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1 **Enhanced antitumor immunity by targeting dendritic cells with**
2 **tumor cell lysate-loaded chitosan nanoparticles vaccine**

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2 **Abstract**

3 Whole tumor cell lysates (TCL) have been implemented as tumor antigens for
4 cancer vaccine development, although clinical outcomes of TCL-based antitumor
5 immunotherapy remain unsatisfactory. In order to improve the efficacy of TCL-based
6 vaccines, biomaterials have been employed to enhance antigen delivery and
7 presentation. Here, we have developed chitosan nanoparticles (CTS NPs) with surface
8 mannose (Man) moieties for specific dendritic cells (DCs) targeting (Man-CTS NPs).
9 The Man-CTS NPs were then loaded with TCL generated from B16 melanoma cells
10 (Man-CTS-TCL NPs) for *in vitro* and *in vivo* assessment. Potency of the
11 Man-CTS-TCL NPs as cancer vaccine was also assessed *in vivo* by immunization of
12 mice with Man-CTS-TCL NPs followed by re-challenge with B16 melanoma cell
13 inoculation. We have shown here that Man-CTS-TCL NPs promote bone
14 marrow-derived dendritic cells (BMDCs) maturation and antigen presentation *in vitro*.
15 *In vivo* evaluation further demonstrated that the Man-CTS-TCL NPs were readily
16 taken up by endogenous DCs within the draining lymph node (DLN) following
17 subcutaneous administration accompanied by increase in serum IFN- γ and IL-4 levels.
18 Tumor growth was also significantly delayed in mice primed with Man-CTS-TCL
19 NPs vaccine, attributable at least in part to cytotoxic T lymphocytes response.
20 Moreover, Man-CTS-TCL NPs vaccine also exhibited therapeutic effects in mice with
21 melanoma. Thus, we report here the Man-CTS-TCL NPs as effective anti-tumor
22 vaccine for cancer immunotherapy.

1 **Keywords:** Dendritic cell targeting; Immunotherapy; Tumor cell lysates;

2 Nano-vaccine; Chitosan

3

1 **1. Introduction**

2 Cancer immunotherapy is now considered a promising therapeutic approach against
3 melanoma, leukemia, prostate and breast cancer [1-3]. The main purpose of this
4 strategy is to prime naïve T cells and evoke long-term memory CD8⁺ T cells that
5 attack tumor cells. Sufficient tumor antigen presentation, often by the antigen
6 presenting cells (APCs) via the major histocompatibility complex (MHC) class I or II
7 pathways, is critical in eliciting effective activation of both CD8⁺ and CD4⁺ T
8 lymphocytes and thus determines the therapeutic efficacy of antitumor
9 immunotherapy [4-6].

10 Dendritic cells (DCs) are professional antigen presenting cells (APCs) and are
11 experts in priming T cells-mediated immunity [7,8]. DCs-based vaccine has been
12 extensively investigated as a feasible approach to enhance antigen-specific immune
13 responses [6]. Most previous studies aimed at developing tumor vaccines that activate
14 cytotoxic T lymphocytes (CTLs) responses and antibody secreting B cells through
15 DCs mediated antigen presentation [9,10]. However, despite promising preliminary
16 data, clinical outcomes of tumor vaccines have been disappointing [11,12]. One major
17 challenge of the current antitumor immunotherapy is inefficient antigen delivery and
18 subsequent induction of T cell mediated immune responses. In addition, the
19 electrofusion process of DCs and tumor cells is time-consuming and laborious [13].
20 Moreover, external pulsing of DCs with selective peptides only results in limited
21 stimulation of T cells due to the rapid turnover of class I peptides complex on the cell
22 surface [14].

1 It has been recently shown that the use of whole tumor cell lysates preparation can
2 overcome the above drawbacks and offers a comprehensive source of potential tumor
3 antigens by inducing CTL responses and CD4⁺ T helper cell activation [15,16].
4 Several clinical trials have used tumor cell lysates for *in vitro* DC priming [17,18].
5 However, soluble tumor lysates containing antigens and cytokines are inherently
6 unstable and tend to result in poor DCs uptake, inefficient antigen cross-presentation,
7 and limited induction of CTL response [19]. Recent strategies for developing
8 prophylactic or therapeutic vaccines have mainly focused on improving *in vivo*
9 antigen delivery to specific DCs and prolonging their activation.

10 Biomaterial encapsulation of antigens has been proposed as a promising strategy to
11 enhance immunogenicity during vaccination [20]. Not only could biomaterial
12 encapsulation protect the antigens from degradation during *in vivo* administration, it
13 also enables controlled release of antigens in a desired manner [21-24]. Moreover,
14 surface of biomaterials could be modified with ligands or antibodies that are
15 specifically recognized by DCs and used for DC targeting [25].

16 Chitosan is a cationic polysaccharide primarily derived from the exoskeletons of
17 crustaceans and extensively used as vaccine delivery vehicles [21,26,27]. Earlier
18 studies have reported chitosan-based nanoparticles, with particle size ranging from
19 100 to 500 nm, as feasible vehicles for *in vivo* delivery of proteins or peptides [28].
20 Moreover, chitosan nanoparticles have been reported to elicit significant adjuvant
21 effect by stimulating innate immune responses [29]. Given the potential
22 pro-inflammatory property of chitosan, we have developed chitosan nanoparticles

1 with surface-decorated mannose (Man-CTS NPs) for specific DC targeting. Indeed,
2 studies have shown that nanoparticles with surface-decorated mannose are effective
3 delivery vehicles for APCs targeting [30-32]. Since the mannose receptor is expressed
4 by immature dendritic cells, the mannose moiety on the surface of the Man-CTS NPs
5 can be detected by DCs, via which antigen uptake would be enhanced [33,34]. Thus,
6 in the present study, targeted delivery of Man-CTS-TCL NPs was assessed both *in*
7 *vivo* and *in vitro*. In addition, to evaluate the potential of Man-CTS NPs as cancer
8 vaccine delivery vehicle, we also encapsulated tumor cell lysates generated from B16
9 melanoma cells (Man-CTS-TCL NPs) and *in vivo* assessment of Man-CTS-TCL NPs
10 on tumor prevention was performed.

11 **2. Material and methods**

12 **2.1. Reagent and antibodies**

13 Chitosan (CTS, Mw = 50,000, DD (degree of deacetylation) > 95%) was supplied
14 by Ao'xing Biotechnology Co., Ltd. (Zhejiang, China), and used without further
15 purification. Sodium alginate (ALG, viscosity: 160 mpa·s, 20 °C, 1% aqueous solution)
16 was supplied by Qingdao Crystal Rock Biology Development Co., Ltd. (Qingdao,
17 China). Tetrabutylammonium (TBA) hydroxide and 4-Aminophenyl
18 α -D-mannopyranoside (MAN) were purchased from Aladdin (Shanghai, China).
19 2-chloro-1-methylpyridinium iodide (CMPI) was obtained from Alfa Aesar (Tianjin,
20 China). Sodium sulfate, polysorbate 80 (Tween 80) and acetic acid were purchased
21 from Sigma (St. Louis, MO). The MicroBCA™ Protein assay kit was supplied by
22 Thermo Fisher Scientific Inc. (Rockford, IL USA). Phosphate Buffered Saline (PBS)

1 (1×), RPMI-1640 medium, heat inactivated fetal bovine serum (FBS), trypsin EDTA
2 0.05%, penicillin/streptomycin (PEST) 10,000 Unit/mL/10,000 µg/mL, sodium
3 pyruvate 100 mM, HEPES 1 M, 2-mercaptoethanol 50 mM, ACK lysing buffer and
4 AlamarBlue[®] reagent were purchased from Life Technologies (Carlsbad, CA, USA).
5 All the other chemicals were of analytical grade.

6 Anti-mouse ELISA kits IFN-γ, IL-4, IgG and IL-12p70 were purchased from
7 eBioscience. Recombinant mouse GM-CSF and IL-4 were purchased from Peprotech
8 (Rocky, Hill, USA). Fluorochrome-labeled anti-mouse monoclonal antibodies (CD3e,
9 CD4, CD8a, CD80, CD86, MHCI, MHCII, CD11c, CD40 and CCR7) were purchased
10 from eBioscience (CA, USA).

11 **2.2. Cell lines and animals**

12 Female C57BL/6 (6-8 weeks old) and female Balb/c mice (6-8 weeks old) were
13 purchased from (Academy of Military Medical Sciences, Beijing, China). All animal
14 procedures were reviewed and ethically approved by Center of Tianjin Animal
15 Experiment ethics committee and authority for animal protection (Approval No.:
16 SYXK (Jin) 2011-0008). Mouse B16 melanoma tumor cell line [35] was purchased
17 from the Cell Bank of China Academy of Sciences, and cultured according to the
18 manufacture's guidelines.

19 **2.3. Generation and isolation of tumor cell lysates**

20 Tumor cell lysates (TCL) were generated as previously described [36]. Briefly, B16
21 melanoma tumor cell pellets were re-suspended in ice cold phosphate-buffered saline
22 (PBS) at a 1×10^7 /ml cell density and subjected to five freeze-thaw cycles of rapid

1 freezing in liquid nitrogen (for 5 min) and thawing at 37 °C (for 5 min). The lysates
2 were then centrifuged at 2,000 g for 10 min to remove cellular debris. Protein
3 concentrations were measured by BCA assay and the concentrated protein solution
4 was diluted to appropriate concentration for *in vivo* and *in vitro* experiments.

5 **2.4. Preparation of mannose-modified alginate (Man-ALG)**

6 Tetrabutylammonium-alginate (ALG-TBA) was synthesized as previous report [37].
7 500 µg (1.190 mmol) ALG-TBA was dissolved in 50 mL of anhydrous
8 dimethylformamide (DMF). 152.1 mg CMPI (0.595 mmol) was added to activate the
9 ALG-TBA in nitrogen at 0 °C. After 1 h, 4-Aminophenyl α-D-mannopyranoside
10 (0.357 mmol) was added at room temperature and leave to react for 24 h. The reaction
11 mixture was precipitated in absolute ethanol, repeatedly dissolved and precipitated for
12 three times. The Man-ALG solution was transferred to a dialysis bag (MW=3500 Da)
13 to dialyze in distilled water for 2 days. The final product of Man-ALG was obtained
14 after freeze-drying. The chemical structure of Man-ALG was confirmed by ¹H NMR
15 spectroscopy (Varian Mercury 400, USA) and IR spectroscopy (Spectrum Instruments
16 Co., Ltd. Brook Germany). The substitution degree (SD) of Man was defined as the
17 ratio of reacted sugar unit to the total sugar unit of ALG.

