

Immortalized nanodendritic cells decorated with immune-checkpoint inhibitors for personalized cancer immunotherapy

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ABSTRACT

In our study, we have designated artificial nanoDCs (anDCs) by transferring the whole DC membrane onto the gold nanoparticles (Au NPs) to overcome the limitation of DC therapy. The anDCs were optimized by varying membrane-to-substrate ratio, exhibiting similar features with natural DC in having a T cell stimulation and tumor regression even after long-term stability of up to 30 days. This versatile anDCs platform allows engineering immune-boosting agents onto their surface on demand. We further functionalized one of the immune checkpoint inhibitors (ICI), α CTLA-4, on anDCs, leading to complete tumor clearance compared to DC membrane vesicles. Surprisingly, α CTLA-4 conjugated anDC (α CTLA-4-anDC) dramatically blocked the CTLA-4 pathway and resulted in increased frequency and effector cytokine functions in cytotoxic CD8⁺ T cells. Thus, our anDC system can effectively induce a tumor-specific T cell immune response by sustainable and potent T cell priming ability based on the high stability of anDCs, suggesting a great potential platform for next-generation personalized cancer immunotherapy.

1. Introduction

Tumors are derived from the uncontrolled proliferation of host cells, and they can trigger immune responses and escape immunosurveillance to survive by employing diverse mechanisms [1], remaining one of incurable diseases due to their characteristics. To clearly cure tumors, there have been investigated surgery, chemotherapy, radiation therapy, or immunotherapy, and these methods were properly operated on according to the cancer types [2]. Among these, cancer immunotherapy has received enormous attention as a prospective future cancer therapy by harnessing host immune systems to fight cancer with fewer side effects [3,4]. Immunotherapy strategies, including cancer vaccines, dendritic cell (DC) vaccines, NK cell therapy, immune checkpoint therapy, and engineered T cell therapy, have been achieved to prevent cancer by establishing immune memory or directly combat cancers by raising immune responses [5–8]. In particular, DC-based immunotherapy has been highlighted due to the fact that it has clinically potential

therapeutic actions for cancer by means of stimulating T cell activation with specific interaction of major histocompatibility complex (MHC) and co-stimulatory molecules [5]. However, the activated DCs *in vivo* showed a short lifetime and removal before delivering to the lymph node or tumor site [3]. An insufficient number of DCs in the target site revealed poor access to T cell activation, resulting in limited antigen-presenting capabilities in clinical trials [5,9,10]. In addition, the storage and retrieval of DCs with high viability rates are inconvenient and challenging from the manufacturing point of view [11].

Over the last several decades, nanosized particles (NPs), containing liposomes, polymer, and inorganic nanoparticles, have been used as versatile cancer immunotherapy platforms because the NPs can provide sustainable targeting efficiency and versatility for functionalization and incorporation with various therapeutic drugs [12–16]. Compared to conventional cell-based immunotherapy, which involves expensive and complicated manipulation techniques, nanomaterials and NPs can result in a dramatic improvement not only in safety but also in the efficacy of

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cancer immunotherapy by the protection of cargos from degradation *in vivo* and delivering to the target site without damages [4,17]. For example, NPs containing antigens, cytokines, antibodies, mRNA, anti-cancer drugs, immunologic adjuvants, and immune checkpoint inhibitors (ICIs) have been engineered and have exhibited enhanced

tumor regression and prevention effects by employing host immune response [18–26]. P. Huang *et al.* reported nanoparticle for mRNA vaccine which is prepared by condensation of ortho-hydroxy tertiary amine and mRNA in microfluidic system without inflammation side effect [27]. Another example of DC-like therapy, T. Fadel and

