Electronic Supplementary Material (ESI) for Nanoscale.

Supporting information

The conjugation strategy affects antibody orientation and targeting properties of nanocarriers

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1 Material and Methods

Culturing of Dendritic Cells

Murine DC2.4 Dendritic Cells (Merck, Germany) were cultured in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich, U.S.A), supplemented with 5% fetal bovine serum (FBS), 1% Penicillin (100 U mL^{-1}) and Streptomycin (100 mg mL^{-1}), as well as 1% 2-Mercaptoethanol (100X), in an incubator with 37 °C and 5% CO_2 humidity (CO_2 Incubator C200, Labotect). The confluence of the cells was checked under the microscope. When reaching a confluence greater than or equal to 80% the cells were passaged. The consumed media of the cells was discarded, and the cells were briefly washed with 10 mL PBS. For the detachment of the cells, 10 mL of cold PBS containing 2 mM EDTA were applied for 10 minutes at 4 °C. Then, 10 mL of FBS containing medium were added to the flask and pipetted up and down to detach all cells. The resulting cell suspension was transferred into a 50 mL tube and centrifuged at 300 g for five minutes. The supernatant was discarded, and the cell pellet was resuspended in 10 – 20 mL of FBS containing medium. Cell viability was checked with trypan blue, by mixing 20 µL of cell suspension to 20 µL of trypan blue solution of which 10 µL were pipetted onto a counting slide and the live count was measured by an automated cell counter (TC10, Bio-Rad). Dependent on the live count, the cell suspension was used for experimental procedures or for keeping the cells in culture.

Nanocarrier Functionalization

The BNF-Starch-redF nanocarriers (micromod, Germany) were functionalized with either the Sulfo-SMCC linker (Thermo, Waltham/U.S.A.) for thiol-maleimide chemistry or the DBCO-PEG_4-NHS linker (Bioscience, Germany) for copper-free click chemistry. For the thiol-maleimide chemistry, nanocarrier to linker ratios were calculated based on 3 nmol mg^{-1} amino groups on the nanocarrier (micromod data sheet). Therefore, the number of NH_2 groups per mg nanocarriers were multiplied by the molecular weight of the Sulfo-SMCC linker and the desired linker ratio (example calculation below). The linker solutions had to be prepared freshly

for each experiment and were not stored over a longer period due to the risk of oxidation. Before starting the linker reaction, the media of the nanocarriers was exchanged to 1 mM PBS-EDTA pH 7.4 utilizing magnetic force. Subsequently, the respective amount of maleimide-linker was added to the nanocarriers and incubated for 1 h at room temperature under shaking. Afterwards, the mixture was washed three times with 1 mM PBS-EDTA pH 7.4 by applying a magnet (strong neodymium magnet, Germany) to separate unbound linker moieties. The remaining pellet was resuspended in 1 mM PBS-EDTA pH 7.4 and quantified by fluorescence calibration. For the copper-free click approach, 10 mg of the DBCO-PEG_4-NHS linker were resuspended in 500 µL of DMSO to obtain a stock solution with 20 mg mL^{-1}. This stock solution could be used for several months being stored at -20 °C. As before, linker ratios were calculated based on 3 nmol mg^{-1} amino groups on the nanocarrier. For example, 1 mg of nanocarriers (100 µl) was reacted with 1.95 µg DBCO-PEG_4-NHS linker (1 µL of 2 mg mL^{-1} DBCO-PEG_4-NHS linker stock solution) for a 1:1 ratio, 19.5 µg DBCO-PEG_4-NHS linker (9.75 µL of 2 mg mL^{-1} DBCO-PEG_4-NHS linker stock solution) for 1:10 ratio and so forth. The reaction was incubated overnight under constant shaking at room temperature. The next day, the mixture was washed three times with PBS by applying the neodymium magnet to separate unbound linker moieties. The remaining pellet was resuspended in PBS and quantified by fluorescence calibration.

Nanocarrier Quantification

To determine the amount of nanocarrier and nanocarrier-conjugates, a fluorescence calibration of the nanocarrier was conducted. The unfunctionalized nanocarriers were utilized as a standard and the samples diluted based on the initial mass used. All probes, including the standard, were prepared as duplicates in deionized water. Fluorescence was measured by the Infinite M1000 plate reader (Tecan) with an excitation wavelength of 552 nm and emission of 580 nm.

Traut's Modification of Antibody-Lysine Residues

The procedure for thiolation of the antibodies (mouse α-hamster CD11c clone N418, and α-hamster IgG isotype control clone HTK888, both Biolegend, U.S.A) lysine residues was partially adapted from the Traut's reagent and Micromod's Technote 201 manufacturer's instructions. In summary, a buffer exchange to 1 mM PBS-EDTA pH 8.0 was conducted for the antibodies with a Zeba™ Desalting Column after the manufacturer's instructions. Subsequently, the antibody concentration was quantified by Pierce 660 nm and the respective amount of Traut's reagent was calculated dependent on the obtained antibody concentrations. The Traut's reagent was prepared as a stock solution in 1 mM PBS-EDTA pH 8.0 (14 mM stock = 2 mg mL^{-1}). For example, 147 µg (490 µL) of antibody were reacted with 7 µL of the 14 mM Traut's stock solution for a 100x molar excess of Traut's reagent. The modification of

antibodies with the Traut's reagent was conducted for 1 h at room temperature. Conclusively, the Traut's reagent was removed from the modified antibody by performing another buffer exchange, followed by a final determination of the concentration by Pierce 660 nm. The modified antibodies were directly tested for thiolation or conjugated to functionalized nanocarriers via thiol-maleimide chemistry.

Enzymatic Modification of Antibody Glycans

The azide modification of the antibodies (α-CD11c, α-IgG isotype) was conducted according to the manufacturer's instructions (Site Click™ Antibody Azido Modification Kit). Briefly, the carbohydrate domain at the Fc region of the concentrated antibody was modified by cleaving the galactose residues via ß-galactosidase for 6 h at 37 °C. Afterwards, the azide group was attached by applying the GalT (Y289L) enzyme in combination with the UDP-GalNAz donor in an overnight reaction at 30 °C. Finally, the azide-modified antibody was purified and concentrated for further experiments, including Pierce 660nm Protein Assay, testing for azide-modification as well as conjugation to functionalized nanocarriers via copper-free click chemistry.

Antibody Quantification

The total amount of antibody or protein concentrations were quantified by using the Pierce 660nm protein assay according to the manufacturer's instructions. BSA (bovine serum albumin) was used as a standard by preparing a dilution series in demineralized water. The Infinite M1000 plate reader (Tecan) measured all samples at an optical density of 660 nm, including the standard, in duplicates.

Detection of Antibody Modification

Comparison between modified and unmodified antibodies in combination with a linker of known mass was achieved by applying SDS-PAGE in combination with either silver staining or Coomassie staining. For each chemistry, a specific linker was applied. The MeO-PEG-Mal (5 kDa) linker (Sigma, U.S.A.) for thiol-maleimide chemistry and the DBCO-mPEG (5 kDa) linker (Iris Biotech, Germany) for copper-free click chemistry. In case of the thiol-maleimide chemistry, the thiolation detection was calculated based on the assumption of five possible thiol groups to be conjugated. For the copper-free click chemistry, the azide detection was calculated based on four azide groups to be linked. Conjugation with the respective detection linker was conducted overnight at room temperature under shaking. Analysis by SDS-PAGE was conducted either under reduced or non-reduced conditions. Samples were prepared in a total volume of 40 or 60 µL. The desired antibody amount was adjusted with demineralized water to a total volume of 26 µL for the 40 µL approach (all volumes can be adjusted to the 60 µL approach by multiplying the volume with 1.5). Further, 4 µL of NuPAGE™ sample reducing agent (4 µL demineralized water for non-reduced

samples) and 10 µL of NuPAGE™ LDS sample buffer were added and incubated at 70 °C for 10 min. The samples were transferred into the chambers of a Bolt™ 10% Bis-Tris Plus Gel submerged in 1X NuPAGE™ MES SDS Running Buffer and run for 1 – 6 h at 110 – 120 V. SeeBlue™ Pre-Stained Standard was used as molecular weight marker. The visualization of the proteins was conducted by applying either silver staining (SilverQuest™) or Coomassie staining (SimplyBlue™ SafeStain) according to the manufacturer's instructions.

Antibody to Nanocarrier Conjugation

The Sulfor-SMCC linker with its maleimide group was binding the terminal sulfhydryl groups of the modified antibody to form the conjugate via thioether bonding. In case of the DBCO-linker, the copper-free click reaction between the alkyne group of the DBCO and the azide group of the modified antibody was employed to generate the conjugate with a stable triazole formation. Maleimide functionalized nanocarriers were synthesized with the thiolated antibodies for three hours at room temperature under shaking (in 0.1 mM PBS-EDTA pH 7.4). DBCO-functionalized nanocarriers were overnight conjugated with the azide-modified antibodies at room temperature under shaking (in PBS). After incubation, the maleimide or DBCO conjugates were washed three times with 0.1 mM PBS-EDTA pH 7.4 or PBS, respectively, by using a magnet. Finally, the conjugates were resuspended in the respective media and utilized for further experiments or stored at 4 °C. Before testing the nanocarrier conjugates in the dendritic cell uptake experiments, they were analyzed by flow cytometry to detect the covalently bound antibodies. This was performed by utilizing a secondary FITC labeled anti-hamster antibody and the unfunctionalized nanocarriers as a negative or positive control, respectively.

Detection of Unbound Antibodies

For the detection of unbound antibodies after the Antibody to Nanocarrier Conjugation, each magnetically separated wash fraction was collected and analyzed in the DC2.4 cell line via flow cytometry. For this purpose, the supernatant and washes were collected and concentrated to a final volume of 350 µL in PBS-EDTA (thiol-maleimide chemistry) or PBS (copper-free click chemistry) using the Savant DNA120 SpeedVac Concentrator (Thermo Fisher). The supernatants and washes of the CD11c and IgG conjugates with an antibody amount of 7.5 µg were analyzed. For the detection of unbound antibodies, 1.5 $x10^5$ cells mL^{-1} were used for each condition in 100 µL PBS in 1.5 mL tubes. All samples were analyzed in triplicates (n = 3). In the first step, the cells were centrifuged at 300 g for 5 min and the cell pellets were resuspended in 100 µL of the supernatants and/or the three washes. After an incubation time of 30 min at 4°C, the cells were centrifuged again at 300 g for 5 min. Then, the cell pellets were resuspended in 100 µL PBS containing 0.5 µg (1 µL, 1:100) of the secondary FITC Goat anti-hamster (Armenian) IgG antibody (polyclonal antibody clone Poly4055, Biolegend). After an

incubation time of 30 min at 4°C, the cells were centrifuged at 300 g for 5 min. Finally, the cell pellet was resuspended in 1 mL PBS and analyzed by flow cytometry

Dendritic Cell Uptake Experiments

For the cell uptake experiments, 1.5 x 10^5 cells mL^{-1} were seeded per well in a 24-well plate and left to attach overnight in a humidified incubator at 37 °C and 5% CO_2. The next day, dependent on the experiment, the FBS containing medium was exchanged to medium without FBS and left for one to two hours so that the cells could adapt to the serum-free environment. In case of FBS dependent experiments, the medium was exchanged to a new FBS containing medium and left for one to two hours. All samples were prepared in triplicates if not stated otherwise and incubated for 2 h with a concentration of 75 µg mL^{-1} in 250 µL of medium per well. For the mouse plasma incubation, each sample was pre-incubated for one hour at 37 °C shaking in a 1:1 ratio with the plasma source. Afterwards, the plasma was exchanged to IMDM without FBS by using a magnet and 75 µg mL^{-1} in 250 µL of medium were incubated with the dendritic cells. After the two-hour incubation, the cells were washed with 1 mL PBS and detached via 250 µL 2 mM PBS-EDTA for 10 min at 4 °C. Afterwards, the cells were transferred from the well into a 1.5 mL micro tube by adding 250 µL of FBS containing medium to the cells in the well and pipetting it up and down to detach as many cells as possible. For the cell viability assay, the cells were centrifuged at 300 g for 5 min and the cell pellet was resuspended in 100 µL Zombie Agua (Biolegend, U.S.A/ previously diluted 1:500 in PBS). After 15 min of incubation at 4 °C in the dark, the cells were centrifuged again at 300 g for 5 min and the cell pellet was resuspended in 1 mL PBS ready to be analyzed by flow cytometry.

Blocking Experiments

All blocking experiments were performed in 250 µL IMDM medium without FBS for 30 minutes at 4°C. The CD11c (monoclonal antibody clone N418, Thermo Fisher) blocking was first tested with four different antibody conentrations (S6). After blocking, a fluorescence-conjugated CD11c antibody (CD11c monoclonal antibody clone N418, PE-Cyanine7, Thermo Fisher) was used to check for free or blocked CD11c integrins on the cell surface, respectively. This validation resulted in a final CD11c blocking concentration of 7.5 µg mL^{-1} before the antibody nanocarrier conjugates cell uptake. The antibody nanocarrier conjugates cell uptake was conducted with a sample concentration of 75 µg mL^{-1} for 30 – 40 min at 4°C for both, the CD11c and non-blocking reference.

Flow Cytometry

Quantification of nanocarriers or conjugates taken up by cells as well as the detection of the attached antibodies by secondary antibody testing was analyzed by flow cytometry. The red fluorescence of the nanocarriers was detected by the YL1 channel with an excitation laser of 561 nm and a 585/16 nm band pass filter for emission. Cell viability with the Live/Dead fixable

Zombie Agua (Biolegend, U.S.A) was performed by using the VL2 channel with an excitation laser of 405 nm and a 512/25 nm band pass filter for emission. Data analysis was performed by applying AttuneTM NxT Software. Here, the cell population was selected with a FSC/SSC scatter plot, excluding cell debris populations. The gated events of viable cells were evaluated by the fluorescent signal expressed as median fluorescence intensity (MFI) or as the percentage of gated events/cells. For the secondary antibody testing, 2 µg of sample were added to 1 µg of secondary antibody in 20 µL PBS and incubated for 30 min at 4 °C in the dark. Unfunctionalized nanocarriers with and without the secondary antibody were applied as controls. After incubation, the samples were added up to 1 mL PBS and analyzed by flow cytometry. The FITC detection of the secondary antibody was performed by using the BL1 channel with an excitation laser of 488 nm and a 530/530 nm band pass filter for emission.

Visualization of Intracellular Localization by cLSM

In order to demonstrate the internalization effect of antibody functionalized nanocarriers in cells, confocal laser scanning microscopy (cLSM) was employed. Experiments were conducted on the LSM SP5 STED Leica Laser Scanning Confocal Microscope (Leica, Germany), composed of an inverse fluorescence microscope DMI 6000CS equipped with a multi-laser combination using a HCX PL APO CS 63 x 1.4 oil objective. Nanocarriers were excited with the excitation laser 561 nm and detected with an emission filter at 570 – 599 nm. The cell membrane was stained with CellMask DeepRed (5 mg mL^{-1}, Thermo) using the excitation laser 633 nm, detected at 660 – 700 nm. For the visualization of the CD11c blocking experiment, 5 x 10^4 cells in 200 µL IMDM with 5% FBS were seeded in a well of a 15 µ-Slide 8 well glass bottom (ibidi). After an overnight incubation at 37 °C and 5% CO_2 in the incubator, the cells were blocked with 7.5 µg mL^{-1} CD11c antibodies (monoclonal antibody clone N418, Thermo Fisher) in IMDM medium without FBS. After the incubation, the wells were washed with PBS and an antibody nanocarrier conjugate uptake with 75 µg mL^{-1} was performed for 30 min at 4°C. Then, the wells were washed with PBS and the cells fixed for 15 min with 4% paraformaldehyde (PFA). Afterwards, the cells were stored in PBS at 4 °C until being further processed for image acquisition. For the visualization of intracellular localization, 5 x 10^4 cells in 200 µL IMDM with 5% FBS were seeded in a well of a 15 µ-Slide 8 well glass bottom (ibidi). After an overnight incubation at 37 °C and 5% CO_2 in the incubator, the cells were treated with the respective sample in medium with 5% FBS as well as pre-incubated with mouse plasma proteins. After the two-hour incubation with a concentration of 75 µg mL^{-1}, the wells were washed with PBS and the cells fixed for 15 min with 4% paraformaldehyde (PFA). Afterwards, the cells were stored in PBS at 4 °C until being further processed for image acquisition. Cell membrane staining was conducted shortly before the cLSM analysis for 5 min in the dark (1:1.000 in PBS).

Incubation and Visualization of Mouse Plasma

SDS-PAGE and silver staining visualized adsorbed mouse plasma proteins on nanocarrier conjugates. First, the samples were incubated with the mouse plasma (GeneTex, California, U.S.A) at a 1:1 volume ratio for 1 h at 37 °C under shaking. After the incubation, the plasma was removed and the samples were washed three times with PBS, using a magnet. For the protein desorption, the washed pellet was resuspended in 100 µL desorption buffer (2% (w/v) SDS + 62.5 mM Tris-HCl in deionized water) and incubated for 10 min at 70 °C under shaking. Afterwards, the magnet was applied to separate desorbed proteins from the nanocarriers. The denatured proteins containing supernatants were quantified by Pierce 660nm Protein assay and 3 µg were used for SDS-PAGE, visualized by silver staining.

Size and Charge of Antibody Nanocarrier Conjugates

The determination of size and charge of synthesized conjugates is important to estimate differences between unfunctionalized nanocarriers and those that are conjugated to a linker with and without the antibody. Light scattering measurements were performed on an ALV spectrometer consisting of a goniometer and an ALV-5004 multiple-tau full-digital correlator (320 channels) which allows measurements over an angular range from 30° to 150°. A He-Ne Laser (wavelength of 632.8 nm) is used as light source. For temperature controlled measurements the light scattering instrument is equipped with a thermostat from Julabo. 1 µL of unfiltered dispersion was diluted with 1 ml PBS which was previously filtered through membrane filters with a pore size of 0.20 µm (GS Millipore). Measurements were performed at 20°C at 7 (dynamic) angles ranging from 30° to 150°. The zeta (ζ) potential of the nanocarriers and synthesized conjugates (10 µL sample) was measured with a Zetasizer Nano – Z Series (Malvern, Germany) in 1 mM potassium chloride solution (1 mL KCl). The results of the measurements are listed in the table S1.

TEM Measurements

The morphology of the particles was investigated using a FEI Tecnai F20 transmission electron microscope operating at voltage of 200 kV. The different samples were diluted to a 75 µg mL^{-1} particle concentration in the solvent of interest, 4 µL were deposited on a carbon coated grid and the excess solvent was blotted away with filter paper.

In solution digestion

First, SDS was removed from protein solutions by applying Pierce detergent removal columns (Thermo Fisher, Germany). The digestion was based on previously established protocols [1, 2]. Briefly, ProteoExtract protein precipitation kit (CalBioChem, Germany) was used to precipitate proteins overnight according to the manufacturer's instructions. Proteins were isolated by centrifugation (14.000 g, 10 min), followed by several washing steps and resuspension in

RapiGest SF (Waters Cooperation, Germany) dissolved in ammonium bicarbonate (50 Mm) buffer. Then, the samples were incubated at 80°C for 15 min. Protein disulfide bonds were reduced using Dithiothreitol (Sigma, Germany) at a final concentration of 5 mM. The reaction was executed for 45 min at 56°C. Iodoacetamide (final concentration 15 mM, Sigma, Germany) was used for alkylation of proteins in the dark for 1 h. A protein:trypsin ratio of 50:1 was chosen for digestion and the reaction was performed over 14 h at 37°C. The reaction was quenched by adding 2 µL hydrochloric acid (Sigma, Germany). Ultimately, degradation products of RapiGest SF were removed by centrifugation of the peptide samples at 14.000 g for 15 min (4°C).

Liquid chromatography-electrospray ionization mass spectrometry (LC-MS)

For absolute protein quantification, all specimen were diluted with 0.1% formic acid and spiked with 50 fmol μL^{-1} Hi3 E. coli standard (Waters Cooperation) [3]. In a nanoACQUITY UPLC system, the tryptic peptides were transferred to a C18 analytical reversed phase column (1.7 µm, 75 µm x 150 mm) and a C18 nanoACQUITY trap column (5 µm, 180 µm x 20 mm). For separation, two mobile phases with (A) consisting of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid with acetonitrile and a gradient of 2% to 37% of mobile phase (B) were applied for a time period of 70 min. The nanoACQUITY UPLC system was connected to a Synapt G2-Si mass spectrometer and electrospray ionization (ESI) was performed in positive ion mode using a NanoLockSpray source. A sample flow rate of 0.3 µl min^{-1} was selected and Glu-Fibrinopeptide, as a reference component, was infused with 150 fmol μl^{-1} at a flow rate of 0.5 µl min^{-1}. The Synapt G2-Si was operated choosing the resolution mode and data-independent acquisition (MSE) experiments were conducted. The data was acquired for a time of 90 min with a mass to charge range of 50 – 2000 Da, a scan time of 1 s and ramped trap collision energy from 20 to 40 V. Data acquisition and processing was performed using MassLynx 4.1.

Protein identification

The continuum data was post lock mass corrected and further processed by Progenesis QI (2.0) applying a reviewed mouse data base (Uniprot) for peptide and protein identification. Noise reduction thresholds were set to 120, 25, and 750 counts by using several processing parameters for low energy, high energy and peptide intensity. For absolute quantification, the Hi3 *E. coli* standard sequence information was added to the mouse data base. Following criteria were chosen for protein and peptide identification: one missed cleavage, maximum protein mass 600 kDa, fixed carbamidomethyl modification for cysteine, variable oxidation for methionine and protein false discovery rate of 4%. At least two assigned peptides and five assigned fragments are required for protein identification. Peptide identification relies on three assigned fragments. Identified peptides with a score parameter below 4 were discharged. The

TOP3/Hi3 approach provided the amount of each protein in fmol [4]. A detailed list of the identified proteins can be found separately in the ESI (excel sheet).

Statistical Analysis

Using GraphPad Prism 5, two-way analysis of variance (ANOVA) was performed with Bonferroni's post-hoc-test choosing a confidence interval of 99.9% (P<0.001***) and 95% (P<0.05*) significance.

2 Supporting Figures and Tables

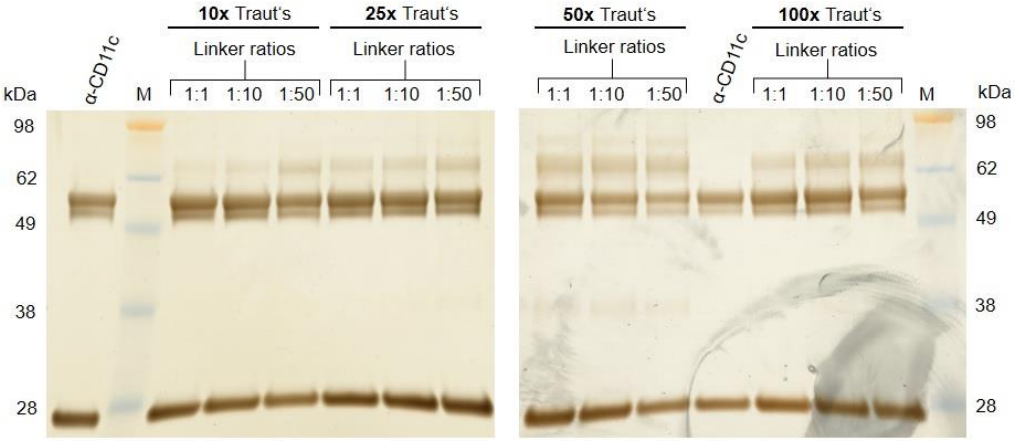

Figure S1: Evaluation of thiolated and linker-reacted CD11c antibodies under reduced conditions. For the analysis, 10 µL of α-CD11c (21 µg) were reacted with 1; 2.5; 5; and 10 µL of Traut's reagent (1.4 mM) for 1h at room temperature, representing a Traut's excess of 10; 25; 50 and 100 mol to α-CD11c, respectively. For the overnight coupling, 4 µg of modified α-CD11c-SH (20 µL) were reacted for each Traut's group in an α-CD11c-SH to maleimide linker ratio of 1:1; 1:10 and 1:50 (0.65 µg $µL^{-1}$ linker stock = 1:1 ratio of linker to α-CD11c). All samples were prepared according to reduced SDS-PAGE instructions (40 µL approach) and boiled for 10 min at 70 °C. The molecular weight marker (M) was applied with 5 µL and each sample at 2 µg onto a 10% Bis-Tris Plus gel and run for 111 min at 120 V. Visualization was conducted by silver staining.

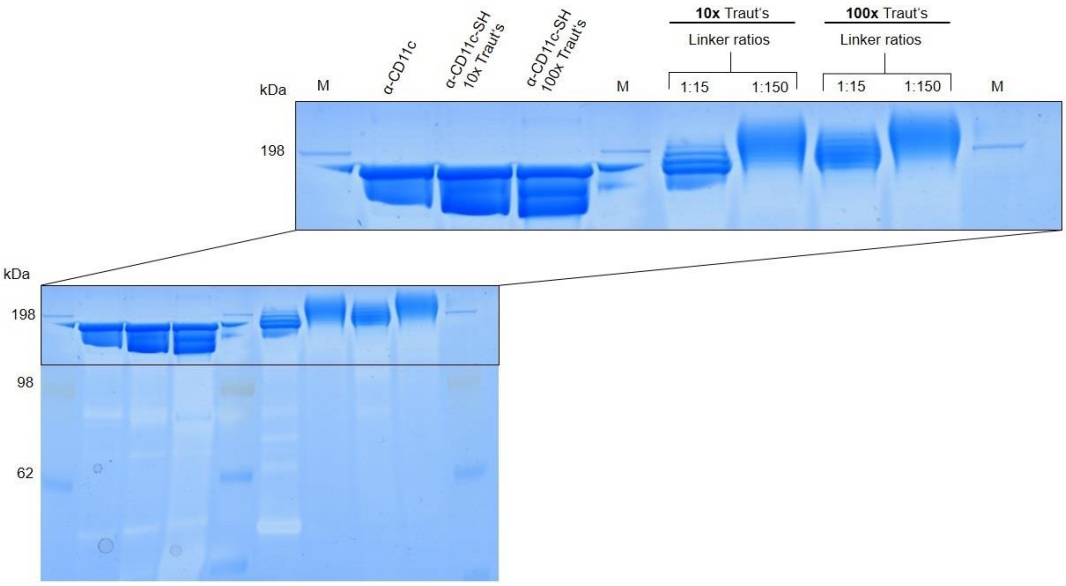

Figure S2: Evaluation of thiolated and linker-reacted CD11c antibodies. For the Traut's modification, 74 µg of α-CD11c (~245 µL) were reacted with 0.4 and 4 µL of Traut's reagent (14 mM) for 1 h at room temperature, representing a Traut's excess of 10 and 100 mol to α-CD11c, respectively. For the overnight coupling, 7 µg of modified α-CD11c-SH (~30 µL) were reacted for each Traut's group in an α-CD11c-SH to maleimide-linker ratio of 1:15 and 1:150 (1.25 µg μL^{-1} linker stock = 1:1 ratio of linker to α-CD11c-SH). Modified samples were prepared according to SDS-PAGE instructions (60 µL approach). Unmodified α-CD11c was prepared at a 40 µL approach. All samples were boiled for 10 min at 70 °C. The molecular weight marker (M) was applied with 5 µL, the unmodified and thiolated α-CD11c groups with 7 µg and the linker-reacted groups with 3.5 µg onto a 10% Bis-Tris Plus gel and run for ~6 h at 120 V. Visualization was conducted by Coomassie Staining.

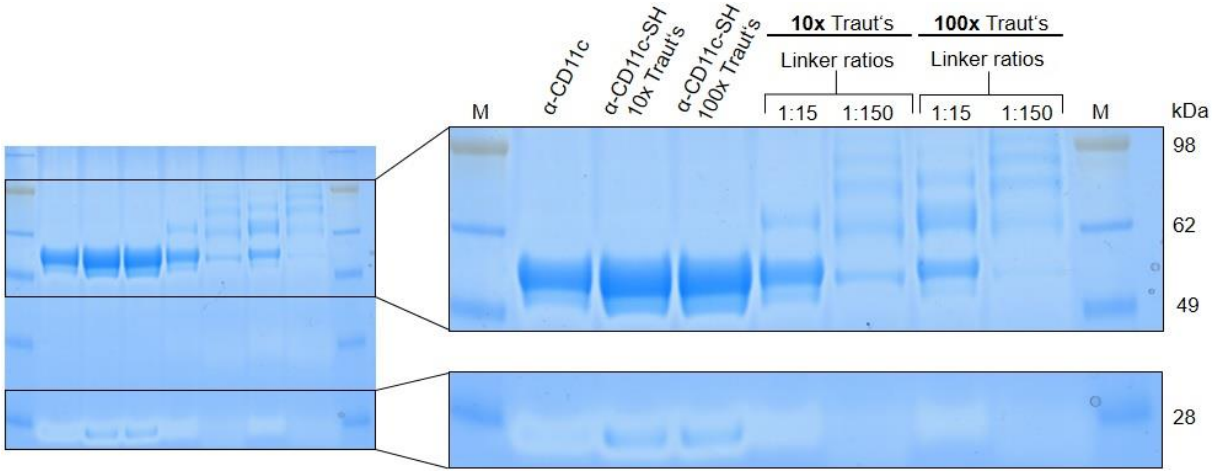

Figure S3: Evaluation of thiolated and linker-reacted CD11c antibodies under reduced conditions. For the Traut's modification, 74µg of α-CD11c (~245 µL) were reacted with 0.4 and 4 µL of Traut's reagent (14 mM) for 1 h at room temperature, representing a Traut's excess of 10 and 100 mol to α-CD11c, respectively. For the overnight coupling, 7 µg of modified α-CD11c-SH (~30 µL) were reacted for each Traut's group in an α-CD11c-SH to maleimide-linker ratio of 1:15 and 1:150 (1.25 µg μL^{-1} linker stock = 1:1 ratio of linker to α-CD11c-SH). Modified samples were prepared according to SDS-PAGE instructions (60 µL approach). Unmodified α-CD11c was prepared at a 40 µL approach. All samples were boiled for 10 min at 70 °C. The molecular weight marker (M) was applied with 5 µL, the unmodified and thiolated α-CD11c groups with 7 µg and the linker-reacted groups with 3.5 µg onto a 10% Bis-Tris Plus gel and run for 111 min at 120 V. Visualization was conducted by Coomassie staining.

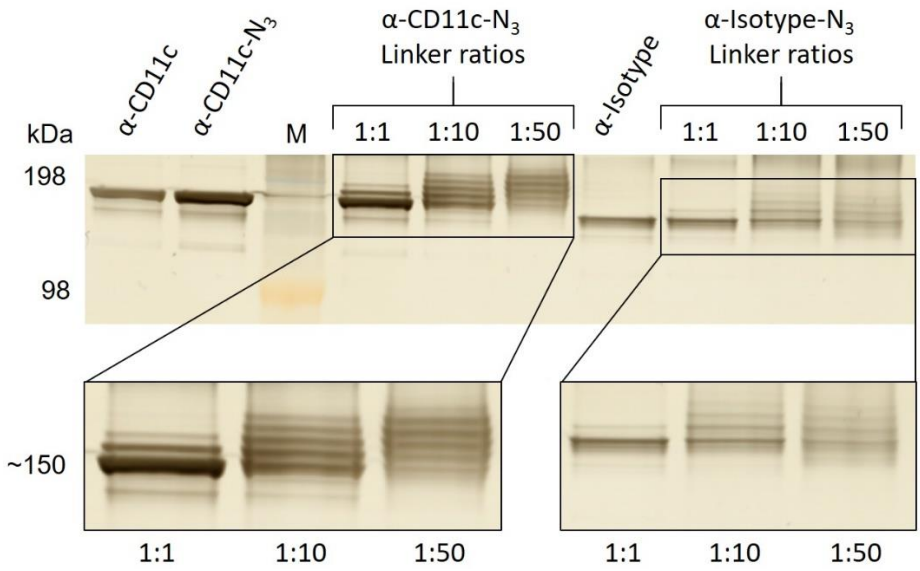

Figure S4: Detection of azide-modified antibodies. For the overnight coupling**,** 1 µg azide-modified antibody (1 µl of α-CD11c-N_3 or α-isotype-N_3) was reacted with the respective amount of DBCO-mPEG 5 kDa linker (0.15 µg μL^{-1} linker stock = 1:1 ratio of linker to antibody). Three linker ratios were tested for each modified antibody. All samples were prepared according to 40 µL SDS-PAGE instructions and boiled for 10 min at 70 °C. The molecular weight marker (M) was applied with 5 µL, the unmodified α-CD11c with 1 µg, the modified α-CD11c-N_3 with ~2µg, the modified α-isotype-N_3 with ~1 µg and the linker coupled samples with ≤0.5 µg onto a 10% Bis-Tris Plus gel and run for ~5 h at 110 V. Visualization was conducted by silver staining.

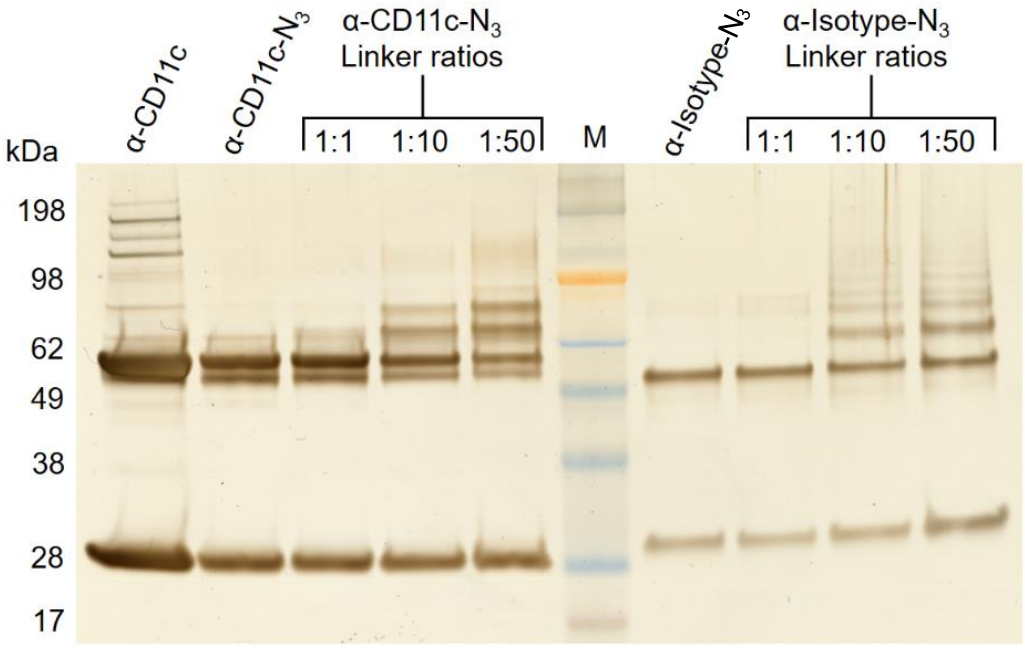

Figure S5: Detection of azide-modified antibodies under reduced conditions. For the overnight coupling**,** 1 µg azide-modified antibody (1 µL of α-CD11c-N_3 or α-Isotype-N_3) was reacted with the respective amount of DBCO-mPEG 5 kDa linker (0.15 µg μL^{-1} linker stock = 1:1 ratio of linker to antibody). Three linker ratios were tested for each modified antibody. All samples were prepared according to 40 µL reduced SDS-PAGE instructions and boiled for 10 min at 70 °C. The molecular weight marker (M) was applied with 5 µL, the unmodified α-CD11c with 1 µg, the modified α-CD11c-N_3 with ~2 µg, the modified α-Isotype-N_3 with ~1 µg and the linker coupled samples with ≤0.5 µg onto a 10% Bis-Tris Plus gel and run for 70 min at 110 V. Visualization was conducted by silver staining.

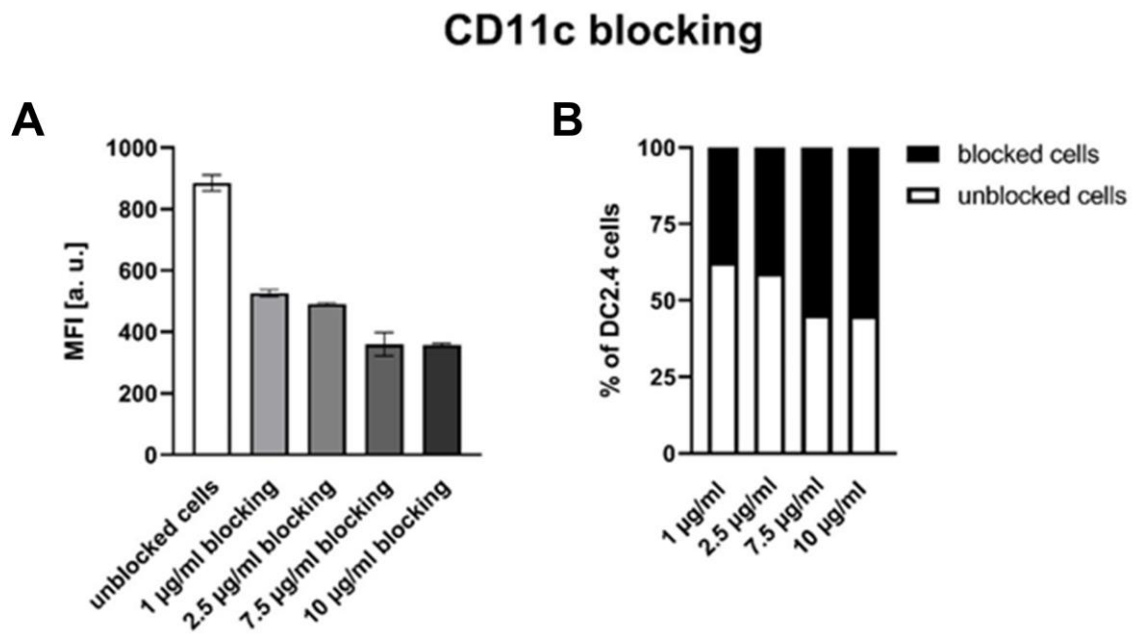

Figure S6: Concentration-dependent blocking of CD11c integrins on dendritic cells. DC2.4 dendritic cells were initially blocked with CD11c antibodies (unlabeled) for 30 min at 4°C, followed by the detection of free CD11c integrins by fluorescence-labeled CD11c antibodies also for 30 min at 4°C. Only viable cells are gated and analyzed by flow cytometry (**A**). Values are given as mean ± SD (n = 2). MFI [a.u.] = median fluorescence intensity [arbitrary units]. For a percentage representation of the results, the MFI value of unblocked cells was used as 100% and the individual groups were calculated accordingly (**B**).

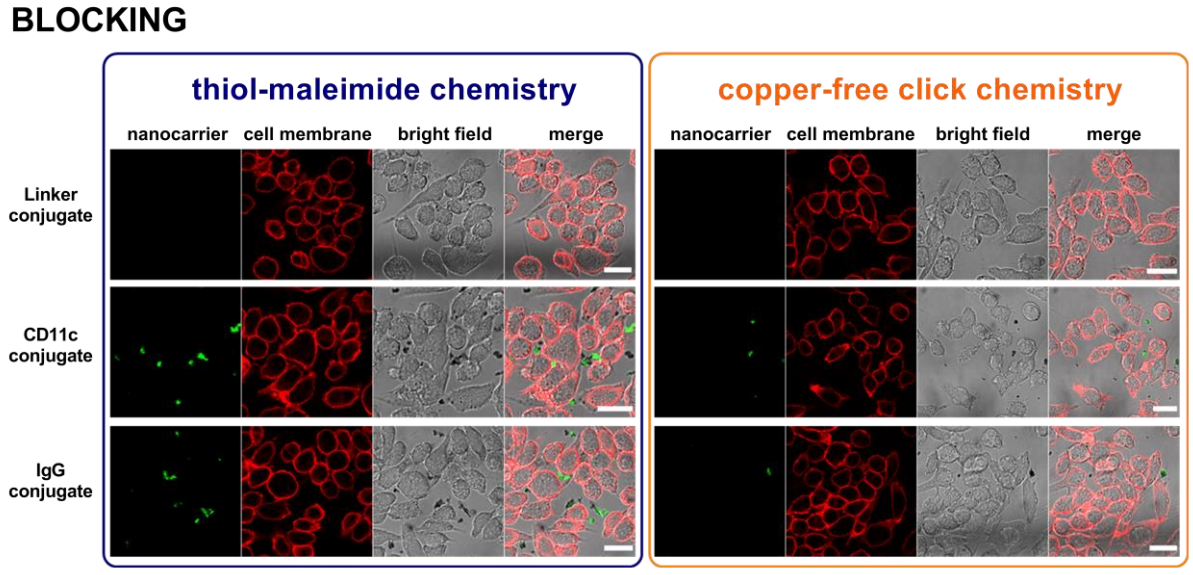

Figure S7: Visualization of blocking CD11c prior to an antibody nanocarrier conjugate uptake. For the image acquisition, 5 x 10^4 cells per well were pre-treated with 7.5 µg mL^{-1} CD11c antibodies in 200 µL IMDM without FBS for 30 min at 4°C. After the incubation, the wells were washed with PBS and the antibody nanocarrier conjugates uptake was performed with 75 µg mL^{-1} in 200 µL IMDM without FBS for 30 min at 4°C. After the incubation, the wells were washed with PBS and the cells were fixed for 15 min with 4% paraformaldehyde (PFA). Nanocarriers were excited with the laser 561 (emission filter 570 – 599 nm) and the cell membrane was excited with the laser 633 (emission filter 660 – 700 nm). All scale bars represent 20 µm.

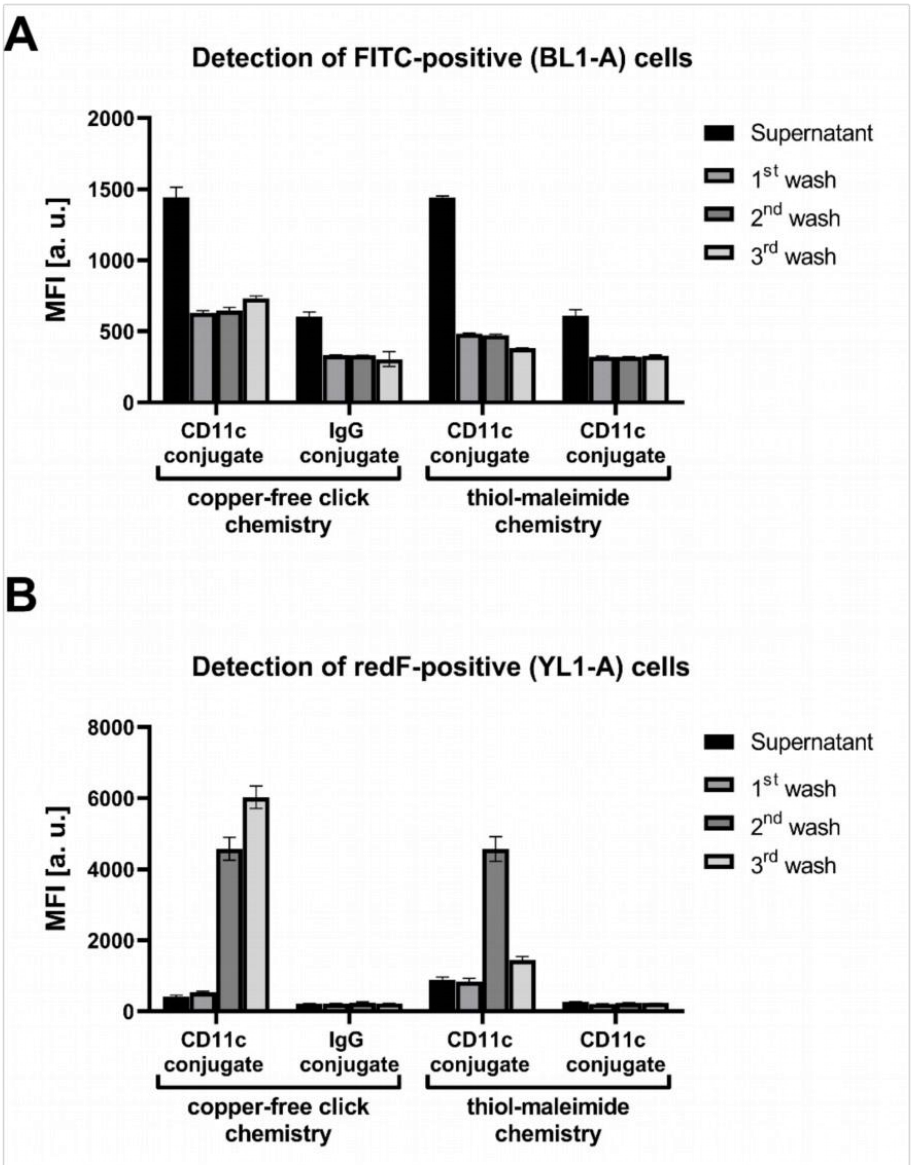

Figure S8: Detection of unbound antibodies and free nanocarriers in each magnetically separated fraction. For the detection of unbound antibodies and nanocarriers in each wash fraction, 1.5 x 10^5 cells were incubated (30 min, 4°C) in 100 µL of the respective supernatant or one of the three washes. Unbound CD11c or IgG isotype antibodies were detected by a secondary FITC anti-hamster antibody (30 min, 4°C). The CD11c and IgG conjugates with an antibody amount of 7.5 µg are analyzed by flow cytometry. The detection of FITC-positive (BL1-A) cells represents unbound antibodies in each wash fraction that bound to the cell surface and are detected by the secondary FITC antibody (**A**). The detection of redF-positive (YL1-A) cells represents free nanocarriers in each wash fraction that were not magnetically purified and attached to the cells (**B**). Values are given as mean ± SD (n = 3). MFI [a.u.] = median fluorescence intensity [arbitrary units].

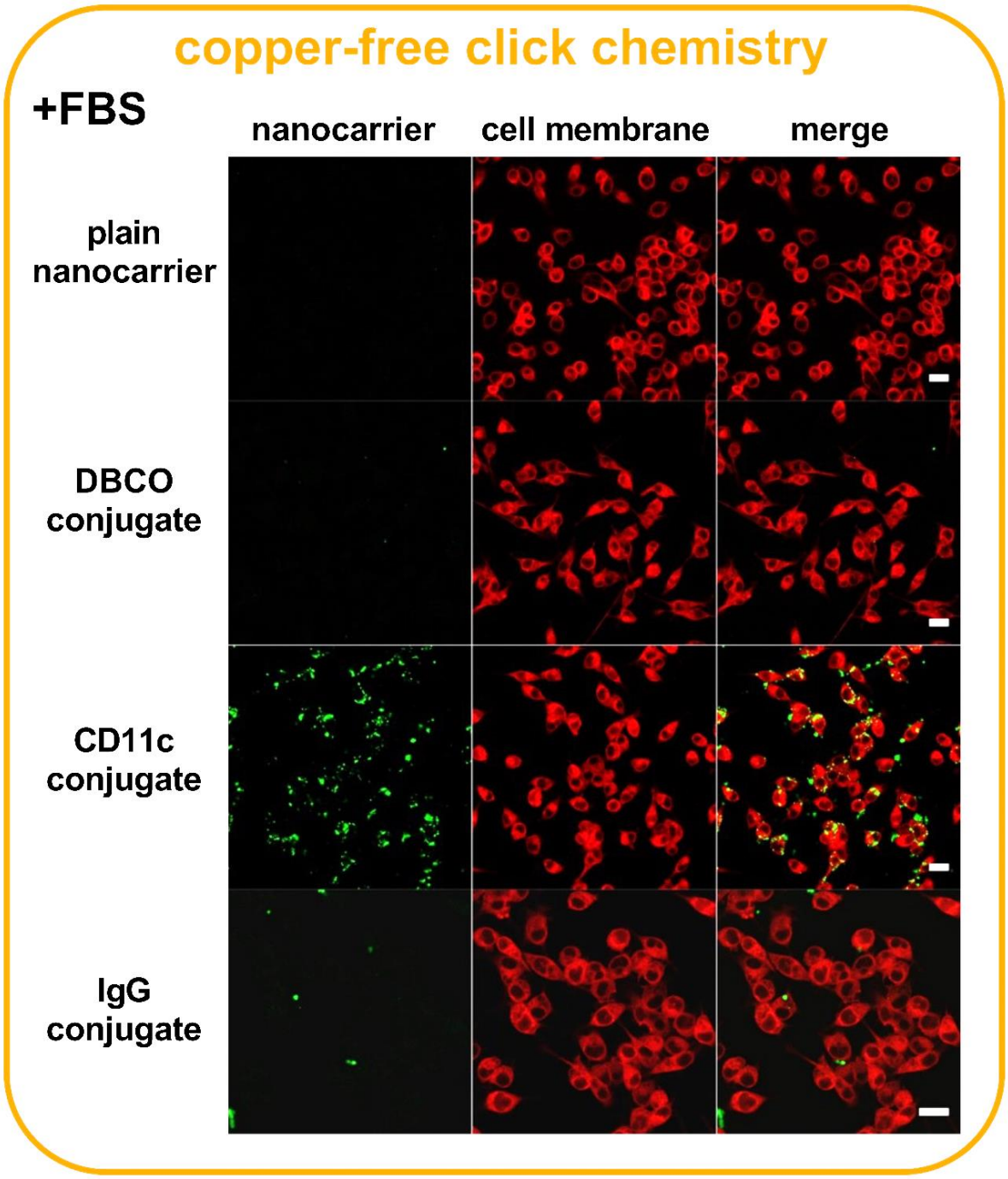

Figure S9: Site-directed targeting of dendritic cells by antibody nanocarrier conjugates. For the image acquisition, 5 x 10^4 cells per well were treated with 75 µg mL^{-1} samples in 200 µL IMDM with 5% FBS. After the two-hour incubation, the wells were washed with PBS and the cells fixed for 15 min with 4% paraformaldehyde (PFA). Nanocarriers were excited with the laser 561 (emission filter 570 – 599 nm) and the cell membrane was excited with the laser 633 (emission filter 660 – 700 nm). All scale bars represent 20 µm.

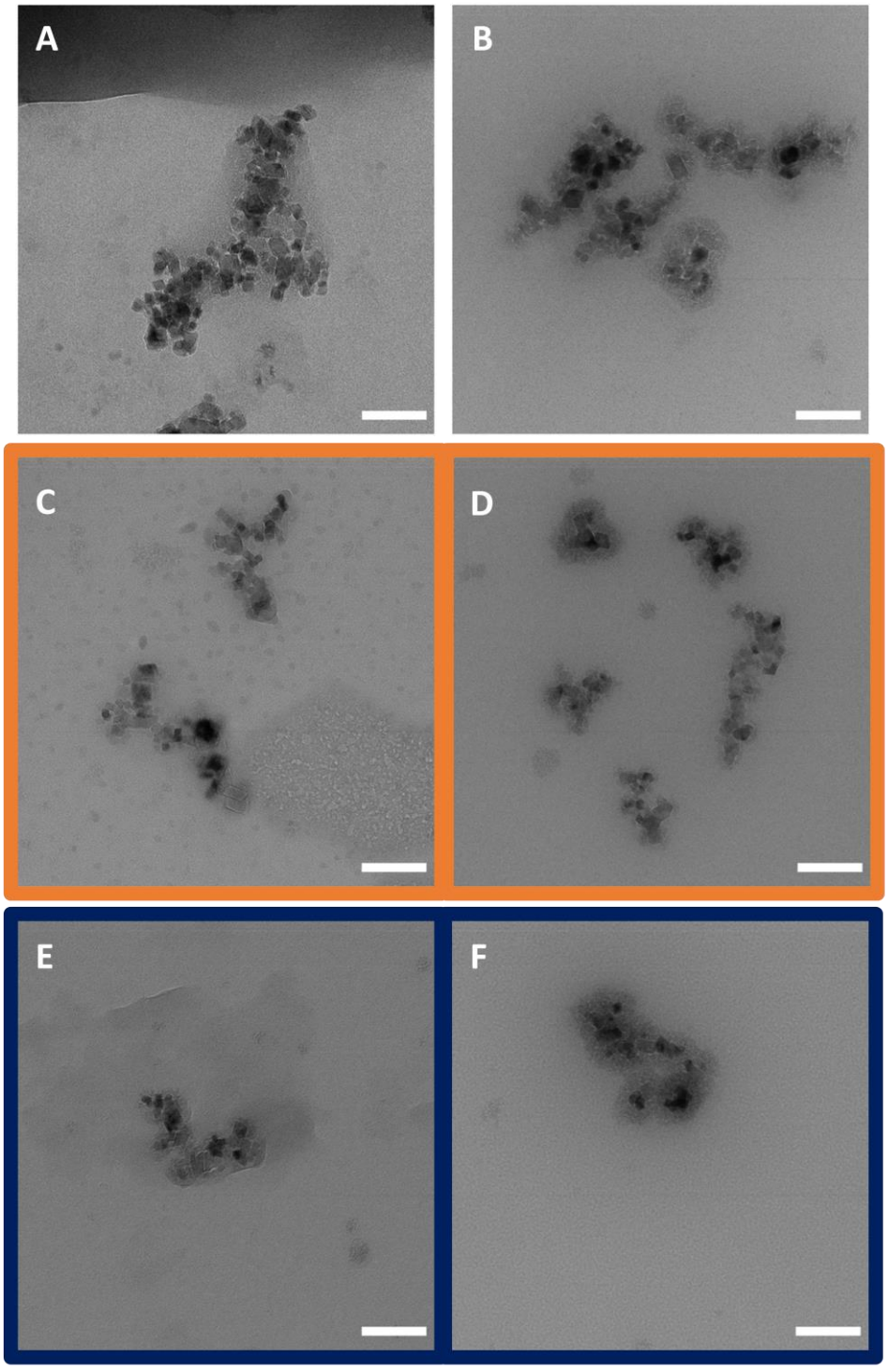

Figure S10: TEM images of antibody-modified nanocarriers in cell culture medium. The images show small clusters of the nanocarriers in different conditions with a sample concentration of 75 µg mL^{-1}. Nanocarriers in water (**A**) and in IMDM with 5% FBS (**B**) are shown. Antibody-modified nanocarrier conjugates (7.5 µg antibody amount for all tested conjugates) obtained via copper-free click chemistry in orange with CD11c (**C**) or IgG (**D**) antibody and those obtained via thiol-maleimide chemistry in blue with CD11c (**E**) or IgG (**F**) antibody are shown. All scale bars represent 100 nm.

Table S1: Average size and charge of synthesized nanoconjugates. Light scattering measurements were performed on an ALV spectrometer with 10 µL of sample in 1 mL PBS (n = 1, with an experimental error of 10%). The ζ-potential was measured at the Zeta-Sizer – Z (Malvern) in 1mM KCl (1mL) with an average of three measurements (n = 3). Thiol-maleimide generated conjugates were synthesized with a nanocarrier to linker ratio of 1:150 and copper-free click conjugates with a nanocarrier to linker ratio of 1:10. Different nanocarrier to antibody ratios/amounts were investigated (20:1 [25 µg]; 67:1 [7.5 µg]; and 200:1 [2.5 µg].

Sample name	**Average diameter in PBS[a]**	**PDI in PBS [a]**	**ξ-potential [mV]**	**ξ-deviation [mV]**
BNF Starch redF (pristine nanocarrier)	210 nm	0.148	-5.77 ± 1.73	4.38 ± 0.46
Thiol-maleimide generated conjugates:				
Maleimide linker conjugate	244 nm	0.216	-15.5 ± 0.17	3.59 ± 0.39
α-CD11c conjugate [25.0 µg]	298 nm	0.396	-11.3 ± 0.61	4.10 ± 1.29
α-CD11c conjugate [7.5 µg]	190 nm	0.266	-14.37 ± 0.15	3.46[b] ± 0.42[b]
α-CD11c conjugate [2.5 µg]	198 nm	0.150	-12.8 ± 1.82	7.53 ± 7.08
α-IgG conjugate [25.0 µg]	/[c]	/[c]	-10.62 ± 3.63	3.74 ± 0.85
α-IgG conjugate [7.5 µg]	234 nm	0.205	-11.40 ± 2.44	5.24 ± 1.05
α-IgG conjugate [2.5 µg]	230 nm	0.122	-13.00 ± 0.26	4.38 ± 0.58
Copper-free click generated conjugates:				
DBCO linker conjugate	210 nm	0.200	-10.3 ± 0.1	5.39 ± 3.47
α-CD11c conjugate [25.0 µg]	202 nm	0.080	-9.75 ± 0.65	3.81 ± 0.60
α-CD11c conjugate [7.5 µg]	204 nm	0.060	-9.35 ± 0.19	3.81 ± 0.60
α-CD11c conjugate [2.5 µg]	204 nm	0.168	-9.09 ± 1.41	3.94 ± 0.62
α-IgG conjugate [25.0 µg]	238 nm	0.260	-8.67 ± 0.54	5.03 ± 0.92
α-IgG conjugate [7.5 µg]	228 nm	0.169	-8.97 ± 1.10	5.70 ± 0.23
α-IgG conjugate [2.5 µg]	208 nm	0.168	-9,82 ± 0.68	3.50 ± 0.36

[a] Determined by multi angle DLS (at scattering angles of 30° to 150°)

[b] (n = 2)

[c] not measurable (macroscopic aggregates)

3 References

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