18 **2.5. Preparation and characterization of mannose-decorated chitosan** 19 **nanoparticle encapsulation of tumor cell lysates (Man-CTS-TCL NPs)**

20 Chitosan and tumor cell lysates (TCL) were dissolved in 1% acetic acid and
21 ultrapure water, respectively. One mg/mL of chitosan solution and 1 mg/mL of TCL
22 were obtained. The TCL solution was added drop-by-drop into chitosan solution and

1 mixed at 1:1 (w/w). The mixture was then agitated at 300 rpm for 30 min to obtain
2 chitosan/tumor cell lysates complex, which were collected by centrifugation and
3 dissolved in PBS for experimental use. The Man-ALG solution (1 mg/ml) was added
4 drop-by-drop into CTS-TCL NPs suspension to obtain mannose decorated CTS-TCL
5 NPs (Man-CTS-TCL NPs) through electrostatic interaction. Man-CTS-TCL NPs were
6 collected by centrifugation and suspended in PBS (pH 7.4) for further use. The same
7 protocol was followed for CTS-TCL NPs preparation except alginate (1 mg/ml) was
8 added instead of Man-ALG. The method for CTS NPs and Man-CTS NPs is the same
9 with Man-CTS-TCL NPs although TCL was not added for CTS NPs and Man-CTS
10 NPs.

11 Morphology of the nanoparticles was determined by transmission electron
12 microscopy (TEM, Philips-FEI TecnaiT10, USA). The average particle size, size
13 distributions, and surface charges of nanoparticles were measured respectively by a
14 90plus particle sizer and Zeta potential analyzer (Nano-ZS 90, Malvern Instrument,
15 UK).

16 **2.6. Assessment of antigens release from Man-CTS-TCL NPs**

17 The amount of TCL released from Man-CTS-TCL NPs was measured under acidic
18 (pH 5.0) and neutral (pH 7.4) condition. Four mg of Man-CTS-TCL NPs were
19 suspended in 4 mL of PBS and incubated at 37 °C with continuous agitation in an
20 orbital shaker. The suspension was centrifuged at 12000 rpm for 10 min at designated
21 time points. Three mL of supernatants were collected and stored at -80 °C for assaying
22 later. Then 3 mL of fresh PBS was added to the original tube for further incubation.

1 Supernatant samples were tested for total protein using BCA protein assay according
2 to the manufacturer's instruction. Absorbance at 562 nm was measured using a
3 microplate reader (Varioskan LUX multimode reader, Thermo Scientific, USA);
4 proteins concentration was calculated according to standard curve. Cumulative release
5 was studied for 72 h. The amount of proteins released at different time points was also
6 calculated respectively.

7 **2.7. Generation and stimulation of bone marrow derived dendritic cells (BMDCs)**

8 *in vitro*

9 BMDCs were isolated from hind limb bones of mice using published protocol
10 [38,39]. Briefly, both distal bone ends were excised and the marrow cells were flushed
11 using RPMI 1640 (Gibco, Grand Island, NY). The red blood cells were lysed and the
12 remaining cells were centrifuged at 1500 rpm for 10 min. 2×10^6 /mL cells were
13 aliquoted in RPMI 1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 1
14 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, 20 ng/mL of
15 GM-CSF (PeproTech) and 10 ng/mL of IL-4 (PeproTech) at 37 °C. Cells were cultured
16 in six-well plates in complete medium with cytokines at 4×10^6 cells/2 mL/well. At day
17 5 of culturing, TCL, Man-CTS NPs or Man-CTS-TCL NPs were added to make final
18 concentration of 10 μ g/ml, and cultured for 2 more days. On day 7, most of the
19 non-adherent cells have acquired typical dendritic morphology, and these cells were
20 used as the source of DCs in subsequent experiment. At this time point supernatant
21 from different cultures were collected and stored at -80 °C for cytokines determination.

22 For flow cytometry, cells cultured for 7 days were rinsed three times with PBS and

1 briefly trypsinized to form a single cell suspension. Cells were stained for 30 min on
2 ice with following fluorescence-labeled antibodies ($1 \mu\text{g}/2 \times 10^6$ cells): Anti-mouse
3 MHC I (H-2Kb)-FITC (clone: AF6-88.5.5.3), Anti-mouse MHC II (clone: AF6-120.1),
4 Anti-mouse CD11c PE-Cy7 (clone: N418), Anti-mouse CD86 APC (clone: GL1),
5 Anti-mouse CD80 FITC (clone: 16-10A1), Anti-mouse CD40 PE (clone: 1C10),
6 Anti-mouse CCR7 PE (clone: 4B12). Cells were then washed twice with cold PBS
7 and analyzed on a four color BD Accuri C6. Data analysis was performed using BD
8 Accuri C6 software (BD biosciences, San Jose, CA).

9 **2.8. Flow cytometry analysis of antigens uptake by BMDCs and the intracellular** 10 **localization of antigen in BMDCs**

11 The methods for FITC-labeled TCL (TCL-FITC), TCL-FITC loaded CTS
12 nanoparticles (CTS-TCL-FITC NPs) and TCL-FITC loaded Man-CTS nanoparticles
13 (Man-CTS-TCL-FITC NPs) preparation are detailed in supporting information.

14 Immature BMDCs were cultured with TCL-FITC, CTS-TCL-FITC NPs or
15 Man-CTS-TCL-FITC NPs at 37°C for 2, 4, 6, 12, 24 and 48 h. The surface binding of
16 TCL-FITC by BMDCs was determined by measuring mean fluorescence intensity
17 (MFI) using flow cytometry.

18 BMDCs (1×10^6 cells/mL) were cultured with TCL-FITC, CTS-TCL-FITC NPs or
19 Man-CTS-TCL-FITC NPs at 37°C for 4 h. At the end of the experiment, the cells
20 were labeled with 50 nM Lyso Tracker Red DND-99 (Invitrogen, CA, USA) for 60 min
21 to visualize late endosomes and lysosomes. The nuclei were stained with DAPI
22 (Sigma, MO, USA) for 5 min and fluorescent images were recorded by a confocal

1 laser scanning microscopy (TCS SP5II, Leica, Ernst-Leitz-Strasse, Germany).

2 **2.9. *In vivo* trafficking analysis of Man-CTS-TCL NPs**

3 Near-Infrared Cyanine 7 dyes were used to label TCL. The methods of Cy7 labeled
4 TCL (TCL-Cy7), TCL-Cy7 loaded CTS nanoparticles (CTS-TCL-Cy7 NPs) and
5 TCL-Cy7 loaded Man-CTS nanoparticles (Man-CTS-TCL-Cy7 NPs) preparation are
6 included in supporting information.

7 Balb/c mice were injected subcutaneously with TCL-Cy7 (50 μ g), CTS-TCL-Cy7
8 NPs containing 50 μ g TCL-Cy7 or Man-CTS-TCL-Cy7 NPs containing 50 μ g
9 TCL-Cy7 dispersed in 100 μ l PBS. Vaccine kinetics was then studied at several time
10 points after administration by a small animal *in vivo* imaging system (Maestro, CRI
11 USA). Mice were anesthetized by inhalation of isoflurane and fluorescence spectral
12 cubes were acquired using near infrared (illumination light from 700 nm to 900 nm in
13 10 nm steps at 2 s exposure for each step Exp.700 nm to 760 nm, Emission 800 nm
14 long pass) preset filter combinations. Unmixed images in which background signals
15 were subtracted and quantified by using Maestro software. Kinetics was measured by
16 quantifying the fluorescent intensity in pre-set regions of interest (ROI) at the
17 injection site and the draining inguinal lymph node. The fluorescence signal in the
18 injection site at each time point was presented as the percentage of the maximum
19 recorded value, to show percent decrease in time [40].

20 **2.10. *In vivo* immunization, cytokines secretion, and T lymphocytes proliferation** 21 **assay**

22 C57BL/6 mice were immunized 3 times with PBS (control), TCL, CTS NPs,

1 Man-CTS NPs, CTS-TCL NPs and Man-CTS-TCL NPs group by subcutaneous (*s.c.*)
2 injection at days -14, -13 and -7. The dose of TCL in each group was 100 µg/mouse.
3 Blood was collected 7 days after last immunization for cytokines and antibody
4 analysis, cytokines of IFN-γ, IL-4, IL-12p70 and TCL antigens specific IgG antibody
5 in serum was determined by ELISA (eBioscience). For analysis of tumor specific IgG
6 antibody, using 10 µg/mL total tumor cell lysates coating the plate to test tumor
7 antigens specific IgG antibody.

8 For *in vitro* T lymphocytes proliferation assay, splenic T cells were isolated from
9 C57BL/6 mice of all treatment groups 7 days after last immunization and labeled with
10 5 µmol/L 5, 6-carboxyfluorescein acetate N-succinimidyl ester (CFSE, Sigma-Aldrich)
11 according to the manufacturer's instructions. The CFSE-labeled T cells (4×10^6
12 cells/mL) were then incubated with 10 µg/mL soluble TCL antigens for re-stimulation
13 of antigen-specific memory T cells responses and maintained in culture for 5 days. T
14 cells were then collected and T lymphocyte proliferation was assessed by flow
15 cytometry. Percentages of CD4⁺ CFSE^{low} and CD8⁺ CFSE^{low} T cells were analyzed.

16 **2.11. Immunization and tumor challenge**

17 Female C57BL/6 6-8 weeks old mice were randomized into one of the following
18 treatment groups (n=6): (1) Control group, (2) TCL group, (3) CTS NPs group, (4)
19 Man-CTS NPs group, (5) CTS-TCL NPs group and (6) Man-CTS-TCL NPs group.
20 Mice received 3 subcutaneous injections of 100 µL (concentration was 1 mg/mL) of
21 vaccine at days -14, -13 and -7 in the left flank followed by inoculation with 1×10^5
22 B16 cells suspended in 50 µL PBS in the right side. The day of inoculation was

1 counted as day 0 followed by the closely monitored at every other day for
2 pain/distress, tumor volume and body weight. Tumor growth was evaluated by
3 measuring two perpendicular tumor dimensions using a caliper. Tumor volumes (V,
4 mm³) were calculated using the following formula: $V=1/2(W^2 \times L)$, where W (width) is
5 the perpendicular dimension to the length, L (length) is the longest dimension. Mice
6 were sacrificed at day 21. Tumors were removed and weighted. Spleen and lymph
7 nodes were excised and made into single cells suspension for flow cytometer analysis
8 of T cells subpopulations.

9 **2.12. CTL assays**

10 Single cell suspensions of splenocytes were prepared from mice 21 days after B 16
11 melanoma cell inoculation. Cells were seeded onto 6-well plates ($\sim 1 \times 10^7$ cells/well)
12 and co-cultured with B16 cells ($\sim 1 \times 10^6$ cells/well) for 72 hours. The appropriate
13 numbers of effector (E) cells were incubated with 6000 fresh target (T) B16 cells with
14 E:T ratios varied at 10:1 to 50:1 in each well of U-bottomed 96 well plates. The
15 mixture were then incubated for 4 h at 37 °C in 95% O₂/5% CO₂ and the lactate
16 dehydrogenase levels in cell culture supernatants were tested by CTL assay kit
17 following the manufacture's (Promega) instruction [41, 42].