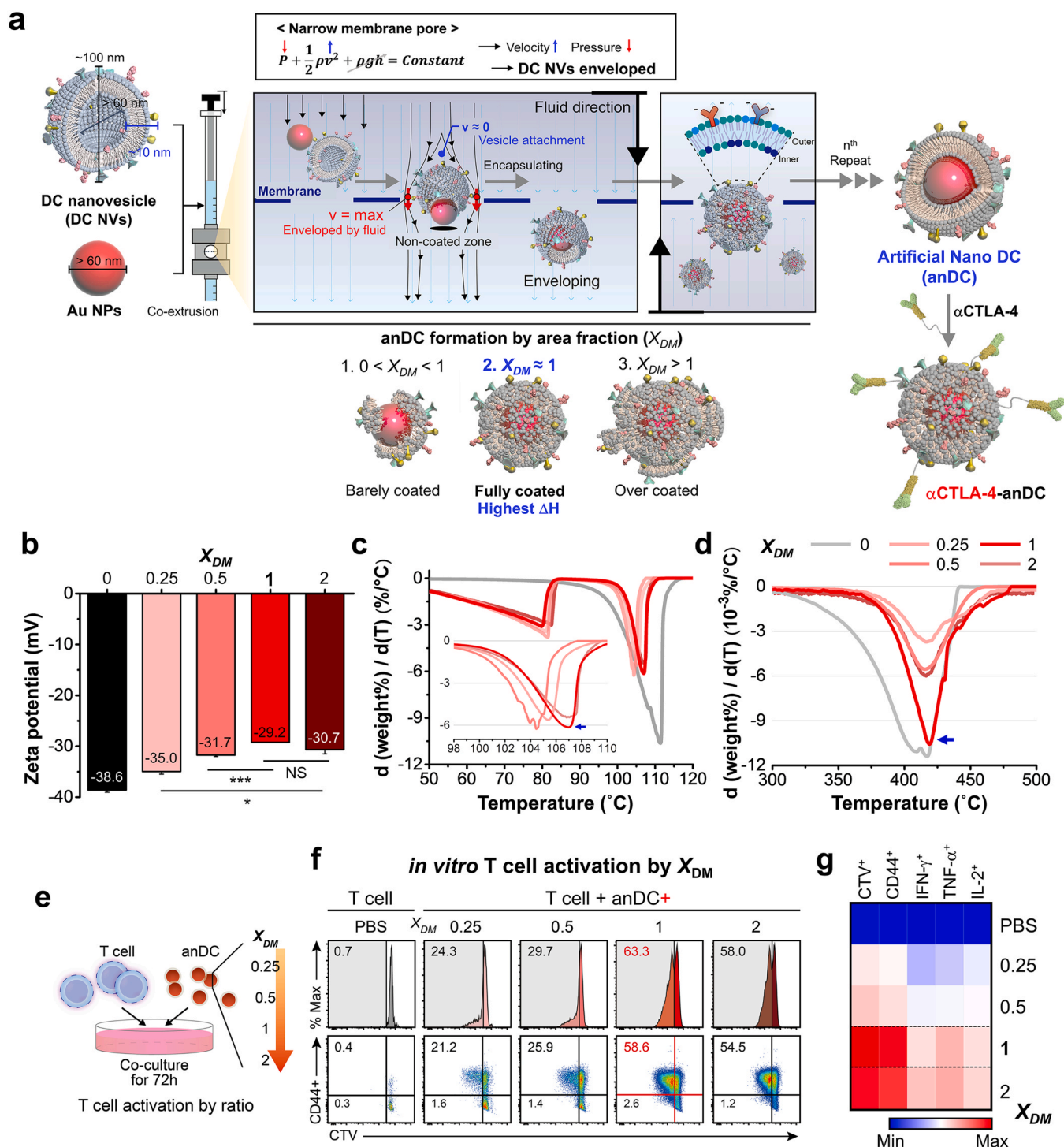


Fig. 1. Physical and biological optimization of anDCs for enhanced thermostability and therapeutic efficacy. (a) Magnified illustration of the overall process and mechanism of DC nanovesicles (NVs) enveloping on the substrate through extrusion to prepare anDCs and $\alpha\text{CTLA-4-anDC}$. (b) Surface zeta potential of anDC. DTG curves of anDCs with different X_{DM} values. (c) The bilayer transition temperature (T_m) and glass transition temperature (T_g) observed at 98–110°C, and (d) the maximum decomposition temperature (T_{dm}) at 350–475°C. The blue arrows indicate the energy changes in anDC when $X_{DM}=1$. (e) Illustration of T cell activation experiment co-culture with anDC by varying X_{DM} . (f) Flow cytometry data showing the proliferation of P14 CD8 $^+$ and CD44 $^+$ T cells and assessed by a CTV dilution assay at different X_{DM} . (g) The frequencies of CTV+, CTV+CD44+, CTV+IFN- γ +, and CTV+TNF- α + in P14 CD8 $^+$ T cells were illustrated by heatmap. The original data is displayed in Fig. S4.

