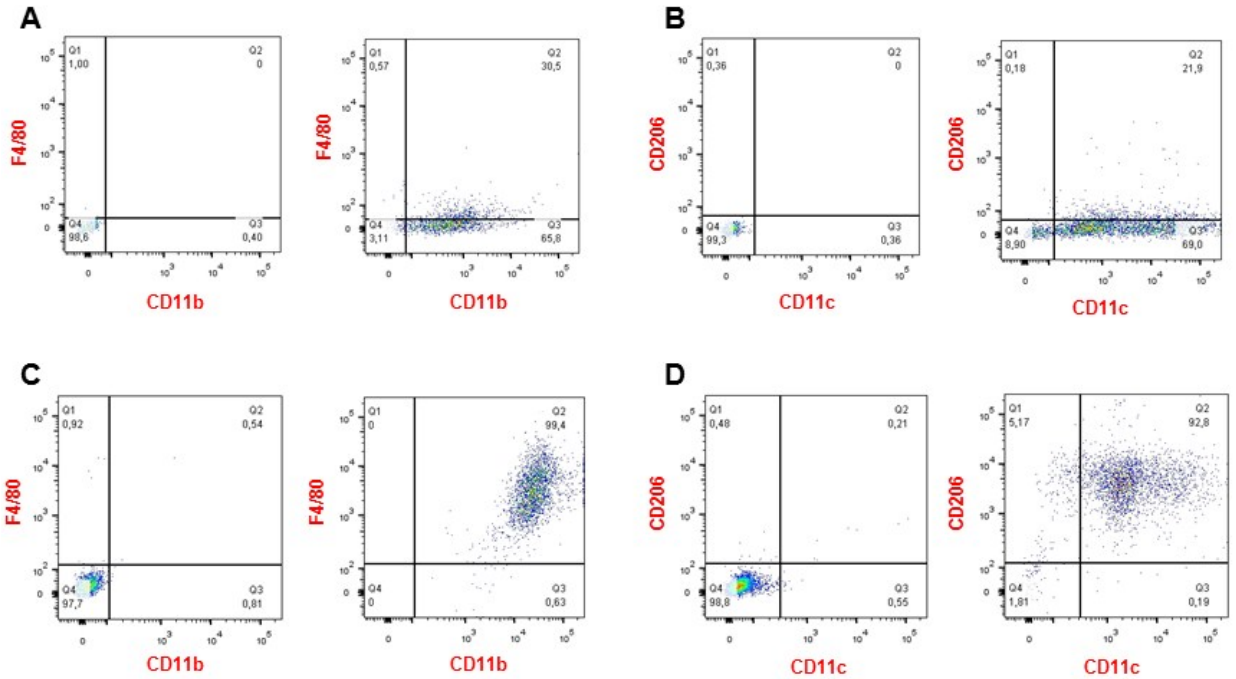


Figure S3. Markers expression of M1– / M2–like macrophages derived from murine bone marrow. Monocytes were collected from murine bone marrow and matured either with GM–CSF (**A**) or M–CSF (**C**) for six days. After that, cells were stimulated with LPS (**B**) and IL–4 (**D**) for two days to obtain M1– /M2–like macrophages. Matured MØ macrophages were characterized according to their CD11b and F4/80 expression. Macrophages were distinguished based on their peculiar CD11c and CD206 surface expression.

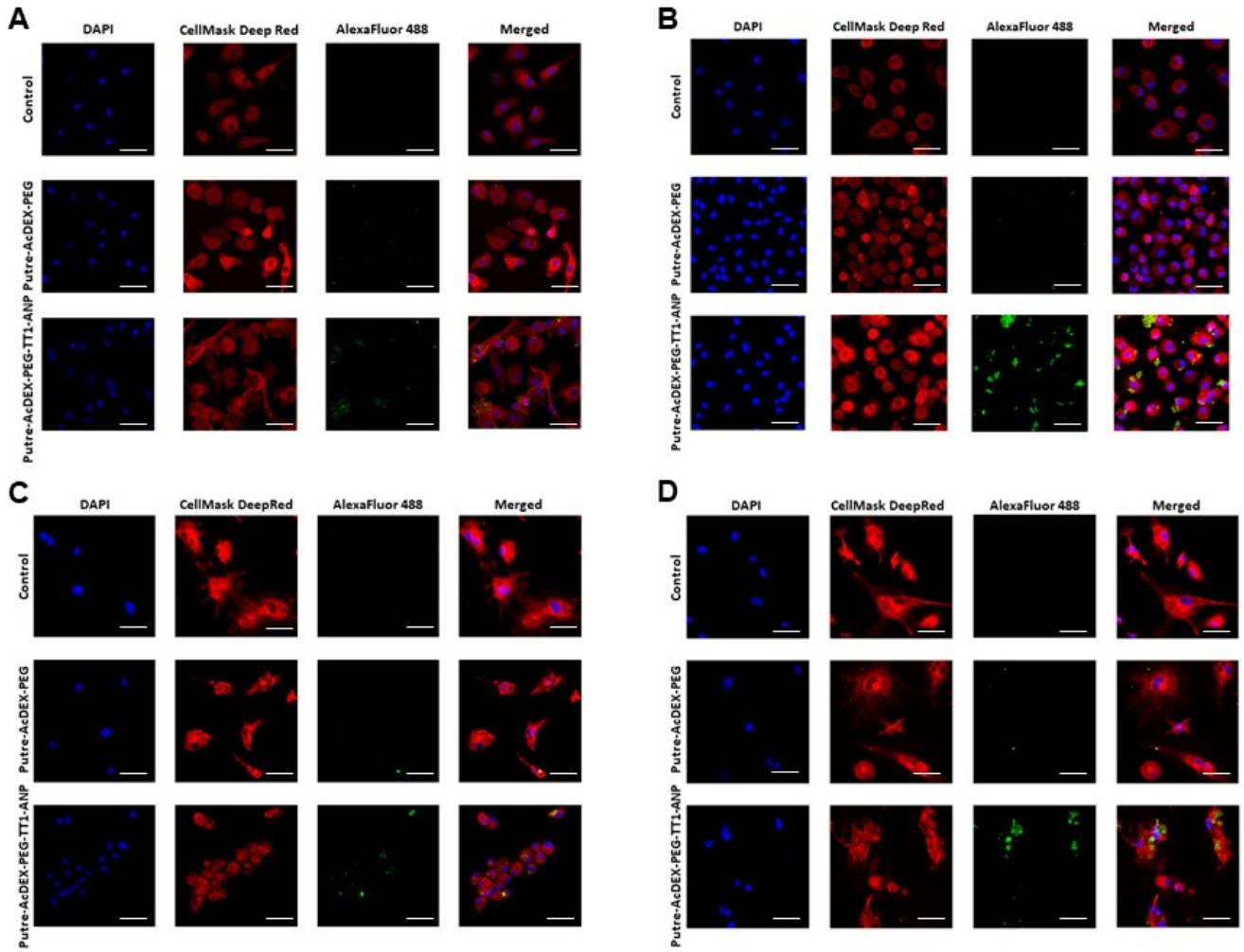


Figure S4. Qualitative cell uptake studies on primary M1– / M2–like macrophages. Fluorescence confocal images, presenting the interactions between Putre-AcDEX nanoparticles and human M1– (**A**) and M2–like (**B**) macrophages and murine M1– (**C**) and M2–like (**D**) macrophages. Cells were incubated 1 hour with the NPs, fixed and stained with DAPI (nuclei) and CellMask Deep Red (membranes), while particles were detected in the AlexaFluor 488 channel. Scale bars are equal to 21,8 μm.

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