18 **2.13. In vivo assessment of antitumor effect of the M-CTS-TCL NPs**

19 Female C57BL/6 6-8 weeks old mice were s.c. injected in the right flanks with $1 \times$
20 10^5 B16 cells suspended in 50 μ L PBS. On day 7, mice were randomly assigned into
21 six groups (n=6 in each group). On day 7, 14, and 21 post tumor inoculation, mice
22 were s.c. immunized with PBS (Group 1), TCL (Group 2), CTS NPs (Group 3),

1 Man-CTS NPs (Group 4), CTS-TCL NPs (Group 5) and Man-CTS-TCL NPs (Group
2 6). The day on which B16 melanoma cell inoculation was performed as day 0. Mice
3 were closely monitored every other day for pain/distress, tumor volume and body
4 weight. Tumor growth was evaluated by measuring two perpendicular tumor
5 dimensions using a caliper. Tumor volumes (V, mm³) were calculated using the
6 following formula: $V=1/2(W^2 \times L)$, where W (width) is the perpendicular dimension to
7 the length, L (length) is the longest dimension. Mice were sacrificed at day 22. Sera
8 were collected for IFN- γ and IL-4 ELISA analysis. Tumors were removed and
9 weighted and spleens were excised for flow cytometer analysis of T cells
10 subpopulations.

11 **2.14. Statistical analysis**

12 Data are presented as mean \pm standard deviations. The differences between the
13 control group and experimental groups were assessed using a student's t-test, and the
14 differences between groups were determined by one-way ANOVA and Tukey's
15 posttest (GraphPad Software, La Jolla, CA, USA).

16 **3. Results and Discussion**

17 **3.1. Preparation and Characterization of Man-CTS-TCL NPs**

18 Due to its anionic property, CTS NPs was synthesized for TCL encapsulation,
19 forming a CTS-TCL complex, which was further coated with mannose-modified
20 alginate (Man-ALG). The synthesis route of Man-ALG is shown in (Fig.1A) and the
21 structure of Man-ALG was confirmed by FT-IR (Fig.1B). As clearly shown in Fig. 1B,
22 the peaks observed at 1616 cm⁻¹ and 1418 cm⁻¹ are characteristic carboxylate of the

1 absorption bands of ALG. After mannose modification, the peaks at 1670 cm^{-1} (amide
2 band I) and 1510 cm^{-1} (amide band II) could be observed, indicating formation of
3 amide bonds. Fig.1C shows the ^1H NMR spectra of ALG and Man-ALG. Small peaks
4 shown at 7.0-7.5 ppm are representative of typical protons of the 4-Aminophenyl
5 α -D-mannopyranoside moiety, demonstrating successful conjugation of Man to ALG.
6 The substitution degree of Man in Man-ALG was 8.4%, i.e. an average of 8.4 Man
7 moieties per 100 sugars units of ALG.

8 Physical characteristics of nanoparticles, including the average particle size, size
9 distributions, and surface charges of nanoparticles were summarized in Supporting
10 Information (Table S1). The average diameter of Man-CTS-TCL NPs was 120 nm and
11 spherical in shape with an overall charge of -12 mV (Fig.1D&E). The protein
12 releasing kinetics was determined in vitro and shown in Fig.1F, elevated and more
13 sustained protein release could be detected when the surrounding pH values was 5.0,
14 suggesting a better releasing property of the Man-CTS-TCL NPs in the acidic
15 environment of the endo/lysosomes.

16 **3.2. Man-CTS-TCL NPs promoted antigen uptake in BMDCs**

17 The efficacy of Man-CTS-TCL NPs on antigen uptake was initially examined in
18 BMDCs. TCL-FITC, CTS-TCL-FITC NPs and Man-CTS-TCL-FITC NPs were
19 incubated with BMDCs for 2, 4, 6, 12, 24 or 48 h. Antigen uptake partly through
20 surface binding was quantified by MFI of FITC as measured flow cytometry. Cellular
21 localization of TCL-FITC was examined by confocal fluorescent microscopy. As
22 shown in Fig. 2B&C, a 3-fold increase of FITC-TCL uptake was observed in BMDC

1 co-cultured with Man-CTS-TCL-FITC NPs as compared controls (MFI: $82.0339 \pm$
2 $9.0000 [\times 10^4]$). As expected, the fluorescence signal of FITC which corresponds to
3 DC antigen uptake decreased over time in all groups. These results suggest that
4 compared to TCL and CTS-TCL NPs, Man-CTS-TCL NPs can significantly increase
5 antigens uptake of BMDCs, partly via enhanced surface binding. In addition, for
6 clarification fluorescence spectra of TCL-FITC, CTS-TCL-FITC NPs and
7 Man-CTS-TCL-FITC NPs are presented in supporting information (Fig. S1).

8 **3.3. Man-CTS-TCL NPs induce BMDC maturation**

9 To further investigate the effect of Man-CTS-TCL NPs on DCs *in vitro*, immature
10 BMDCs were harvest on day 5 after isolation and exposed to Man-CTS-TCL NPs for
11 48 h. Fig.3A&B shows the microscopic views of mature DCs in microscope. DC
12 maturation was characterized by surface marker expression using flow cytometry, and
13 we found that exposure to Man-CTS-TCL NPs significantly enhanced expression
14 levels of CD80, CD86 and CD40, surface markers that indicates DC maturation
15 (Fig.3C&D). Moreover, the expression levels of MHC I, MHC II, and CCR7 were
16 also upregulated in DCs treated with Man-CTS-TCL NPs (Fig.3E&F), all of which
17 are demonstrative of a facilitative role of Man-CTS-TCL NPs on DC maturation.

18 **3.4. *In vivo* imaging of Cyanine 7 labeled Man-CTS-TCL NPs**

19 In order to investigate the effect of Man-CTS-TCL NPs on endogenous DC
20 activities, TCL-Cy7, CTS-TCL-Cy7 NPs and Man-CTS-TCL-Cy7 NPs were
21 administrated by subcutaneous (*s.c*) injection at the tail base site and the presence of
22 TCL-Cy7, CTS-TCL-Cy7 NPs and Man-CTS-TCL-Cy7 NPs were then visualized at

1 the injection site and in the adjacent draining lymph node (DLN), where immune
2 responses are initiated [43]. We selected tail base as injection site which is able to
3 distinguish from the lymph nodes and it drains specifically to the inguinal lymph node
4 (ILN) [44]. Fig.4A shows representative images of fluorescence signals corresponding
5 to TCL-Cy7, CTS-TCL-Cy7 NPs and Man-CTS-TCL-Cy7 NPs at the injection site
6 and the ILN (Fig.4A red arrows) at designated time points. The fluorescence signals
7 were quantified and the average fluorescence were plotted against time and presented
8 in Fig.4B. Improved migratory ability of Man-CTS-TCL-Cy7 NPs to the ILN was
9 evident as compared to TCL-Cy7 and CTS-TCL-Cy7. Furthermore, accumulation of
10 free TCL was observable in the liver of TCL-only group whilst for CTS-TCL NPs
11 group; the fluorescence signal was detected not just within the ILN, but also in
12 adjacent tissues. No signal of Cy 7 could be recorded in the ILN at 24 h following
13 injection in both TCL and CTS-TCL NPs group, in contrast to the Man-CTS-TCL
14 NPs group, where robust and sustained Cy 7 signal was detectable at 24 h following
15 injection. One explanation for the migratory advantage of Man-CTS-TCL-Cy7 NPs is
16 that both active and passive pathways are involved during antigen migration from the
17 tail back to the right inguinal DLN [28]. This is consistent with previous study
18 reporting that nanoparticles with comparatively smaller size facilitate DC uptake by
19 passive cellular endocytosis [45]. Since the average size of Man-CTS-TCL NPs is
20 approximately 100 nm, it is possible that the nanoparticles are taken up by resident
21 DCs passively in addition to active uptake as a result of the mannose interaction.

22 **3.5. Effect of Man-CTS-TCL NPs on endogenous DCs maturation and antigen**

1 **presentation at the draining lymph node**

2 To assess the efficacy of Man-CTS-TCL NPs as potential cancer vaccine, mice
3 were administrated with Man-CTS-TCL NPs, CTS-TCL NPs, Man-CTS NPs, CTS
4 NPs or TCL only by *s.c.* injection at days -14, -13 and -7. Non-treated mice were also
5 included in parallel as controls. On day 0 and day 7, the draining lymph nodes and
6 spleen of each mouse were obtained. As shown in Fig.5A, the ILNs from CTS-TCL
7 NPs group and Man-CTS-TCL NPs group were moderately bigger in size compared
8 to other groups. DC maturation is associated with a wide range of cellular changes,
9 such as increased expression of costimulatory molecules, surface MHC class II
10 molecules, and chemokine receptor (e.g. CCR7), which allow them to migrate to the
11 LNs [4]. To assess the impact of nanoparticle-delivered TCL antigen on DC
12 maturation, expression levels of CD11c, CD80, CD86, MHCII and CCR7 on APCs
13 within the ILNs were analyzed. It is clear from Fig.5B, expressions of CD11c⁺CD86⁺,
14 CD11c⁺CD80⁺, CD11c⁺MHCII⁺, CD11c⁺MHCI⁺ and CD11c⁺CCR7⁺ were
15 upregulated in the Man-CTS-TCL NPs group, significantly elevated as compared to
16 other groups, indicating more robust impact of the Man-CTS-TCL NPs on stimulating
17 resident DC maturation within the LNs. Furthermore, results of antigen recall T-cell
18 proliferation also showed that proliferation of both CD8⁺ and CD4⁺ T cells were
19 elevated from the Man-CTS-TCL NPs exposed group when compared to others.
20 Proliferation of CD8⁺ T cells were significantly increased compared with CTS NPs
21 and Man-CTS NPs, but not the TCL only group. Whilst CD4⁺ T cell proliferation was
22 significantly enhanced from the Man-CTS-TCL NPS when compared with TCL group,

1 CTS NPs and Man-CTS NPs (Fig.5C&D). Similarly, upregulation of IFN- γ and IL-4
2 was also detected in the sera of mice (Fig.5E&F), confirming T cell activation.

3 **3.6. Man-CTS-TCL NPs as a nano-vaccine exhibited TCL-induced antitumor** 4 **immune responses**

5 To examine the antitumor efficacy of Man-CTS-TCL NPs, we immunized C57BL/6
6 mice with Man-CTS-TCL NPs and re-challenged the mice by inoculation of 1×10^5
7 B16 tumor cells. The detailed immunization protocol is included in Supplementary
8 Fig.S4A. There was no significant difference in mice body weight among those six
9 groups (Fig.6A). As shown in Fig.6B, administration of Man-CTS-TCL NPs could
10 significantly delay tumor growth compared with untreated control group, CTS NPs
11 group, Man-CTS NPs group and mice immunized with TCL, shown by considerably
12 smaller tumor size by Day 21. What's more, compared with control group, TCL group,
13 CTS NPs group, Man-CTS NPs group, and CTS-TCL NPs group, the average tumor
14 weight in Man-CTS-TCL NPs group was significantly decreased (Fig.6C).

15 It has been well established that CD8⁺ T cells are important effector cells in vaccine
16 induced anti-tumor responses [46]. In line with that, we found that mice immunized
17 with Man-CTS-TCL NPs showed significantly more CD8⁺ T cells in the LN and
18 spleen than other groups (Fig.6E&F). No difference could be detected regarding CD4⁺
19 T cells following immunization among all treatment groups (Data not shown).
20 Meanwhile, the absolute number of CD3⁺ T cells in spleen was calculated and no
21 significant difference could be deduced between Man-CTS-TCL NPs group and
22 others (Supplementary Fig.S4B). Significant elevation of the absolute number of

1 CD3⁺CD8⁺ T cells in spleen was seen in the Man-CTS-TCL NPs group when
2 compared with others (Supplementary Fig.S4C), all of which emphasize the
3 importance of CD8⁺ T cells in Man-CTS-TCL NPs-induced antitumor activity.

4 **3.7. Man-CTS-TCL NPs potently enhanced cytotoxic T lymphocytes (CTL)** 5 **responses against tumor**

6 To test the efficacy of T cells in mediating tumor-specific CTL responses, cytotoxic
7 T lymphocyte (CTL) assay was performed. First, we co-cultured the effector cells
8 (splenocytes isolated from immunized mice) and the target cells B16 melanoma cells.
9 Lysis of target cells was then tested at the Effector:Target (E:T) cell ratio of 10:1, 20:1
10 and 50:1. Importantly, the effector T cells isolated from mice immunized with
11 Man-CTS-TCL NPs lysed ~35% target melanoma cells, while the lysed target
12 melanoma cells in control group was only ~12% (Fig.6D). This showed that mice
13 immunized with Man-CTS-TCL NPs were more efficient in inducing CTL response
14 against the B16 melanoma target cells.

15 **3.8. Man-CTS-TCL NPs achieved therapeutic effects in melanoma tumor model**

16 To investigate the therapeutic effect of Man-CTS-TCL NPs, C57BL/6 mice were
17 inoculated *s.c.* with 1×10^5 B16 melanoma cells 7 days before vaccination with
18 Man-CTS-TCL NPs. Detailed protocol is shown in Supplementary Fig.S5A. There
19 was no significant difference in mice body weight among all groups (Fig.7A). As
20 shown in Fig.7B, vaccination with Man-CTS-TCL NPs significantly inhibited B16
21 tumor growth, compared with PBS-administrated controls. Consistently, tumor weight
22 in Man-CTS-TCL NPs group was significantly decreased at day 22 compared to

1 control group, although no differences could be observed between other groups and
2 control (Fig 7C). Expression of mouse serum IFN- γ was consistent with the
3 anti-tumor results (Fig.7E). Compared with other groups, mice in Man-CTS-TCL NPs
4 group showed more CD8⁺ T cells in spleen (Fig.7F), with no difference detectable
5 regarding the absolute number of CD3⁺ T cells in spleen (Supplementary Fig.S5B).
6 Significant increase of the absolute number of CD3⁺CD8⁺ T cells in spleen was
7 observed in Man-CTS-TCL NPs when compared to others (Supplementary Fig. S5C).
8 These results suggest that Man-CTS-TCL NPs exhibit therapeutic efficacy in mice
9 with melanoma, and it may provide a translational option as immunotherapy.

10 **4. Conclusion**

11 In summary, we demonstrate here chitosan-based nanoparticles with surface
12 -decorated mannose, Man-CTS NPs as potential vehicle for cancer vaccine delivery.
13 Furthermore, by encapsulating TCL generated from B16 melanoma cells, we further
14 showed the Man-CTS-TCL NPs as potent cancer vaccine for tumor prevention. The
15 robust antitumor effect of Man-CTS-TCL NPs could be partly attributed to effective
16 stimulation of cellular and humoral antitumor immunities by the nanoparticles. In
17 addition, we also demonstrate the possibility of using mannose as effective DC
18 targeting ligand by conjugation of mannose with biomaterials to facilitate DC
19 maturation, antigen uptake and presentation. Thus, we report here chitosan-based
20 nanoparticles with surface-decorated mannose as antigen delivery vehicle that
21 improves the efficacy of antitumor immune responses than CTS-TCL NPs and TCL
22 alone. The Man-CTS-TCL NPs could therefore be considered a feasible therapeutic

1 approach as development of cancer vaccine.

2 **Acknowledgements**

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22 **Figure Legend**

23 **Figure 1 Preparation and characterization of mannose decorated chitosan**
24 **nanoparticle encapsulation of tumor cell lysates.** (A) Scheme of Man-ALG
25 preparation. (B) FT-IR spectra of Man-ALG. (C) ¹H-NMR spectra of Man-ALG in
26 D₂O. (D) The size distribution of Man-CTS-TCL NPs. (E) The TEM image of
27 Man-CTS-TCL NPs. (F) Protein released from Man-CTS-TCL NPs in PBS at
28 different pH values.

1 **Figure 2 Mannose-decorated CTS-TCL NPs enhance bone marrow dendritic**
2 **cells uptake of antigens *in vitro*.** (A) DCs were incubated with FITC-labeled TCL,
3 CTS-TCL NPs, and Man-CTS-TCL NPs for 4 h, then labeled with LysoTracker-Red to
4 identify late endosomes and lysosomes, and labeled with DAPI for cell nucleus. The
5 uptake of nanoparticles was detected by confocal microscopy. Scale bars, 25 μ m. Data
6 are representative of three independent experiments. (B-C) DCs were incubated with
7 FITC-labeled TCL, CTS-TCL NPs, and Man-CTS-TCL NPs for 2, 4, 6, 12, 24 and 48
8 h, the histogram of mean fluorescence intensity (MFI) of BMDCs was detected by
9 flow cytometry. Data are representative of three independent experiments. * P <0.05,
10 ** P <0.01 and *** P <0.001, Man-CTS-TCL NPs group compared with other groups.

11 **Figure 3 Effect of mannose-decorated CTS-TCL NPs on mouse bone marrow**
12 **dendritic cells maturation and antigen presentation *in vitro*.** (A-B) Representative
13 microscopic views of BMDCs maturation stimulated with TCL (A), Man-CTS-TCL
14 NPs (B) for 48 h. Scale bar: 25 μ m. (C-D) BMDCs were analyzed for expression of
15 CD80, CD86, CD11c and CCR7 by flow cytometry after stimulated with TCL,
16 Man-CTS NPs or Man-CTS-TCL NPs for 48 h. Data are representative of three
17 independent experiments. (E-F) BMDCs were analyzed for expression of MHCI,
18 MHCII, CD11c and CD40 by flow cytometry after stimulated with TCL, Man-CTS
19 NPs or Man-CTS-TCL NPs for 48 h. Data are representative of three independent
20 experiments, * P <0.05, ** P <0.01 and *** P <0.001, Man-CTS-TCL NPs group
21 compared with other groups.

22 **Figure 4 *In vivo* tracking of Cyanine 7 labeled TCL, CTS-TCL NPs and**

1 **Man-CTS-TCL NPs in the injection site and draining lymph node.** (A) Presence
2 of TCL, CTS-TCL NPs and Man-CTS-TCL NPs in the injection site and the right
3 inguinal draining lymph node, based on quantification of the fluorescent signal of
4 near infrared coupled to the Cyanine 7. The lymph node is indicated by a red arrow.
5 Repeated measurements in time plotted on the same scale of fluorescence. (B) The
6 mean density of Cyanine 7 in the draining lymph node was calculated. All images are
7 overlays of bright photographs with fluorescence intensity measurements indicated on
8 the color scale. DLN, draining lymph node; ILN, inguinal lymph node. Data are
9 representative of three independent experiments.

10 **Figure 5 Effect of Man-CTS-TCL NPs induces immune responses in normal mice.**

11 (A) Draining lymph node was isolated at day 0 after s.c. injection of PBS, TCL, CTS
12 NPs, Man-CTS NPs, CTS-TCL NPs and Man-CTS-TCL NPs in the right flank of
13 C57BL/6 mice at day -14, -13 and -7, images of inguinal lymph node in different
14 treatment groups. (B) Draining lymph node was made into single cell suspensions,
15 and the expression of major DCs surface markers was analyzed by flow cytometry.
16 (C-D) Antigen presentation in draining lymph node after immunized with PBS, TCL,
17 CTS NPs, Man-CTS NPs, CTS-TCL NPs and Man-CTS-TCL NPs for 3 times *in vivo*.
18 MHC I and MHC II antigen presentation was determined by measuring CFSE labeled
19 CD8⁺ T and CD4⁺ T cell proliferation (defined as CFSE^{low}), respectively. (E) IFN- γ
20 released from mice serum was tested by ELISA. (F) IL-4 released from mice serum
21 was tested by ELISA. Data are representative of three independent experiments.
22 Differences between Man-CTS-TCL NPs and other groups are analyzed using

1 one-way ANOVA analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Man-CTS-TCL NPs
2 group compared with other groups.

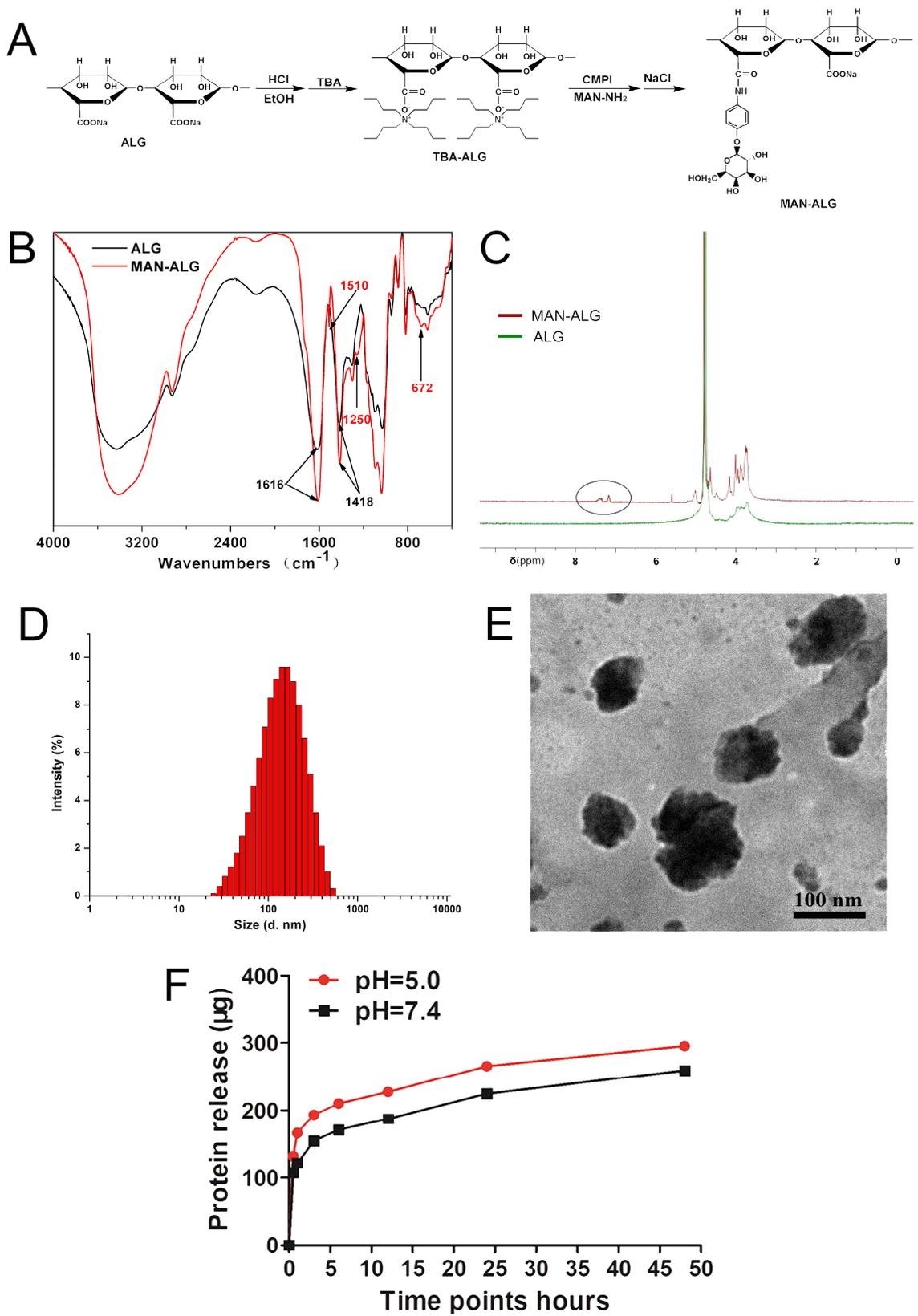
3 **Figure 6 Man-CTS-TCL NPs vaccines induce protection effect in prophylactic**
4 **tumor model.** (A) Mean body weight during the total immunization course. (B) Mean
5 tumor growth curves given by tumor volume. (C) The tumor weight was measured 21
6 days after last immunization. (D) Splenocytes were isolated 21 days after B16 tumor
7 cells inoculation, co-cultured with B16 cells, and 3 days later used as effector cells for
8 cytotoxic T lymphocytes (CTL) response assay. (E) Phenotype analysis of CD3⁺CD8⁺
9 T cells in mice lymph nodes 21 days after B16 tumor cells inoculation. (F) Phenotype
10 analysis of CD3⁺CD8⁺ T cells in mice spleen 21 days after B16 tumor cells
11 inoculation. Data are representative of three independent experiments. Bars shown are
12 mean \pm SD (n=6), and differences between PBS control group and other groups are
13 determined using one-way ANOVA analysis and Student's t test. Relative to control
14 groups: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

15 **Figure 7 Man-CTS-TCL NPs vaccines confer therapeutic protection against**
16 **melanoma.** (A) Mean tumor growth curves given by tumor volume. (B) The tumor
17 masses of each mouse on 22 days after B16 tumor cells inoculation. (C) The tumor
18 weight was measured 22 days after tumor challenge. (D) Images of excised tumors
19 from each mouse in each treatment group by the end of the assay. (E) IFN- γ released
20 from serum of tumor bearing mice control, TCL, CTS NPs, Man-CTS NPs, CTS-TCL
21 NPs and Man-CTS-TCL NPs groups was tested by ELISA. (F) Phenotype analysis of
22 CD3⁺CD8⁺ T cells in mice spleen 22 days after B16 tumor cells inoculation. Data are

1 representative of three independent experiments. Bars shown are mean \pm SD (n=6),
2 and differences between PBS control group and other groups are determined using
3 one-way ANOVA analysis and Student's t test. Relative to control group: * P <0.05,
4 ** P <0.01 and *** P <0.001.

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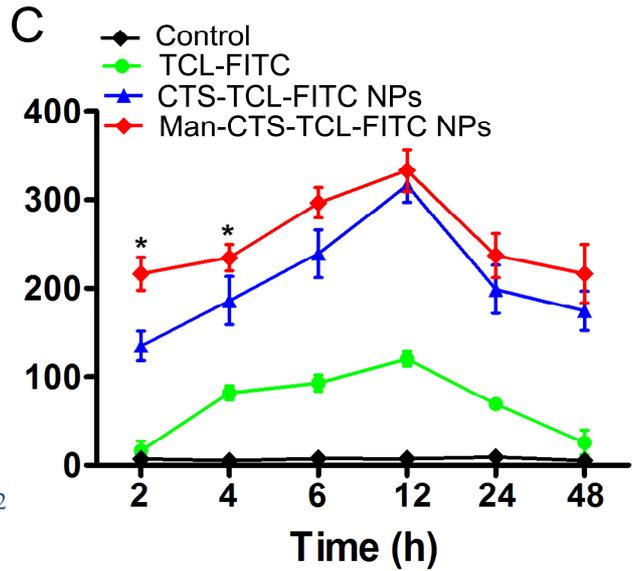
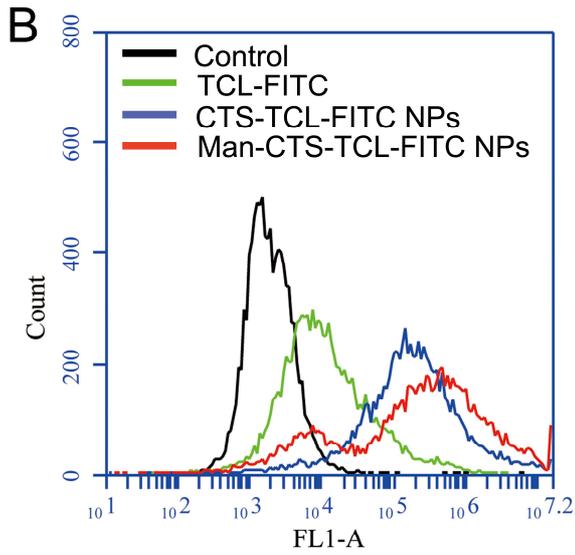
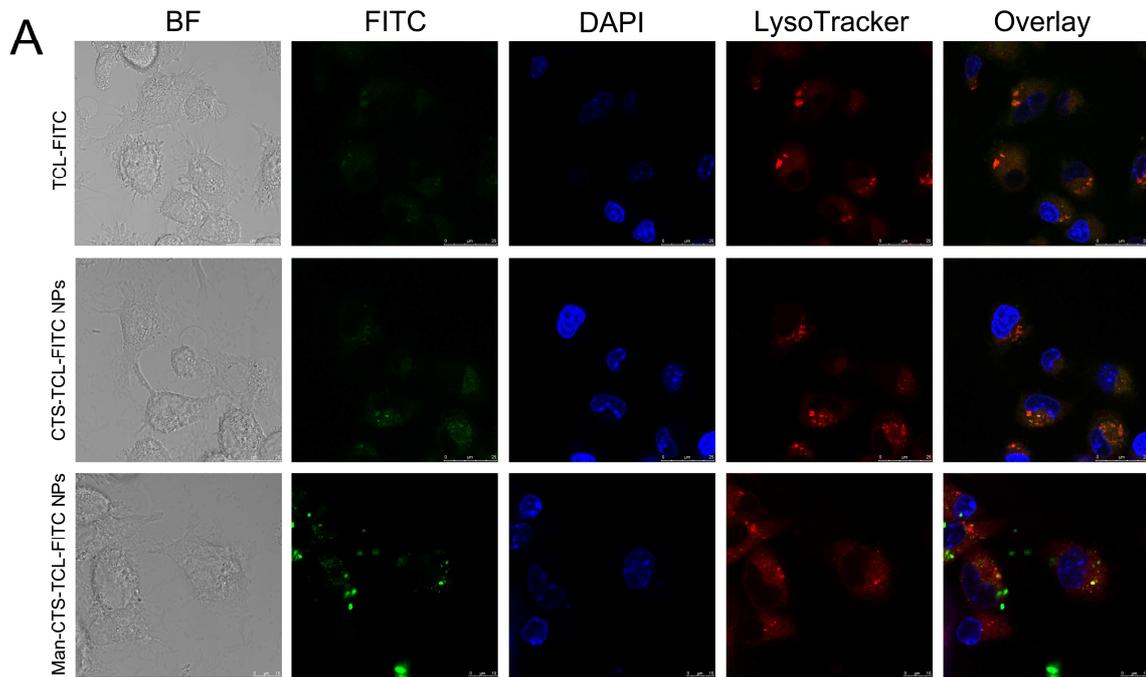
1 **Figure 1**



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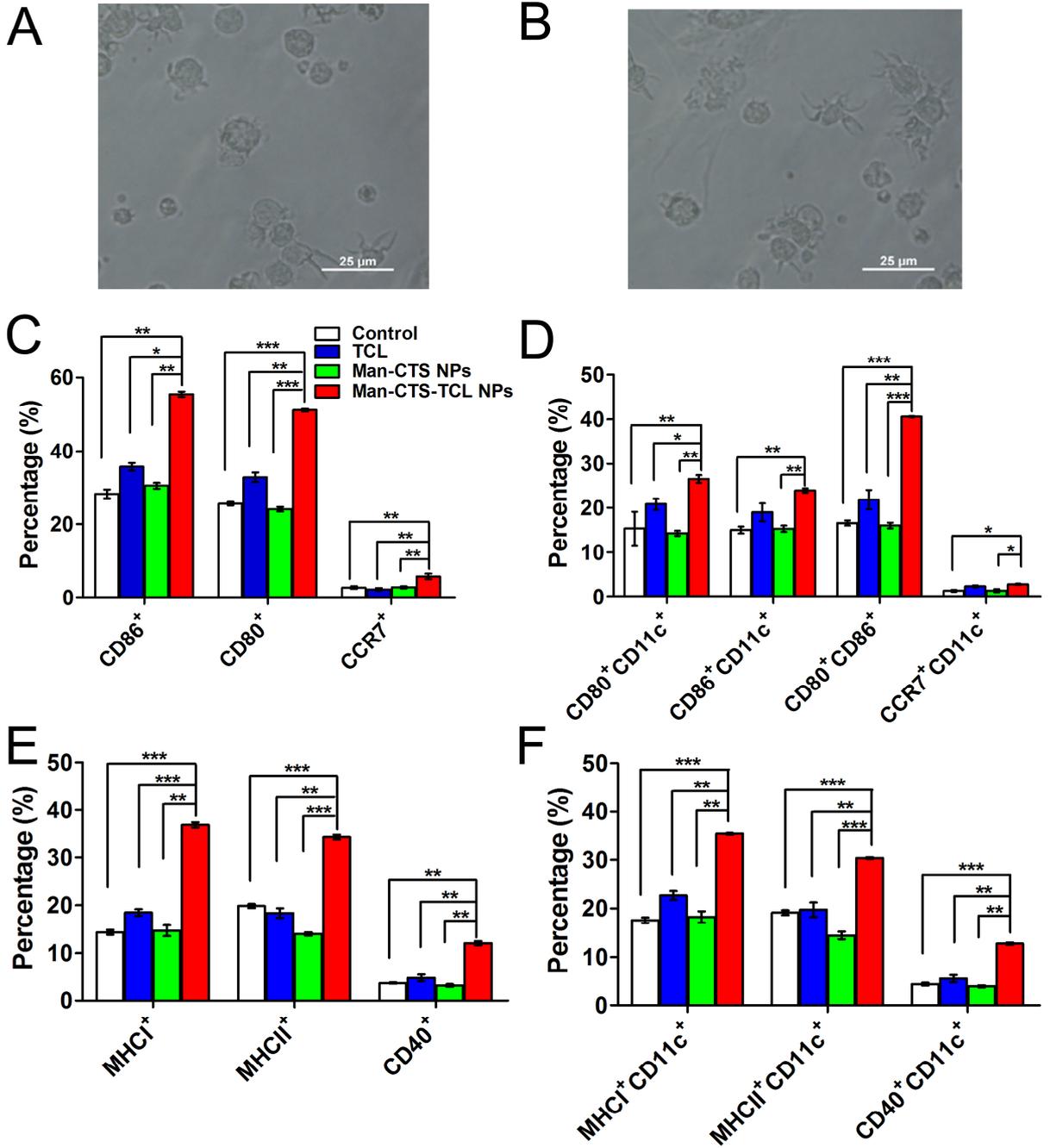
1 **Figure 2**



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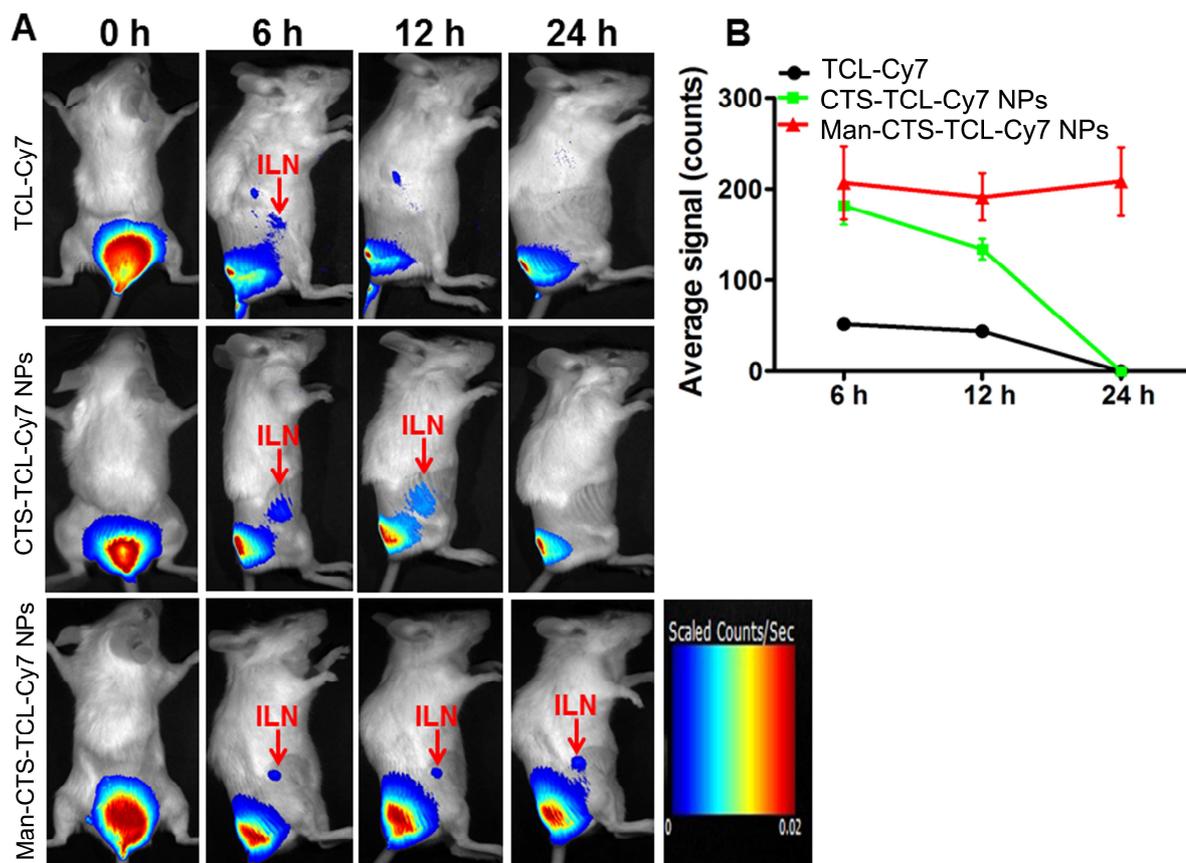
1 Figure 3



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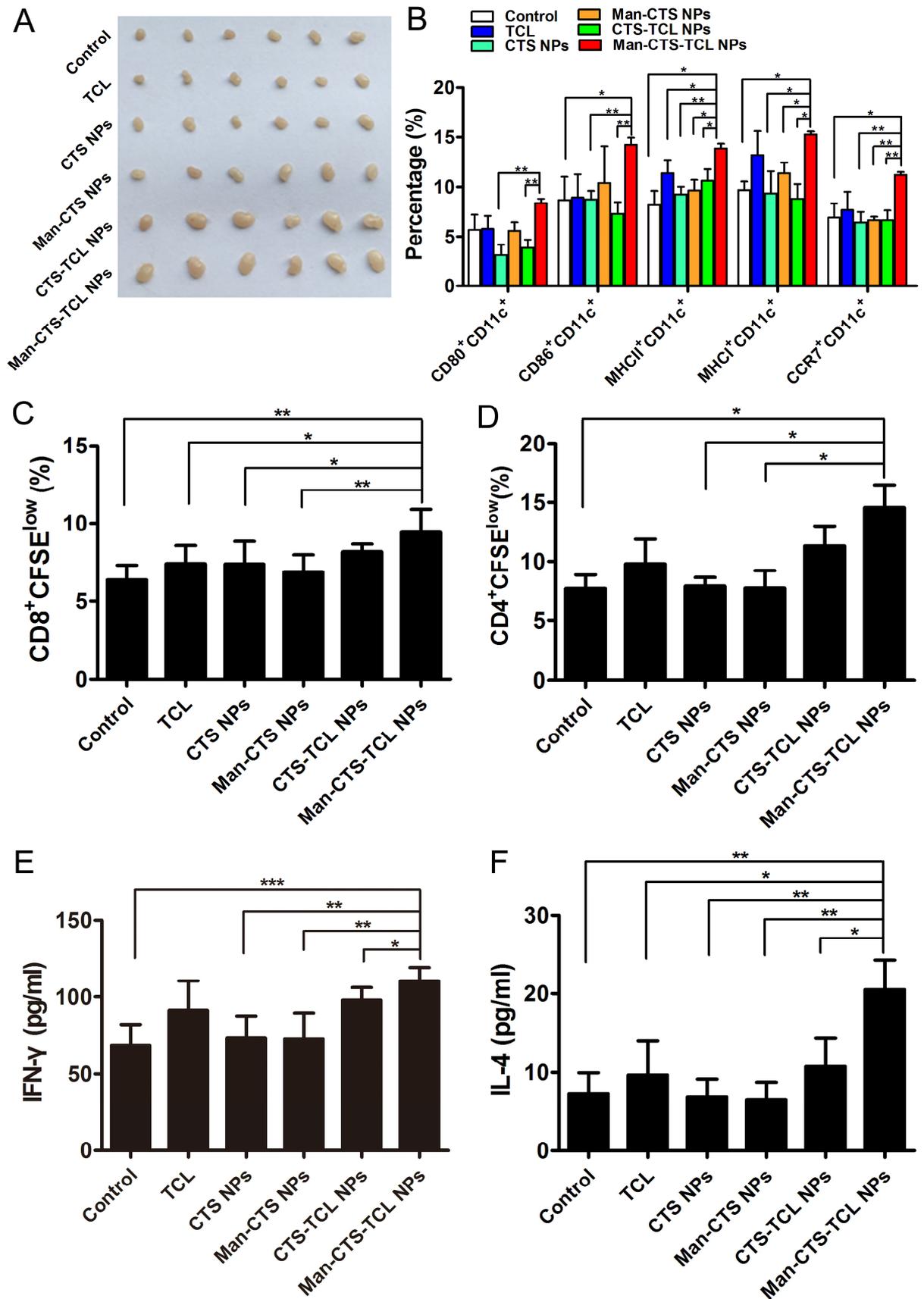
1 **Figure 4**



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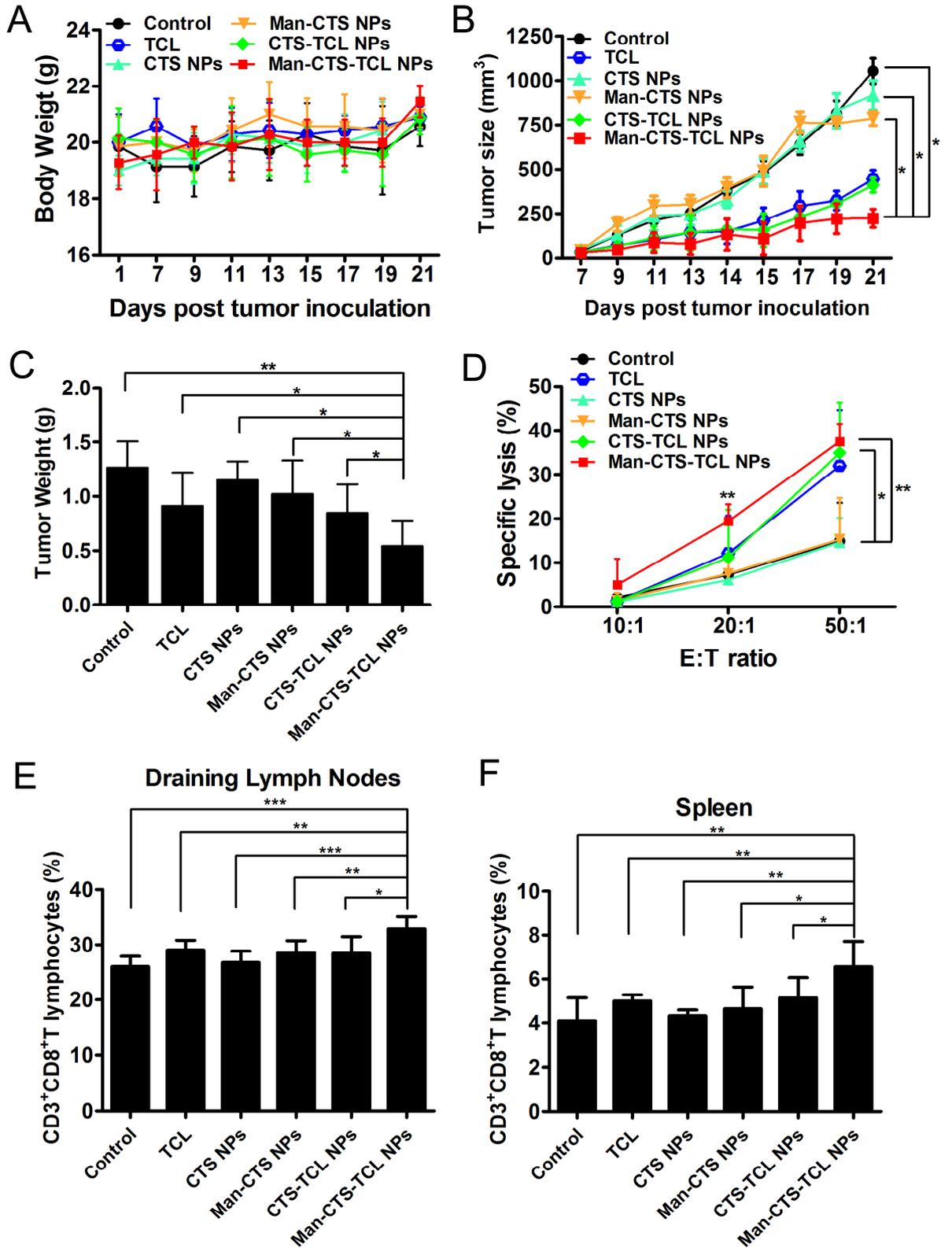
1 **Figure 5**



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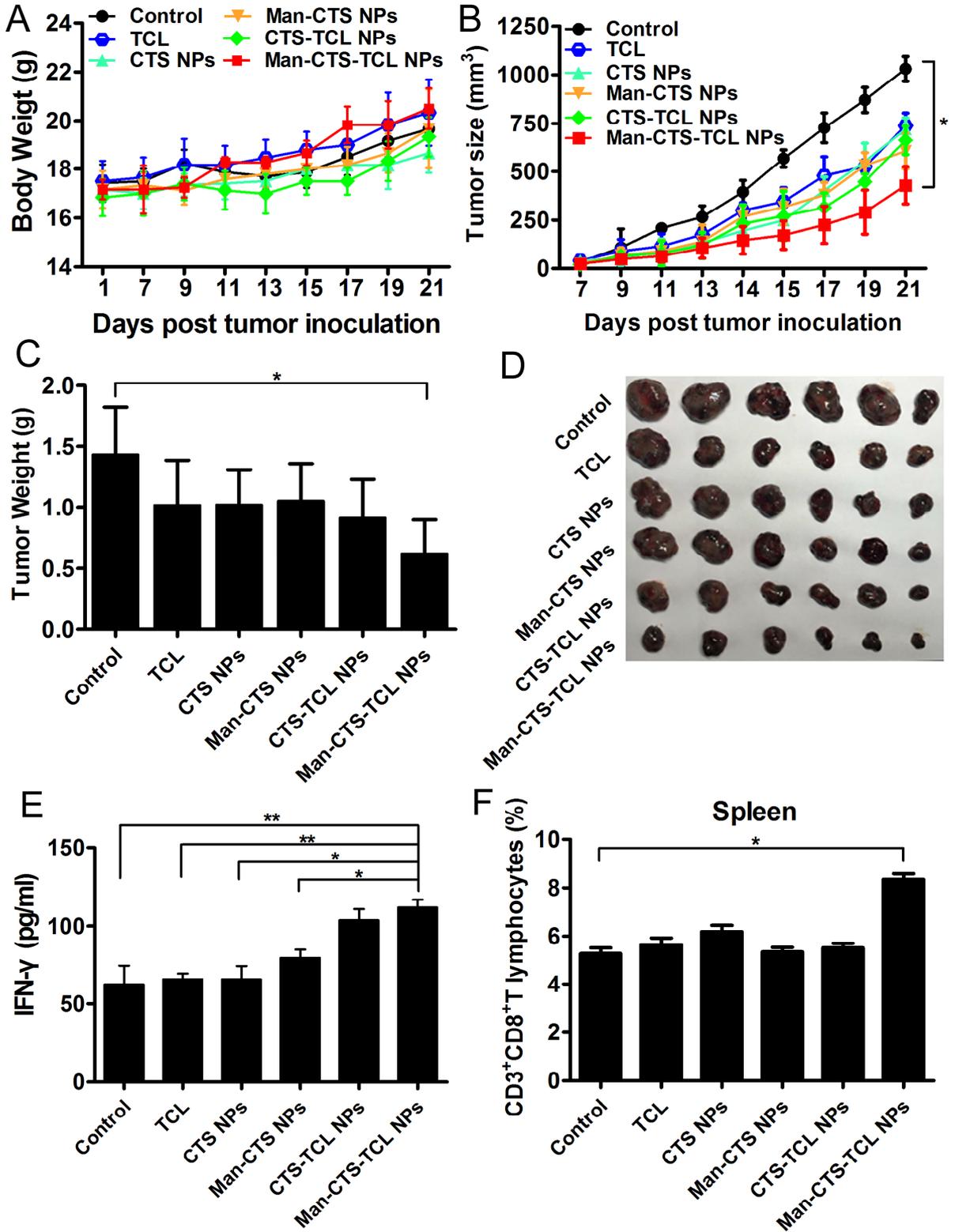
1 Figure 6



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1 Figure 7



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1 Supporting Information for

2 **Enhanced antitumor immunity by targeting dendritic cells with**
3 **tumor cell lysate-loaded chitosan nanoparticles vaccine**

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3 **Experimental section**

4 **Preparation of FITC labeled tumor cell lysates (TCL-FITC)**

5 Two mg of FITC in 1 mL of 20 mmol/L carbonate buffer (pH 9.5) added to a solution
6 of TCL (1 mg/mL, 10 mL). The solution was incubated with continuous stirring at 4
7 °C for 18 h in the dark. The reaction mixture was dialyzed against distilled water
8 (MWCO 1000) to obtain TCL-FITC.

9 **Preparation and characterization of Man-CTS-TCL-FITC NPs**

10 Three mL of TCL-FITC solution was added drop-by-drop into chitosan solution (1
11 mg/mL, 1% acetic acid) and mixed at 1:1 (w/w). The mixture was then agitated at 300
12 rpm for 30 min to obtain TCL-FITC loaded CTS nanoparticles (CTS-TCL-FITC NPs),
13 which were collected by centrifugation and dissolved in PBS for experimental use.
14 The Man-ALG solution (1 mg/mL) was added drop-by-drop into CTS-TCL-FITC
15 NPs suspension to obtain mannose decorated CTS-TCL-FITC NPs
16 (Man-CTS-TCL-FITC NPs) through electrostatic interaction. Man-CTS-TCL-FITC
17 NPs were collected by centrifugation and suspended in PBS (pH 7.4) for further use.

18 **Preparation of Cy7 labeled tumor cell lysates (TCL-Cy7)**

19 Two mg of Cy7-NHS in 1 mL of 20 mmol/L carbonate buffer (pH 9.5) was added to a
20 solution of TCL (1mg/mL, 10 mL). The solution was incubated with continuous
21 stirring at 4 °C for 18 h in the dark. The reaction mixture was dialyzed against
22 distilled water (MWCO 1000) to obtain TCL-Cy7.

1 Preparation and characterization of Man-CTS-TCL-Cy7 NPs

2 Three mL of TCL-Cy7 solution was added drop-by-drop into chitosan solution (1
3 mg/mL, 1% acetic acid) and mixed at 1:1 (w/w). The mixture was then agitated at 300
4 rpm for 30 min to obtain TCL-Cy7 loaded CTS nanoparticles (CTS-TCL-Cy7 NPs),
5 which were collected by centrifugation and dissolved in PBS for experimental use.
6 The Man-ALG solution (1 mg/mL) was added drop-by-drop into CTS-TCL-Cy7 NPs
7 suspension to obtain mannose decorated CTS-TCL-Cy7 NPs (Man-CTS-TCL-Cy7
8 NPs) through electrostatic interaction. Man-CTS-TCL-Cy7 NPs were collected by
9 centrifugation and suspended in PBS (pH 7.4) for further use.

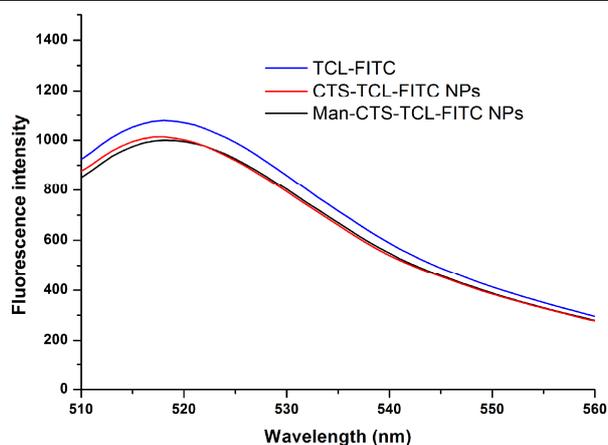
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11 Results section

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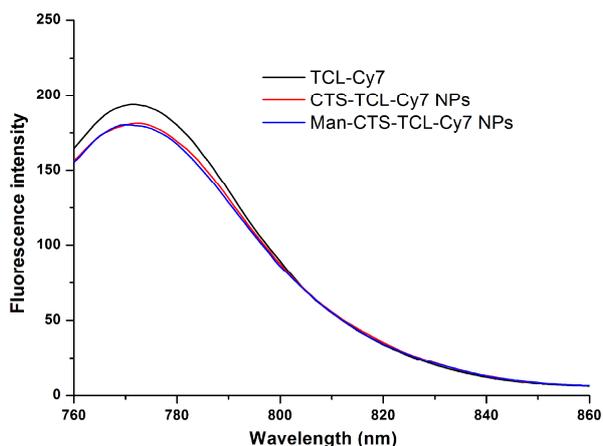
Table S1. Characterization of nanoparticles (n=3).

Sample	Size (nm)	DPI	Zeta potentials (mV)
CTS-TCL NPs	127.46±6.73	0.114±0.031	-14.07±1.22
Man-CTS-TCL NPs	120.15±9.93	0.121±0.049	-12.07±1.36
CTS-TCL-FITC NPs	134.72±2.65	0.103±0.051	-15.41±1.25
Man-CTS-TCL-FITC NPs	136.18±7.63	0.131±0.047	-14.58±1.19
CTS-TCL-Cy7 NPs	139.16±10.03	0.119±0.039	-15.46±1.52
Man-CTS-TCL-Cy7 NPs	135.48±8.97	0.124±0.041	-14.92±1.43

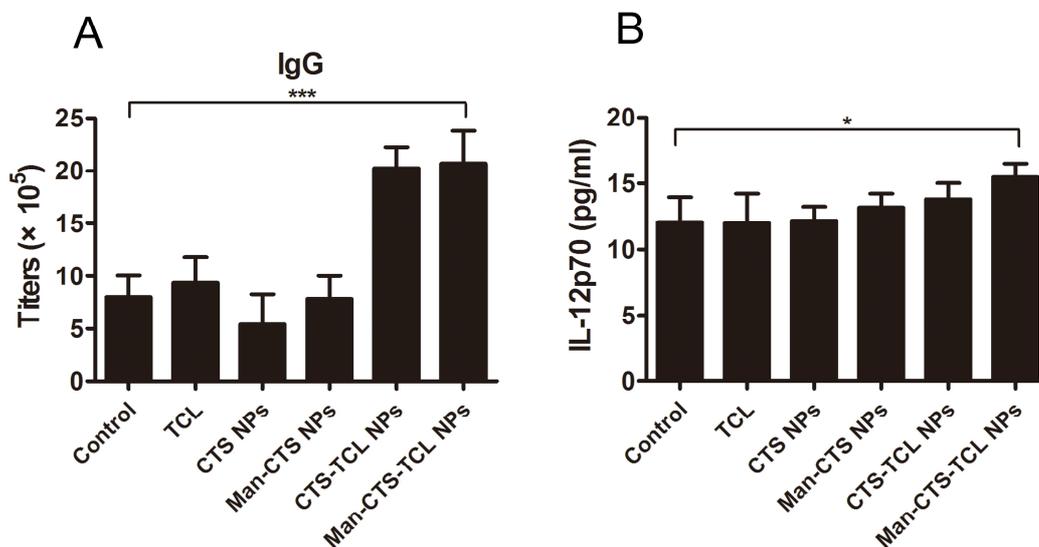


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1 **Figure S1** The fluorescence spectra of TCL-FITC, CTS-TCL-FITC NPs and
 2 **Man-CTS-TCL-FITC NPs** in PBS, and the excitation wavelength is 495 nm.

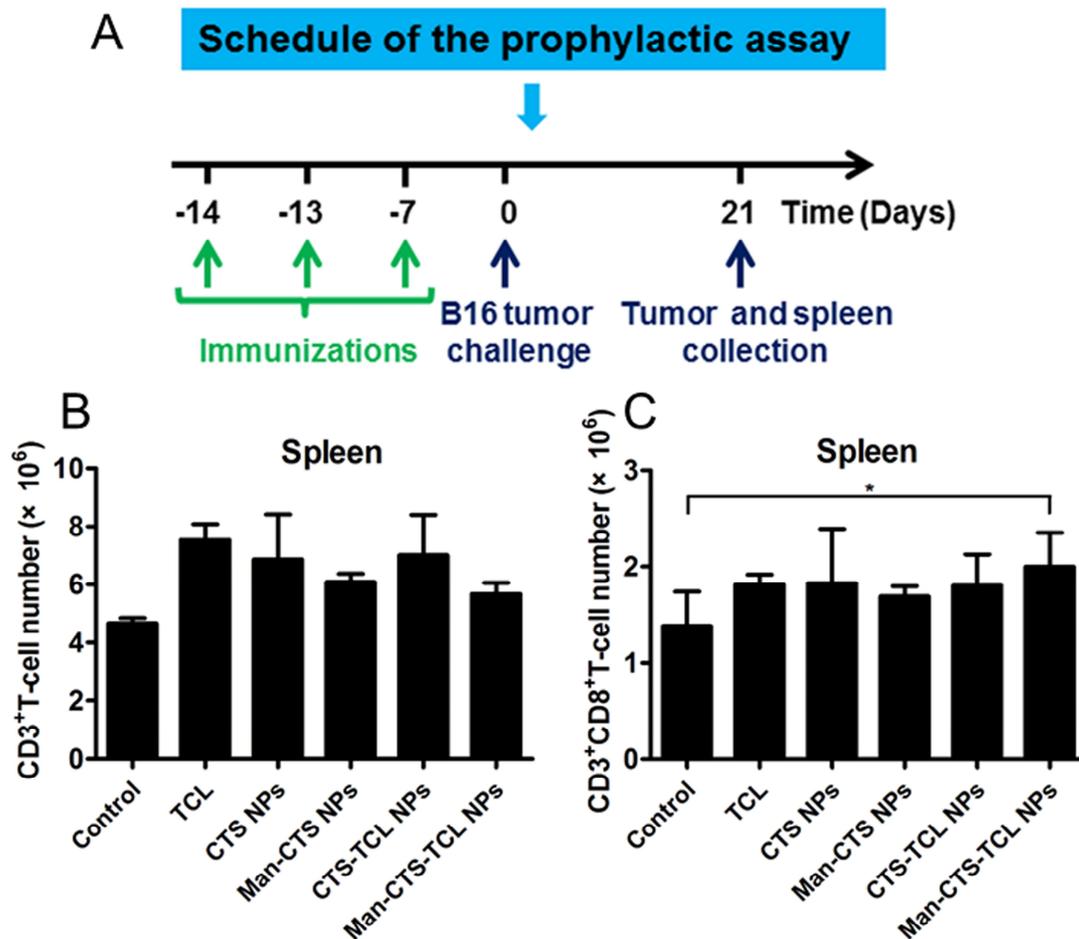


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 4 **Figure S2** The fluorescence spectra of TCL-Cy7, CTS-TCL-C NPs and
 5 **Man-CTS-TCL-Cy7 NPs** in PBS, and the excitation wavelength is 743 nm.

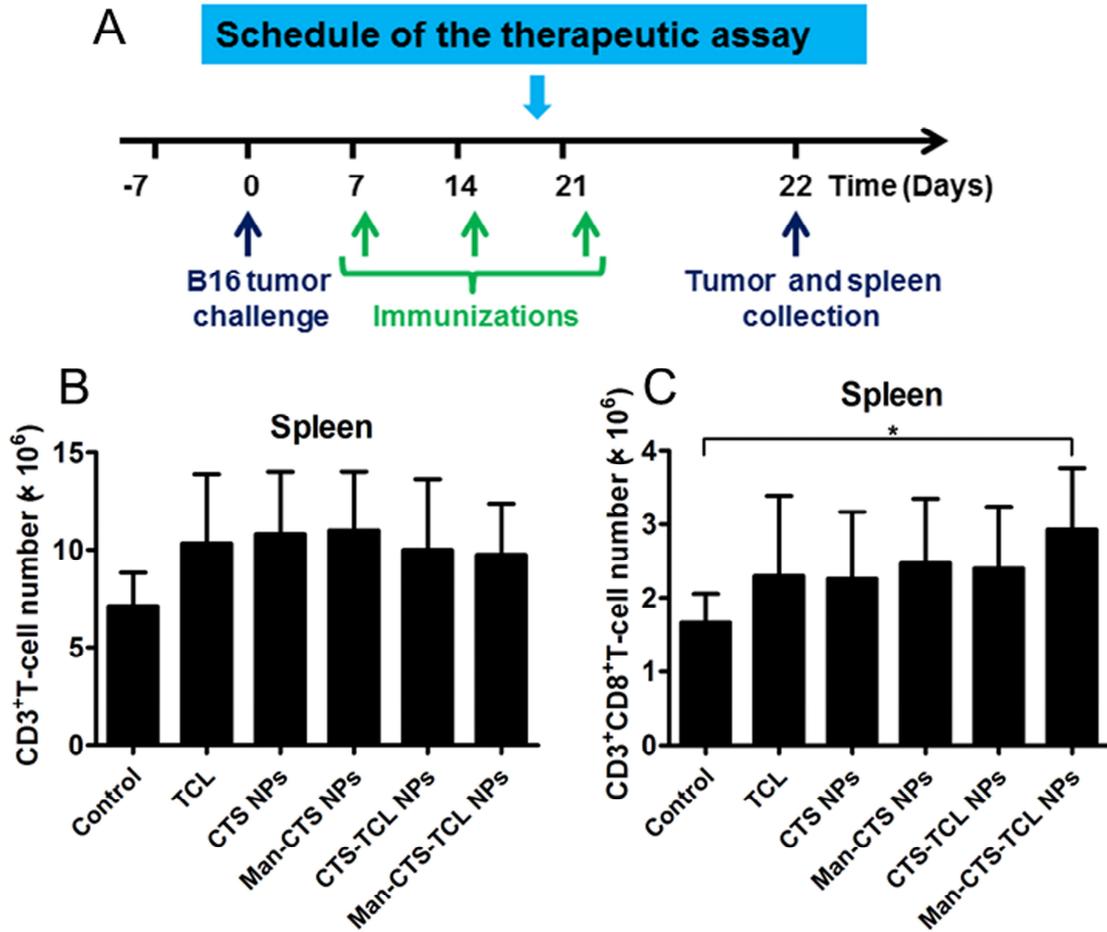


6
 7 **Figure S3** Immunization with Man-CTS-TCL NPs produces both cellular and
 8 **humoral immune responses in mice.** (A) Female C57BL/6 mice (n=6) 6-8 weeks
 9 old were subcutaneously immunized with PBS, TCL, CTS NPs, Man-CTS NPs,
 10 CTS-TCL NPs and Man-CTS-TCL NPs at days -14, -13, and -7. At day 0, serums of
 11 mice were collection and assayed for tumor specific IgG antibody (humoral immune
 12 response) by ELISA. (B) Serums of mice were used for measuring IL-12p70 (cellular
 13 immune response) by ELISA. Data are representative of three independent

1 experiments. Bars shown are mean \pm SD (n=6), and differences between PBS control
 2 group and other groups are determined using one-way ANOVA analysis and Student's
 3 t test. Relative to control group: * P <0.05, ** P <0.01 and *** P <0.001.



4
 5 **Figure S4 Immunization of mice with Man-CTS-TCL NPs enhances the absolute**
 6 **number of CD3⁺CD8⁺ T cells in mice spleen.** (A) Schedule used for the prophylactic
 7 assay. (B) Number of CD3⁺ T cells (C) and CD3⁺CD8⁺ T cells isolated from spleen
 8 21 days after B16 tumor cells inoculation. Data are representative of three
 9 independent experiments. Bars shown are mean \pm SD (n=6), and differences between
 10 PBS control group and other groups are determined using one-way ANOVA analysis
 11 and Student's t test. Relative to control group: * P <0.05, ** P <0.01 and *** P <0.001.



1

2 **Figure S5 In the therapeutic tumor model, Man-CTS-TCL NPs possesses higher**

3 **level of the absolute number of CD3⁺CD8⁺ T cells in mice spleen.** (A) Schedule

4 used for the therapeutic assay. (B) Number of CD3⁺ T cells (C) and CD3⁺CD8⁺ T cells

5 isolated from spleen 22 days after B16 tumor cells inoculation. Data are

6 representative of three independent experiments. Bars shown are mean \pm SD (n=6),

7 and differences between PBS control group and other groups are determined using

8 one-way ANOVA analysis and Student's t test. Relative to control group: * P <0.05,

9 ** P <0.01 and *** P <0.001.

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