co-workers have developed functionalized carbon nanotubes with MHC-1 and α CD28 for T cell stimuli and IL-2 encapsulated nanocomposites for delaying melanoma growth [19,28]. Thus, the NPs for cancer immunotherapy serve as a promising and powerful approach with high stability and versatile manipulation depending on the purpose of therapies.

Recently, the encapsulation technology of NPs with cell-derived membranes, consisting of extracellular vesicles (EVs), cell membranes, or lysate, has been proposed as an interesting strategy for biomimicking the complexity of living cells in function [25,29–31]. As first reported by L. Zhang *et al.*, the translocation of entire cellular membranes onto various types of NPs without biological damage could deliver natural cellular functionalities on the surface in the nanoscale and is highly beneficial for targeting, stability, and storage [32]. Furthermore, the NP core is responsible for the support and stability of EV or cell membrane structure on the surface [33,34], and the membrane surface could be decorated by functional agents or antibodies to boost immune response. For the sake of cancer immunotherapy, cancer cell membrane or lysate could be coated on NPs, resulting in enhanced host immune systems by cancer antigens [25,31,35]. In addition to cancer cell extracts, bacteria membranes or virus could be incorporated for activation of both innate and adaptive immune systems [36,37]. However, membrane derived from the tumor cells displays immunosuppressive molecules such as PD-L1 or CD47, which can lead to cancer immune evasion by regulation of both innate and adaptive immune response [38–40].

Herein, to directly activate T cells for cancer therapy, we developed an immortal DC-based nanotherapeutic drug that can be generated by directly introducing a mature DC membrane onto NPs for cancer therapy *in vivo*, denoted as artificial nano DCs (anDCs) (Fig. 1a). These anDCs are beneficial as a versatile cancer immunotherapy platform because they do not have the viability, stability, and immunosuppressive issues that are limitations of conventional DC therapies and tumor cell-based NPs. Since the stability and coverage of the DC membrane on the substrate can differ depending on the DC membrane/substrate ratio during the preparation process, we are first aimed at optimization of anDC thermodynamically and biologically by adjusting the optimal DC/substrate ratio. The optimized anDCs clearly localized on the T cells and induce T cell stimulation as like mature BMDCs *in vitro* and *in vivo*. Furthermore, the anDCs did not lose their T cell activation properties even after 30 days of storage at room temperature under lyophilization. By means of functionalization diversity for anDCs, we finally engineered anDCs with α CTLA-4 (α CTLA-4-anDC), the ICI, to boost T cell activation in tumors. Therefore, we found that administration of α CTLA-4-anDC to 3 different cancer types resulted in dramatic tumor suppression with strong tumor-specific cytotoxic CD8⁺ T cell activation. Thus, we developed an optimized therapeutic drug with high stability and tumor suppression using simple top-down nanotechnology for personalized cancer immunotherapy.

2. Experimental section/methods

2.1. Mice

Six- to eight-week-old specific pathogen-free female C57BL/6 (B6) and BALB/c mice were purchased from Orient Bio. LCMV GP_{33–41} epitope-specific T cell receptor transgenic P14 mice were provided by Dr. Rafi Ahmed (Emory University, School of Medicine, Atlanta, GA, USA). Experiments were performed with the approval of the International Animal Care and Use Committee at Yonsei University in accordance with the Laboratory Animal Act of the Korean Ministry of Food and Drug Safety, which improves the ethics and reliability of animal testing through the appropriate administration of laboratory animals and animal testing (Permit No. IACUC-A-202005–1069–03).

2.2. BMDC differentiation and BMDC membrane derivation

BMDCs were differentiated from bone marrow cells isolated from the femurs of naïve C57BL/6 and BALB/c mice (6–8 weeks). Red blood cells (RBCs) from the bone marrow were lysed using ACK lysis buffer (Gibco Laboratories). Single-cell suspensions after RBC lysis were cultured in RPMI (Corning) supplemented with 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific), 1% penicillin/streptomycin (P/S) (Thermo Fisher Scientific), and 20 ng/ml recombinant murine GM-CSF (JW Creagene). The culture media were replenished on day 3 and 6. For the maturation of BMDCs, 20 ng/ml LPS (Sigma Aldrich) was added to the culture media for 6 days. Seven days post culture, nonadherent cells were collected and pelleted by centrifugation at 2000 rpm for 5 min at room temperature. Next, the BMDCs were resuspended in 1 × PBS (pH 7.4) containing an EDTA-free Pierce™ protease inhibitor mini tablet (PIT) (Sigma Aldrich, 1 tablet per 10 ml of solution). The BMDC membrane was derived from 1 ml aliquots of a BMDC solution containing 1–2 × 10⁶ cells through three freeze (–70°C)–thaw (room temperature) cycles to break the cell membrane. Aliquots of BMDC suspensions were pelleted by centrifugation at 6000 rpm for 5 min and washed three times with a 1 × PBS solution containing PIT to obtain pure cell membranes. The pelleted BMDC membranes were resuspended in 1 × PBS solution and sonicated at 20% amplitude 60 times (sonication for 3 s, intervals of 2 s on ice, ultrasonic processor, VC-505; Sonics & Materials) for 3 min in a capped glass vial.

2.3. Preparation of anDCs

BMDC membrane vesicles were sequentially extruded through 1 μ m, 400 nm, and 100 nm polycarbonate membrane filters (Whatman) for 12 successive passes in a mechanical mini-extruder (Avanti). Afterward, to form cell membrane-enveloped Au NPs, 100-nm BMDC membrane vesicles suspensions and 60-nm Au NPs were mixed at appropriate ratios in a 1-ml syringe (Hamilton) and extruded through a 100-nm membrane filter in the same manner as described above. The prepared anDCs were stored at –20°C and 4°C with lyophilized powder until use.

2.4. Peptide pulsing for T cell activation

For peptide pulsing, BMDCs or DC NVs, and anDCs were incubated with various MHC class I-restricted peptides, including GP_{33–41} (0.2 μ g/ml), p15E (5 μ g/ml), AH1 (5 μ g/ml), E6 (5 μ g/ml), and E7 (5 μ g/ml), for 30 min at 37°C in RPMI supplemented with 10% FBS-supplemented RPMI. The following peptides were used: GP_{33–41} peptide, H-2D^b-restricted dominant CD8⁺ T cell epitope of lymphocytic choriomeningitis virus (LCMV); p15E peptide, H-2 K^b-restricted CD8⁺ T cell epitope of H-2^b haplotype tumors; AH1 peptide, H-2 L^d-restricted CD8⁺ T cell epitope of H-2^d haplotype tumor; E6 peptide, H-2 K^b-restricted CD8⁺ T cell epitope of human papillomavirus E6 protein; and, E7 peptide, H-2D^b-restricted CD8⁺ T cell epitope of human papillomavirus E7 protein. After 30 min, each peptide-pulsed cell was washed and used for various experiments. For the *in vivo* T cell proliferation experiment, 1 × 10⁶ peptide pulsed BMDCs or anDCs were intravenously injected into recipient mice. For *in vivo* tumor experiments, 1 × 10⁶ peptide-pulsed BMDCs, DC NVs, or anDCs were subcutaneously injected into tumor-bearing mice 5 and 10 days after tumor inoculation.

2.5. Optimization and characterization of anDCs

To optimize the surface-coating coverage, anDCs were fabricated in water or 1 × PBS with membrane-to-nanocore surface area ratios of 0, 0.25, 0.5, 1, and 2. The surface morphology was analyzed via SEM (7610 F-plus, JEOL). The surface zeta potential and hydrodynamic size of the anDCs were determined using DLS (SZ-100, Horiba). To confirm the distribution of DC membranes on particles, 1 μ m PS microbeads (Sigma-Aldrich) were used as templates for enveloping DC vesicles.

Before coating, the membrane was stained with DiD (Thermo Fisher Scientific) for 30 min, followed by washing step. The DiD-stained membrane area around the particles was measured by confocal microscopy (Carl Zeiss). For immunostaining of the DC surface molecules, fluorochrome-labeled antibodies against CD80, CD86, MHC class I, and MHC class II (eBioscience) were used. The anDCs were blocked with 1% bovine serum albumin in PBS solution for 1 h and sequentially stained with anti-antibodies for 45 min at room temperature. After washing with $1\times$ PBS, flow cytometry was performed. The amount of cell membrane coated on the nanosubstrate was analyzed using photoluminescence (PL, FP-8300, JASCO) intensity. Thermostability was measured using a simultaneous DSC/TGA analyzer (SDT 650, TA instruments) with a programmed temperature increment of $10^\circ\text{C}/\text{min}$ from 30°C to 1000°C under N_2 atmosphere. The samples were analyzed in 80 μL pans, and the data were obtained using TA Data Analysis software. The photoabsorption properties of the Au NPs, anDCs ($X_{\text{DM}}=1$), and anDCs pulsed with the GP₃₃₋₄₁ peptide were measured using a UV-vis spectrophotometer (Evolution 300, Thermo Fisher Scientific) at room temperature. The total protein amounts and contents of DC ghosts (before/after sonication), DC NVs, and anDCs were examined determined using a BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. and SDS-PAGE, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to characterize the protein composition and was imaged using Coomassie blue staining.

2.6. CD8⁺ T cell purification and adoptive cell transfer

For CD8⁺ T cell enrichment, CD8⁺ T cells were isolated from the spleens of wild-type P14 mice using the MagniSort Mouse CD8⁺ T cell Enrichment Kit (Thermo Fisher Scientific). For the adoptive transfer of P14 CD8⁺ T cells (Thy1.1/Thy1.1), the cells were isolated from the spleens of naïve P14 mice after CD8⁺ T cell enrichment. After isolation, 5×10^6 purified P14 CD8⁺ T cells were transferred into wild-type C57BL/6 mice via the tail vein.

2.7. In vitro T cell and DC co-culture assays

P14 CD8⁺ T cells were isolated from the spleen of wild-type P14 mice. P14 CD8⁺ T cells were labeled with 2.5 μM CTV (Life Technologies) for 30 min at 37°C and washed with RPMI containing 10% FBS. A total of 5×10^4 labeled T cells were co-cultured in RPMI containing 10% FBS with 1×10^4 BMDCs, DC NVs, $\alpha\text{CTLA-4}$ -DC NVs, anDCs, or $\alpha\text{CTLA-4}$ -anDCs. After 72 h, the cells were harvested, stained, and analyzed using flow cytometry. The colocalization of anDCs and T cells was evaluated using confocal microscopy (Carl Zeiss). After 72 h of co-culture of DiD-stained anDCs and P14 CD8⁺ T cells, unbound anDCs were removed by centrifugation at 2500 rpm for 2 min. P14 CD8⁺ T cells were stained with DAPI (Sigma Aldrich) and FITC-labeled anti-CD8 (BD Biosciences) for 45 min after blocking for visualization.

2.8. Stability test of anDCs

In order to compare the effect of lyophilization on DC NVs and anDC formation, the morphologies of before and after lyophilization were visualized using corrected scanning transmission electron microscopy (Cs-TEM, JEM-ARM-200 F(NEOARM), JEOL). All the measurements were performed using water as the solvent. Polystyrene (PS) microparticles of 800 nm size (Sigma-Aldrich) were used to verify that the cell membrane stably maintained the vesicle formation when the core particles were selectively removed in a situation where the cell membrane was coated on the core particle. The composite DC membrane-coated PS particles were prepared in the equal method as the anDCs were and are abbreviated PS-DCs. The PS cores were selectively extracted from PS-DCs using tetrahydrofuran (THF, Sigma-Aldrich) and observed through Cs-TEM.

A stability test was conducted to confirm whether anDCs maintained their biological functionality even after storage. After preparing the anDCs, the samples were stored for 10, 20, or 30 days as a dispersion in $1\times$ PBS, pellets, or lyophilized powder. Dispersed and pelleted anDCs were stored at 4°C and -20°C , respectively, and the lyophilized powder was stored at room temperature. On the 30th day of storage, BMDCs and anDCs were prepared using the same method and compared as positive controls. Peptide pulsing and *in vitro* T cell co-culture assays were performed in the same manner as described above.

2.9. Surface modification of anDCs

CTLA-4 (CD152) monoclonal antibody (mAb) ($\alpha\text{CTLA-4}$, BioXCell) was coupled to plain anDCs as described [41]. Briefly, for 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (sodium salt) (DSPE-PEG (2000)-COOH, Avanti) conjugated with $\alpha\text{CTLA-4}$, 67.5 μL of 0.25 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC, Alfa Aesar) and 67.5 μL of 0.25 M *N*-hydroxysuccinimide (NHS, Sigma-Aldrich) were mixed with 28.4 μL of MES buffer solution (10 mM MES/150 mM NaCl, pH 5) containing DSPE-PEG (2000)-COOH (10 mg/ml) and kept at room temperature for 30 min. Residual EDC and NHS were removed by dialysis for 3 days. The mixture was then incubated with the desired amount of $\alpha\text{CTLA-4}$ (500 μg) at 4°C overnight with gentle stirring. Fourier transform infrared spectroscopy (FT-IR, FT/IR-4700, Jasco) and Raman spectroscopy (XploRA™ PLUS, HORIBA) were performed for qualitative analysis to verify that DSPE-PEG (2000)- $\alpha\text{CTLA-4}$ was properly synthesized. The molecular weights of DSPE-PEG (2000)-COOH, DSPE-PEG (2000)-COO-succinimide, $\alpha\text{CTLA-4}$, and DSPE-PEG (2000)-CONH- $\alpha\text{CTLA-4}$ were quantitatively measured using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (Autoflex Max, Bruker) with 3,5-dimethoxy-4-hydroxycinnamic acid and 4-hydroxy-3,5-dimethoxy-cinnamic acid (Sigma Aldrich) as the matrix. The mixture of DSPE-PEG (2000)- $\alpha\text{CTLA-4}$ and anDCs was extruded through a 100-nm membrane filter in the same manner as described above and sonicated for 3 min using a bath sonicator. To examine morphology, DC NVs, Au NPs, anDCs, and $\alpha\text{CTLA-4}$ functionalized anDCs ($\alpha\text{CTLA-4}$ -anDCs) were placed on a carbon-coated grid and visualized using energy-filtering TEM (EF-TEM, LIBRA 120, Carl Zeiss). The hydrodynamic size and surface morphologies of the anDCs and $\alpha\text{CTLA-4}$ -anDCs were analyzed by DLS (SZ-100, Horiba) measurements and AFM (NX10, Park Systems), respectively. To confirm the $\alpha\text{CTLA-4}$ insertion, PE-labeled $\alpha\text{CTLA-4}$ (BD Biosciences) was used.

2.10. Mouse tumor model

MC38 cells were obtained from Seung-Woo Lee's laboratory (POSTECH, Seoul, Korea). TC-1-luciferase cells were obtained from Hye Ryun Kim's Laboratory (Yonsei University, Seoul, Korea). These two cell lines were cultured in RPMI (Corning) supplemented with 10% FBS and 1% FBS at 37°C in a 5% CO_2 incubator. MC38 and CT26 cells (5×10^5) in PBS were subcutaneously injected into age-matched (6–8 weeks) C57BL/6 and BALB/c mice, respectively. Tumor sizes were measured at the indicated time points using a caliper, and the following formula was used for all calculations: $1/2 \times (\text{length} \times \text{width}^2)$. Tumors larger than 2000 mm^3 in size were considered dead. TC-1-luciferase cells (5×10^5) in PBS were intravenously injected into age-matched (6–8 weeks) C57BL/6 mice via tail vein.

2.11. Imaging of Luciferase activity

Imaging was performed using an IVIS imaging system (IVIS Spectrum, Perkin Elmer). Xenolight D-luciferin (Perkin Elmer, 122799) was injected at 150 mg/kg intraperitoneally into mice. After 10 min, TC-1-luciferase-bearing mice were anesthetized with isoflurane and placed

in a supine position in the chamber. Photons were quantified using an IVIS imaging system for 1–3 min. Starting after 6 days, the presence of tumors was observed every 4 days, and the number of tumor nodules on lungs was counted on day 18. Later and every 4 days thereafter, the area was observed and palpated for the presence of a tumor nodule. To detect anDC in tumor model, we labeled anDC with the fluorescent dye DiR (Thermo Fisher Scientific). After 5 days post CT26 tumor inoculation, we subcutaneously or intravenously injected DiR-labeled anDC and then analyzed anDC distribution by IVIS imaging at indicated time points.

2.12. Tumor harvest

Tumors were harvested at the indicated time points and homogenized into small pieces before digestion with 1 mg/ml collagenase type VI (Worthington Biochemical Corporation) for 30 min at 37 °C to obtain single-cell suspensions.

2.13. Antibodies and flow cytometry analysis

For the analysis of lymphocytes, cells were plated in 96-well plates (round bottom) in PBS supplemented with 2% FBS and stained with fluorochrome-conjugated antibodies for 20 min at 4 °C. Fluorochrome-conjugated antibodies against CD90.1 (OX-7), IFN- γ (XMG1.2), IL-2 (JES6-5H4), CD80 (16-10A1), and MHCI (AF6-88.5) were obtained from BD Biosciences; antibodies against CD8 (53-6.7), FOXP3 (FJK-16 s), CD25 (PC61.5), CD44 (IM7), CD86 (GL1), and MHC II (M5/114/15/2) were obtained from Thermo Fisher Scientific. The antibodies against CD4 (RM4-5), PD-1 (29 F.1A12), Tim-3 (RMT3-23), Ki-67 (16A8), granzyme B (GB11), and TNF- α (MP6-XT22) were obtained from Biolegend. For intracellular staining, antibodies against TNF- α (MP6-XT22) and IFN- γ (XMG1.2) were used in combination with anti-CD8 antibodies. Dead cell populations were removed using a Live/Dead fixable stain kit (Thermo Fisher Scientific).

To detect intracellular cytokines in T cells, cells were incubated with the GP₃₃₋₄₁ peptide, OVA₂₅₇₋₂₆₄ peptide, p15E (KSPWFTTL) peptide, or PMA-ionomycin. For GP₃₃₋₄₁, OVA₂₅₇₋₂₆₄, or p15E-specific T cell stimulation, T cells were restimulated with GP₃₃₋₄₁ (0.2 μ g/ml), OVA₂₅₇₋₂₆₄ (0.2 μ g/ml), or p15E (5 μ g/ml) for 6 h in the presence of Golgi plug/Golgi Stop (BD Biosciences). All peptides were synthesized using the GenScript software. For polyclonal T cell stimulation, T cells were restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of Golgi plug/Golgi stop (BD Biosciences). Intracellular staining was performed after surface staining using the BD Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences) or Foxp3/ transcription factor staining buffer set (Invitrogen), according to the manufacturer's instructions.

For characterization of antibodies (CD80, CD86, MHCI, and MHCII) on anDCs, the anDCs were stained according to the manufacturer's protocol, and flow cytometry was performed as described previously. Owing to the nanometer scale of anDCs, the anDCs could aggregate more than 2–3 particles together during the staining process. After the measurement, the aggregated anDCs (size > 300 nm) were detected and gated to check the antibody levels.

2.14. Quantification and statistical analysis

All stained samples were analyzed on a FACS Canto II instrument (BD Biosciences) or CytoFLEX LX (Beckman Coulter) using FlowJo software (Tree Star). Statistical analysis was performed using Prism software version 7.0 (GraphPad). Comparisons between multiple groups were performed using one-way ANOVA with Tukey's *post hoc* test. Kaplan-Meier survival curves were analyzed using the Mantel-Cox log-rank test with a 95% confidence interval. