Journal Name

Supplementary Information

Shikimoyl-ligand decorated gold nanoparticles for use in *ex vivo* engineered dendritic cell based DNA vaccination

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Running Title: DC-based DNA vaccination with Shikimoyl-functionalized AuNPs

Synthesis of Shikimoyl-ligand (VII, Figure 1)

Step I. ***Synthesis of (3R,4S,5R)-3,4,5-tri(acetyloxy)-1-cyclohexene-1-carboxylic acid*** **(I, Figure 1):** A mixture of 1 g (5.74 mmol) of (-) shikimic acid, 2.5 mL of dry acetic anhydride and 5 mL glacial acetic acid was taken in a 50 mL RB flask, 4 drops of perchloric acid was added. The resulting reaction mixture was stirred for 15 min at 0 ºC. The temperature of the reaction mixture was raised to room temperature and the mixture was stirred at room temperature for 12 h. The reaction mixture was poured onto crushed ice and extracted with chloroform (3 x 15 mL). The combined organic extracts were washed with brine solution, dried over anhydrous sodium sulphate and filtered. The filtrate, upon rotatory evaporation, produced a white residue which upon several times washing with ice cold pentane (to ensure complete removal of acetic acid), afforded the dried residue (intermediate **I)** as a gummy white solid (1.5 g, 87% yield, R_f = 0.4, 95:5 chloroform: methanol, v/v).

^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm = 2.1 [s, 9H, -CO-C$\underline{H}_3$]; 2.45 [dd, 1H, 6-H_a]; 2.9 [dd, 1H, 6-H_b]; 5.3 [m, 2H, 3-H, 5-H]; 5.7 [t, 1H, 4-H]; 6.85 [d, 1H, 2-H]

ESI-MS *m*/*z*: calcd 300.261 (for $C_{13}H_{16}O_{18}$), found 299 $[M-1]^+$

Step II. ***Synthesis of 6-[(tert-butoxycarbonyl)amino] hexanol*** **(II, Figure 1):** 1 g (8.5 mmol) of 6-aminohexanol was dissolved in 1 N NaOH solution (10 mL) in a 50 mL RB flask. 3.9 mL of $(BOC)_2O$ (17.1 mmol) was added to the alkaline solution and the mixture was stirred at room temperature for 12 h. The reaction mixture was washed with hexane (3 x 20 mL) to remove excess $(BOC)_2O$. Hexane layer was further extracted with saturated $NaHCO_3$ (2 x 20 mL) solution. Combined aqueous layers were acidified with dilute HCl and the target compound was extracted with ethyl acetate (5 x 10 mL). Combined organic layers were collected and dried over anhydrous sodium sulphate and filtered. The filtrate upon rotatory evaporation afforded 1.4 g of intermediate **II** (80% yield, R_f = 0.6, 5:95 methanol:chloroform, v/v) as white solid. As the product obtained was pure, intermediate **II** was directly used for the next reaction without any further purification.

ESI-MS *m*/*z*: calcd 217.261 (for $C_{13}H_{16}O_{18}$), found 240 $[M + Na]^+$

Step III. ***Synthesis of 6 ((tert-butoxycarbonyl) amino) hexyl-4-methylbenzene sulfonate*** **(III, Figure 1)**. The intermediate **II** (1 g, 4.6 mmol, prepared above in step II) was dissolved in CH_2Cl_2 (7 mL) at 0 °C and freshly prepared Ag_2O (1.6 g, 6.9 mmol), KI (0.76 g, 4.6 mmol) and TsCl (1.05 g, 5.5 mmol) were added. The reaction mixture was allowed to stir at 0 °C under nitrogen for 30 min. Excess Ag_2O was filtered off over a pad of Celite and washed with 12:1 CH_2Cl_2/CH_3OH (v/v). The combined organic extracts were concentrated on a rotavapor. The residue upon column chromatography over silica gel (60-120 mesh size) using 1% methanol in dichloromethane (v/v) as eluent afforded pure intermediate **III** as a colorless oil (1.3 g, 75% yield, R_f = 0.8, 5% methanol in dichloromethane. v/v).

ESI-MS *m*/*z*: calcd 371.49 (for $C_{18}H_{29}NO_5S$), found 372 $[M+1]^+$

Step IV. ***Synthesis of SO-(6-((tert-butoxycarbonyl) amino)hexyl) ethane(thioperoxoate)*** **(IV, Figure 1**). KSAc (0.92 g, 8 mmol) and intermediate **III** (1 g, 2.7 mmol, prepared in step III) were dissolved in anhydrous THF. The resulting solution was refluxed for 16 h under argon at 75 °C. THF was removed with rotavapor and the residue was dissolved in ethyl acetate. Decolorizing carbon was added to the ethyl acetate solution and the mixture was filtered. Solvent from the filtrate was evaporated and the residue upon column chromatography over 60-120 mesh size silica gel using 1.5% methanol in dichloromethane (v/v) as eluent afforded pure intermediate **IV** as a reddish oil (0.45 g, 59% yield, Rf = 0.7, 5% methanol in dichloromethane, v/v).

^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm = 1.2 [m, 4H, -CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2]; 1.4 [s, 9H, CH_3]; 1.6 [m, 4H, -C$\underline{H}_2$-

CH_2-CH_2-$C\underline{H_2}$]; 3.3 [dd, 2H, NH-$C\underline{H_2}$]; 3.6 [dd, 2H, O-$C\underline{H_2}$];
ESI-MS *m/z*: calcd 275.41 (for $C_{13}H_{25}NO_3S$), found 276 $[M+1]^+$

Step V. *Synthesis of SO-(6-aminohexyl) ethane(thioperoxoate)* (**V, Figure 1**): TFA (0.6 mL) was added to the ice cold solution (2 mL dry DCM) of the intermediate **IV** (0.4 g, 1.44 mmol) and the mixed solution was stirred for 2 h. Gentle removal of TFA with nitrogen flow afforded 0.2 g (79% yield) pure intermediate **V** as a colorless oil (Rf = 0.3, 5% methanol-chloroform, v/v).
ESI-MS *m/z*: calcd 175.29 (for $C_8H_{17}NOS$), found 176 $[M+1]^+$

Step VI. Synthesis of *5-((6-(acetylthio)hexyl)carbamoyl)cyclohex-4-ene-1,2,3-triyl triacetate)* (**VI, Figure 1**)**:** HOBt (0.28 g, 1.8 mmol) and EDCI (0.35 g, 1.8 mmol) were added sequentially into an stirred ice cold solution (in 5 mL dry DCM/dry DMF 9:1, v/v) of intermediate **I** (0.306 g, 1.02 mmol) under nitrogen atmosphere. After 30 min, intermediate **V** (0.18 g, 1.02 mmol) dissolved in dry DCM (2 mL) was added to the reaction mixture. Di-isopropylethylamine (DIPEA) was added dropwise to the reaction mixture until it was alkaline to litmus. Stirring at room temperature was continued for overnight after which the reaction mixture was diluted with excess DCM, washed sequentially with saturated $NaHCO_3$ (3 x 50 mL) and water (3 x 50 mL). The DCM layer was dried over anhydrous sodium sulfate, filtered and the solvent was removed by rotatory evaporation. The residue upon purification by column chromatography using 60-120 mesh silica gel and 1.5-2% methanol in dichloromethane (v/v) as eluent afforded 1.4 g of pure intermediate **VI** (70% yield, 0.32 g, Rf = 0.5, 95:5 chloroform: methanol, v/v) as yellowish liquid which solidified upon storage at 4 ^oC.
^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm = 1.5 [m, 8H, -$C\underline{H_2}$- $C\underline{H_2}$-$C\underline{H_2}$-$C\underline{H_2}$]; 2.0 [s, 9H, O-$C\underline{H_3}$]; 2.3 [s, H, S-$C\underline{H_3}$]; 2.6 [m, 2H, 6-$\underline{H}$]; 3.3 [t, 4H, S-$C\underline{H_2}$ & NH-$C\underline{H_2}$]; 5.3 [m, 2H, 4-H, 5-H]; 5.7 [t, 1H, 3-H]; 6.4 [d, 1H, 2-H];
ESI-MS *m/z*: calcd 457.54 (for $C_{21}H_{31}NO_8S$), found 480 $[M+Na]^+$

Step VII: Synthesis of *3,4,5-trihydroxy-N-(6-mercaptohexyl)cyclohex-1-ene-1-carboxamide* (**VII, Figure 1**)**:** NaOMe (0.2 g, 1.5 mmol) was added to 2 mL methanol solution of intermediate **VI** (0.35 g, 0.33 mmol) and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was neutralized with Amberlite IR120 (H^+), filtered and the filtrate was dried over anhydrous sodium sulfate, filtered and the organic solvent from the dried filtrate was removed by rotatory evaporation. The residue upon recrystallization from 1:5 (v/v) MeOH:Acetone afforded 0.15 g (60% yield) of white solid target compound **VII** (Rf = 0.3, 10% methanol-chloroform, v/v).
^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm = 1.5 [m, 8H, -$C\underline{H_2}$- $C\underline{H_2}$-$C\underline{H_2}$-$C\underline{H_2}$]; 2.2 [m, 2H, 6-2$\underline{H}$]; 2.5 [t, 4H, S-$C\underline{H_2}$]; 2.7 [t, 2H,NH-$C\underline{H_2}$]; 3.5 [m, 2H, 4-$\underline{H}$, 5-$\underline{H}$]; 4.0 [t, 1H, 3-$\underline{H}$]; 5.4 [s, 2H, 4C-O$\underline{H}$ & 5C-O$\underline{H}$]; 5.7 [s, 1H, 3C-O$\underline{H}$]; 6.45 [d, 1H, 2-$\underline{H}$] (Figure S2);
ESI-MS *m/z*: calcd 289.39 (for $C_{13}H_{23}NO_4S$), found 312 $[M + Na]^+$ (Figure S1)

HPLC Chromatograms of purified target compound **VII** are shown in Figure S3.

Mass spectra were recorded using a commercial LCQ ion trap mass spectrometer (Thermo Finnigan, SanJose, CA, USA) equipped with an ESI source or micromass Quatro LC triple quadrapole mass spectrometer and the ^{1}H NMR spectra were run on either a Varian FT 200 MHz or an AV 300 MHz NMR Spectrometer.

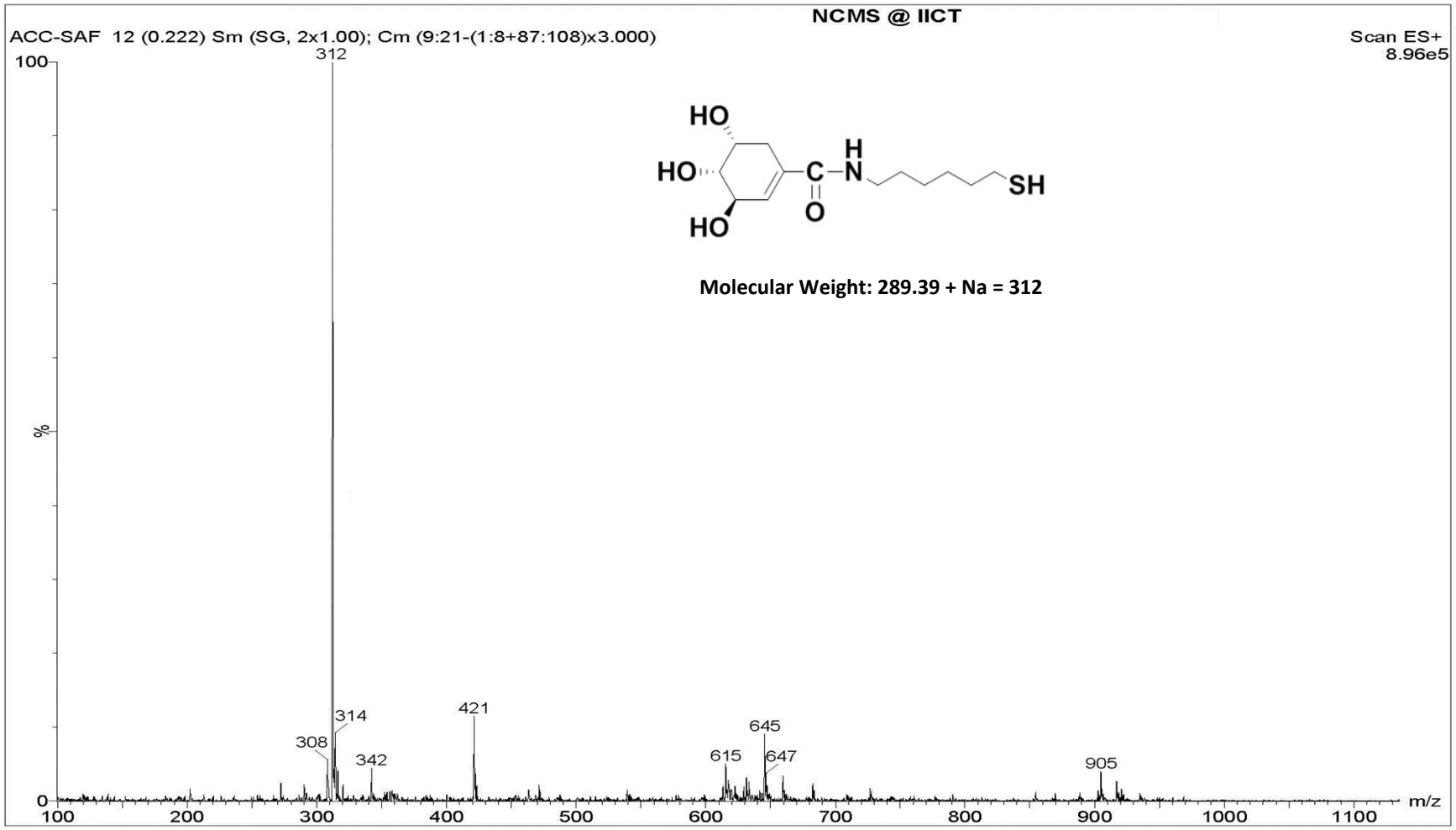


Figure S1. ESI mass spectrum for Shikimoyl-ligand (SL).

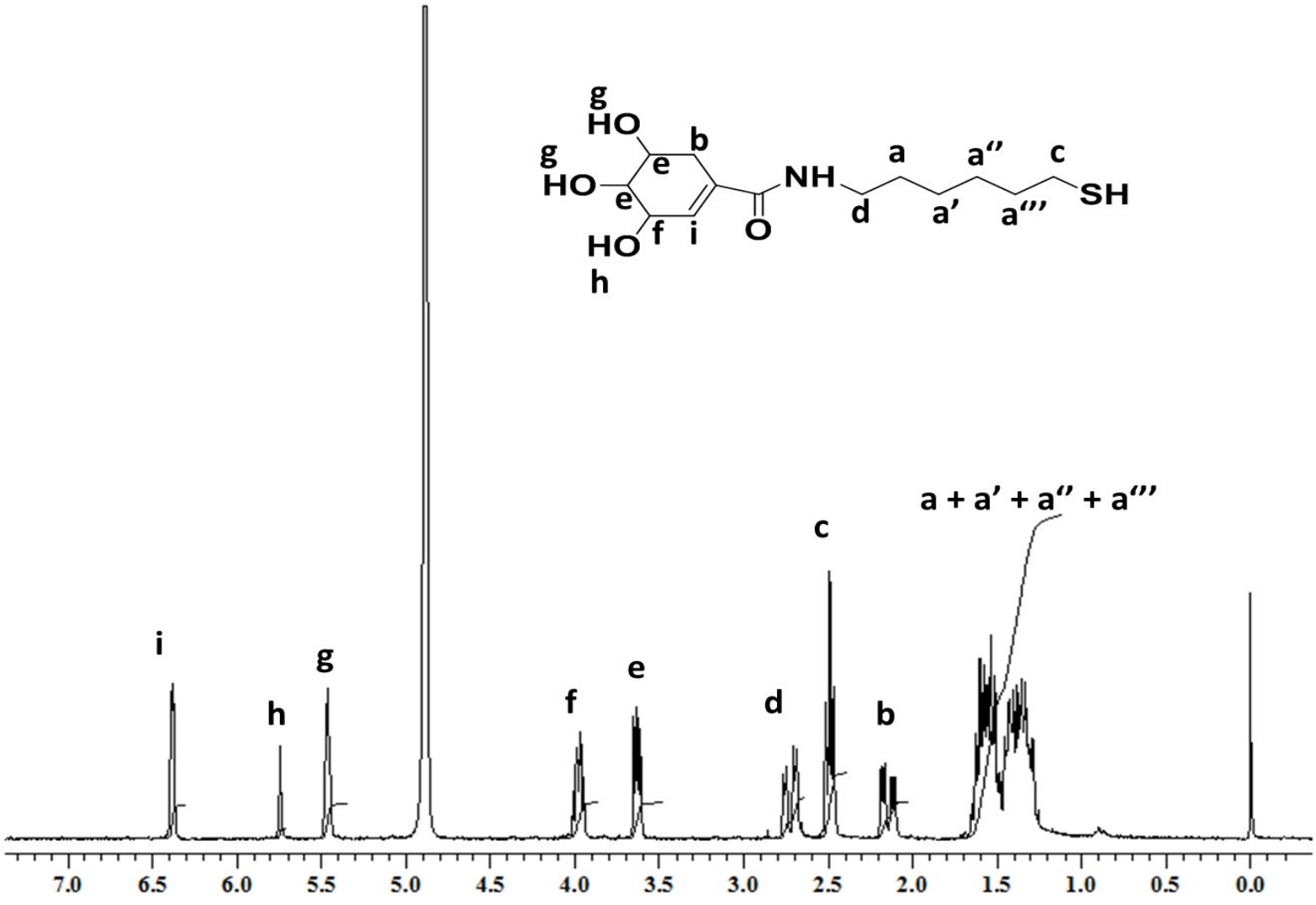
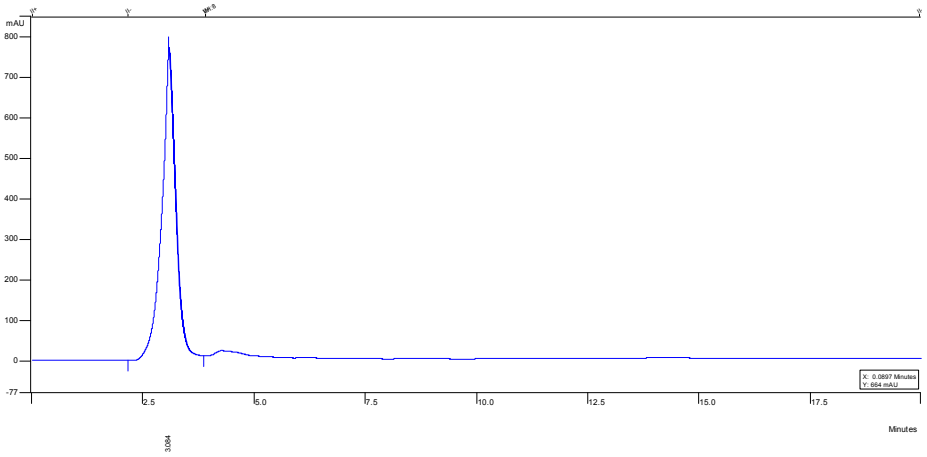


Figure S2: ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD) Spectrum for Shikimoyl-ligand (SL).

a)



b)

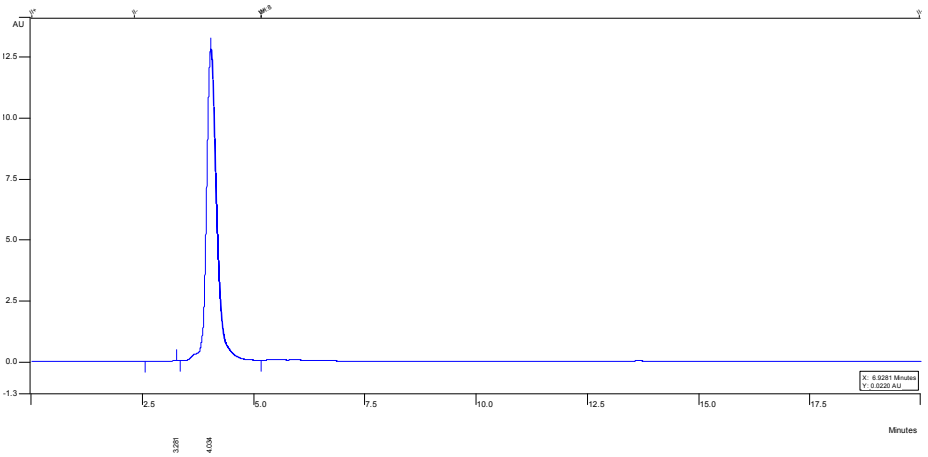
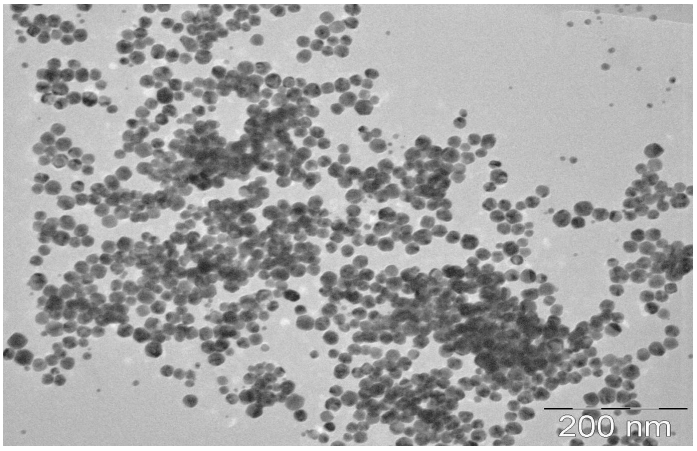


Figure S3. Representative HPLC chromatograms for Shikimoyl-ligand (SL) using pure methanol as mobile phase (a) and using 95:5 methanol:water, v/v, as the mobile phase (b).

HPLC Conditions:

System: Agilent 1100 series
Column: Lichrospher® 100, RP-18e (5 µm)
Mobile Phases: Methanol (**A**); Methanol:Water, 95:5, v/v, (**B**).
Flow Rate: 1.0 mL/min
Typical Column Pressure: 60-65 Bars
Detection: UV at 210 nm.

a)



b)

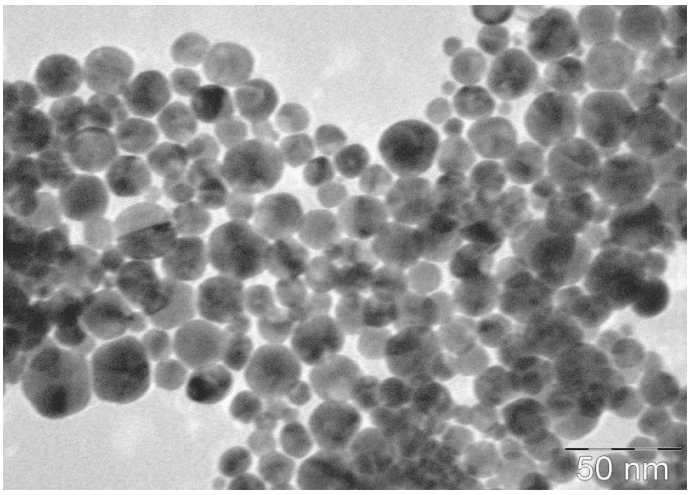


Figure S4: TEM images of AuNPs-I (a) and AuNPs-I-SL (b).

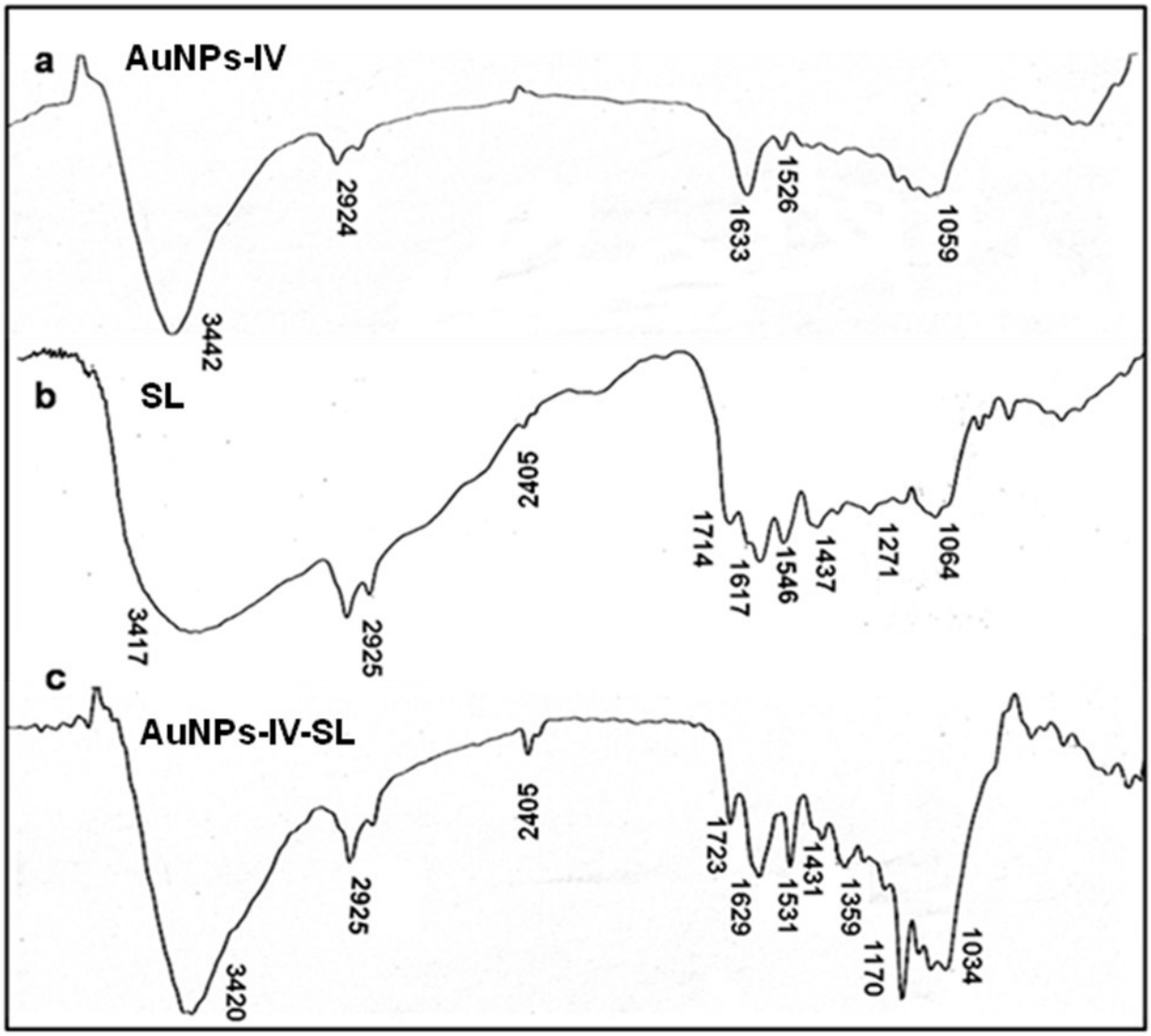


Figure S5: FTIR spectroscopy of AuNPs-II (a), shikimic Acid (b) and AuNPs-Sh-II (c).

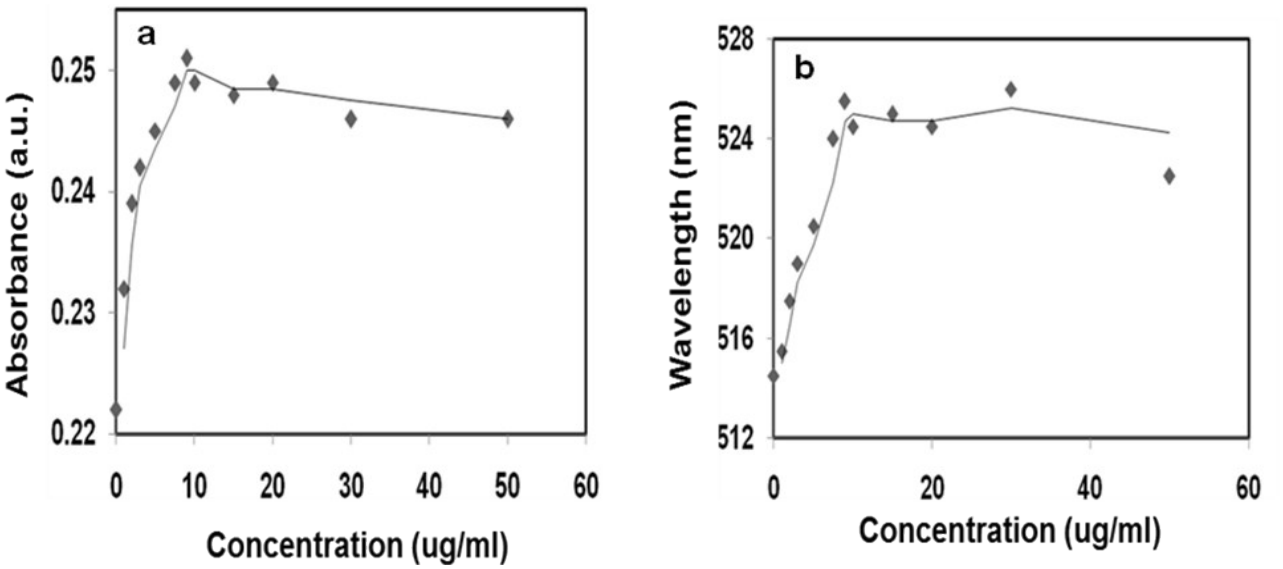
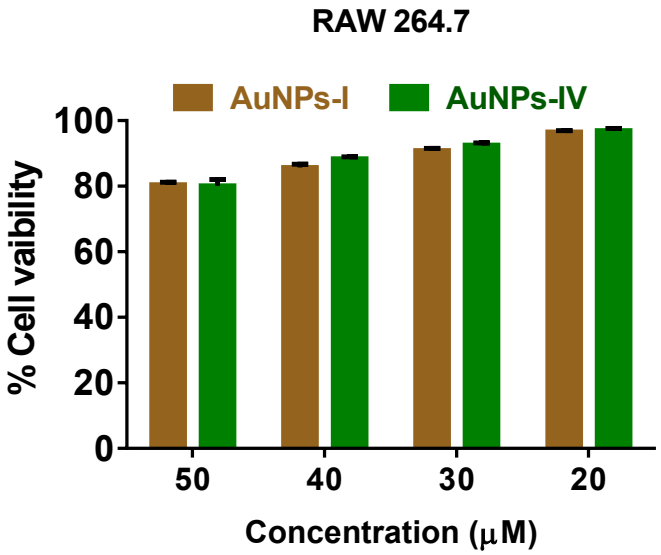


Figure S6: Representative saturation graph for SL ligand conjugation with AuNPs-IV by UV visible spectroscopy. **a** Concentration of SL in AuNPs-IV (µg/mL) vs the absorbance; **b.** Concentration of SL in AuNPs-IV (µg/mL) vs the λ_{max}.

a)



b)

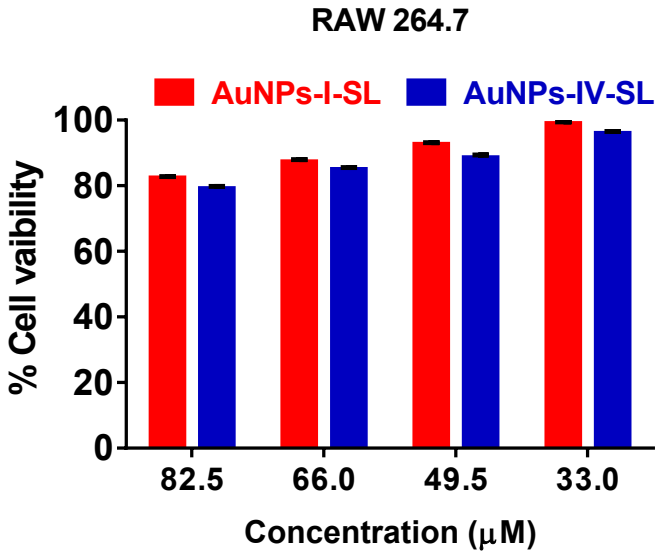


Figure S7. MTT-assay based percent cell viabilities for RAW 264.7 treated with increasing concentrations of (a) AuNPs-I & AuNPs-I-SL (20 μM – 50 μM) and AuNPs-IV & AuNPs-IV-SL (33 μM – 82.5 μM).

IFN-γ and IL-4 ELISA assays

Secreted IL-4 and IFN-γ were measured by ELISA assays as reported earlier.[1] Briefly, two weeks post the last immunization, mice were sacrificed and the splenocytes were isolated by mincing the spleens of the sacrificed mice with a syringe plunger. Erythrocytes were lysed with lysis buffer (1 mL 0.14 M ammonium chloride in 0.02 M Tris HCl, pH 7.2) and the viable cells were counted by a hemocytometer. Immediately thereafter IFN-γ and IL-4 ELISA assays were performed without any *in vitro* restimulation as per the manufacturer's instructions (Endogen mouse IFN-γ and mouse IL-4 ELISA kits, Pierce Biotechnology, USA). Briefly, isolated splenocytes were seeded at $1x10^6$ cells/well in 50 µL complete medium in 96-well plates pre-coated with anti-mouse IFN-γ or anti-mouse IL-4 antibodies. The covered plates were incubated at 37 °C for 12 h in presence of 5% CO_2 and washed with wash buffer (3 x 200 µL). The cells in each plate were then incubated with 50 µL biotinylated secondary antibody for 1 h. The plates were washed with wash buffer (3 x 200 µL) and incubated for 30 min with 100 µL of streptavidin-HRP solution. The plates were finally washed with wash buffer (3 x 200 µL) and incubated in dark for 30 min with 100 µL of TMB substrate solution. 100 µL stop solution was added to each well to stop the reaction and the absorbances were measured using a microplate reader at 450 nm and 550 nm.

Phenotypic analysis of mouse bone marrow derived dendritic cells (mbmDC):

DCs are the most potent antigen presenting cells and are uniquely capable of presenting novel antigens to naive T cells to initiate and modulate immune responses.These cells express highest level of co-stimulatory molecules which are indispensable for proper antigen presentation to T cells.[2] In general, immature DCs, which have a great proclivity for endocytosis, do not effectively induce primary immune responses. However, the expression of MHC and co-stimulatory molecules is important for successful antigen presentation and is enhanced in DCs by maturation signals. DCs strongly express molecules involved with antigen presentation and T-cell activation on their cytoplasmic membrane. Their phenotype is characterized by a high expression of major histocompatibility complex class II molecules and a high expression of adhesion molecules. DCs characteristically express high levels of MHC class I and II molecules, accessory /co-stimulatory molecules B7-1 (CD80), B7-2 (CD86), CD40, adhesion molecules CD11b, CD11c and intracellular adhesion molecules (ICAMs)–ICAM-1 (CD54). They also express receptors for efficient Ag capture and secrete chemokines. DC are characterized by the lack of lineage specific expression markers such as CD3 (T cells), CD19 (B cells), CD14 (monocytes/macrophages), CD56 (NK cells) and CD66b (granulocytes).[3] For DC phenotypic analysis was carried out using widely used cells surface markers unique to dendritic cells such as CD206, MHC II, CD86, CD45R, CD11c, H2kB, f4/80 and CD40. We isolated dendritic cells from mouse bone marrow (femur & tibia) and approximately $2x10^6$ mbmDC were fixed for overnight in 2% formaldehyde solution and centrifuged. Cell pellets were suspended in 3 mL PBS containing 2% FBS and 0.1% sodium azide and incubated for 30 min at 37 °C. The incubated cells were divided equally into two eppendorff tubes, centrifuged and the supernatants were discarded. 100 µL commercially available FITC conjugated mouse monoclonal antibody against various cell surface markers (Chemicon, USA) at a dilution of 1:100 in wash buffer was added to one of the eppendorff tubes. 100 µL wash buffer was added to the second eppendorff tube as control. The cell suspensions were incubated for 2.5 h at room temperature and centrifuged. The resulting cell pellets were washed with wash buffer (3 x 1 mL) and were finally resuspended in 1 mL wash buffer for recording the flow cytometry histograms using a FACS-caliber instrument (Becton Dickinson).

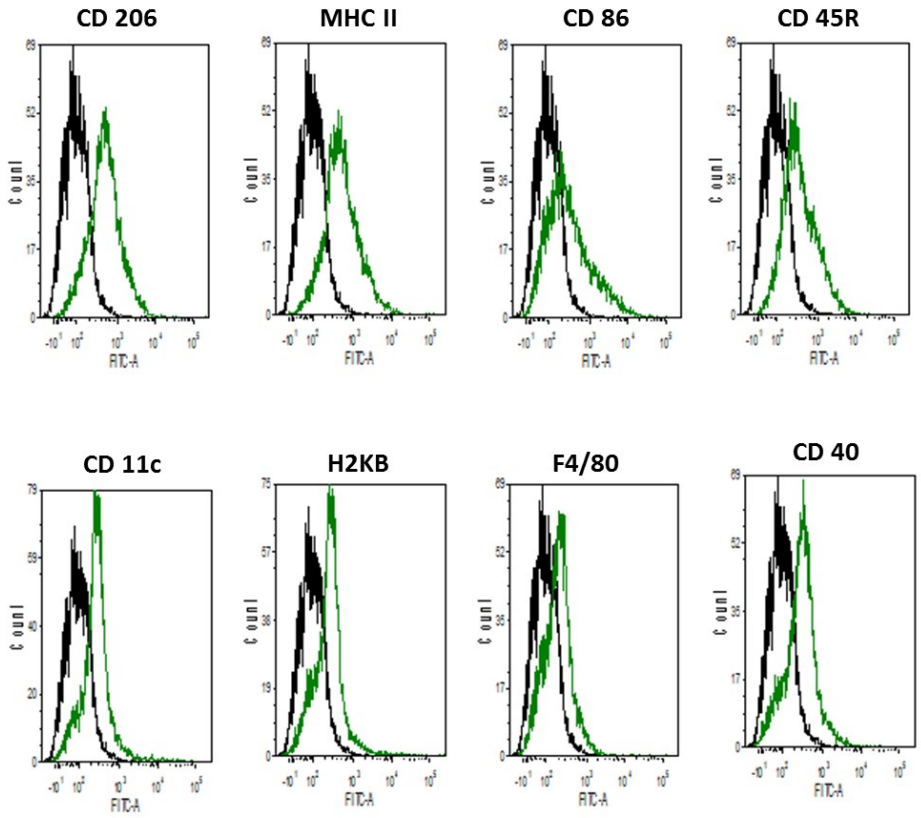


Figure S8: Dendritic cells isolated from mouse bone marrow express several cell surface markers. mbmDCs were stained with FITC conjugated monoclonal antibodies specific for the cell surface markers including CD 206, cell surface MHC II, CD11c, CD86, CD 45R, H2KB, F4/80 and CD40 and analyzed using flow cytometry. mbmDCs (~5 x 10^5) were stained with FITC -conjugated monoclonal antibodies. Each experiment was repeated three times and similar markers profiles were observed in each time.

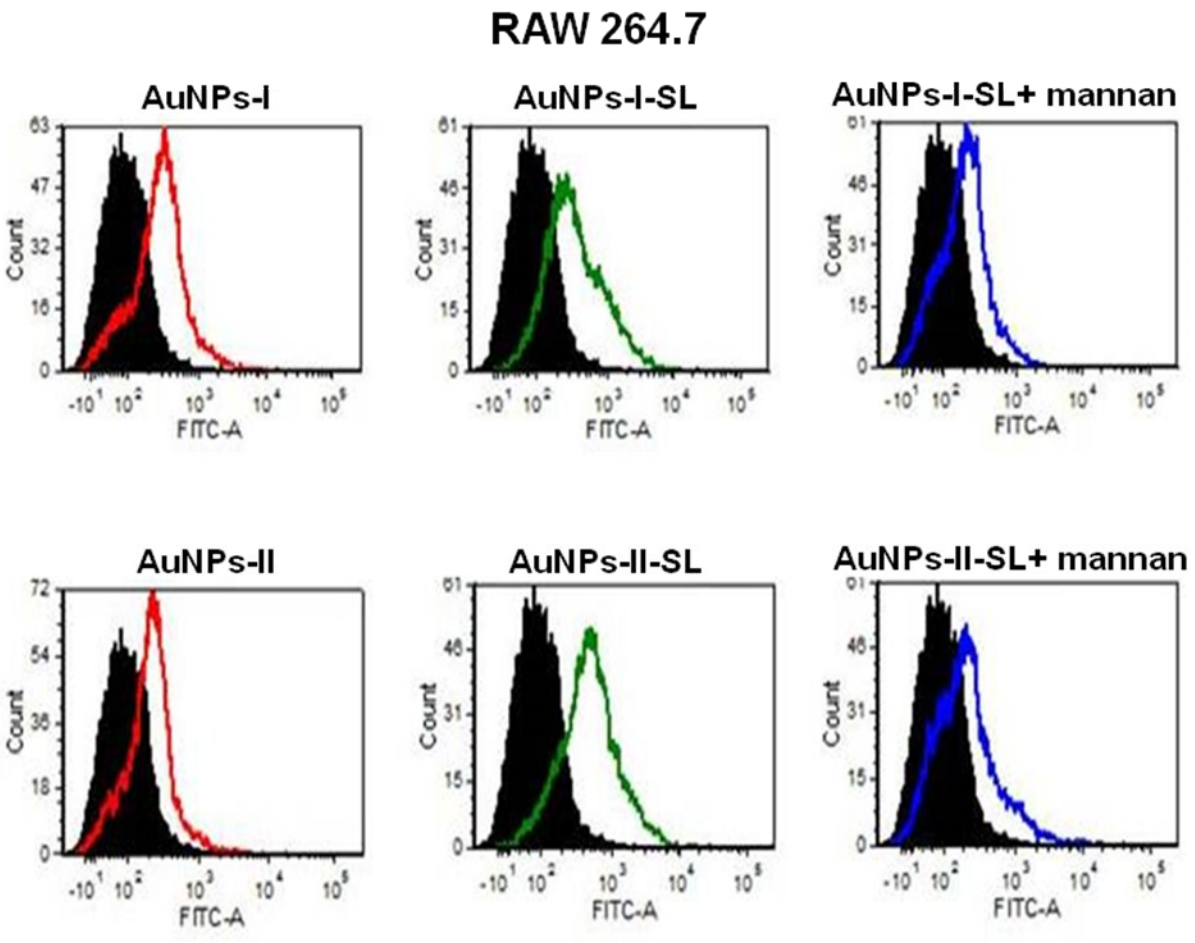


Figure S9. Flow cytometric cellular uptake studies in RAW264.7 cells using p-α5GFP nanoplexes of AuNPs (I & IV) & AuNPs-SL (I & IV). The histograms for the GFP expression profiles in RAW264.7 cells transfected with nanoplexes of gold nanoparticles/nanoconjugates (AuNPs-I, AuNPs-IV and AuNPs-Sh-I & -IV) and pα5GFP plasmid were recorded 24 h post addition of nanoplexes to cells. The third panels in both upper and lower rows show the uptake profiles for the Au-nanoplexes in cells pre-saturated with mannan, a natural ligand of mannose receptor. Untreated RAW264.7 cells were used as control.

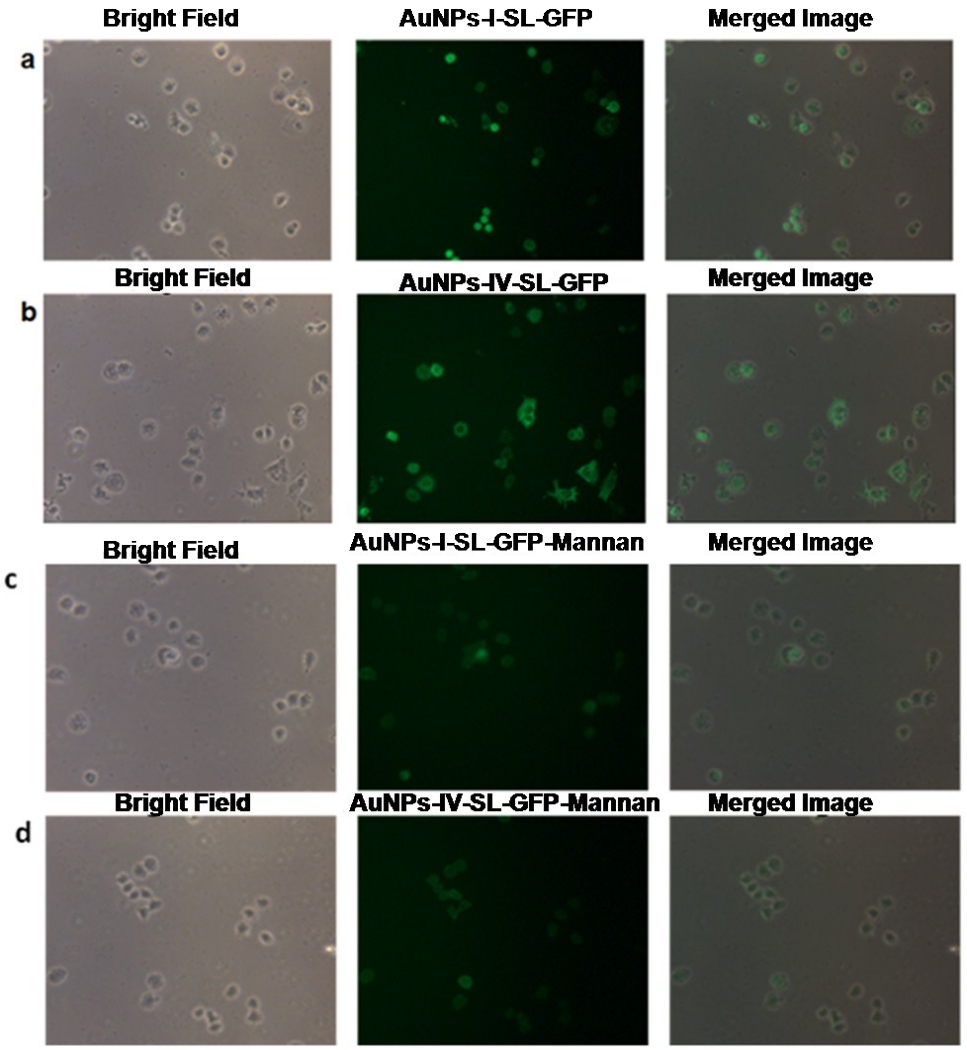


Figure S10. Representative epifluorescence micrographs of mbm-DCs transfected with ligand decorated Au-nanoplexes of pα5GFP. (a-b) The GFP expression profiles in mbmDCs transfected with nanoplexes of gold nanoconjugates (AuNPs-I-SL & AuNPs-IV-SL) and pα5GFP plasmid were recorded 24 h post addition of nanoplexes to cells. (c-d) figure show the representative epifluorescence micrographs for the nanoconjugates (AuNPs-I-SL & AuNPs-IV-SL) and pα5GFP plasmid in DCs pre-saturated with mannan, a natural ligand of mannose receptor.

Reference

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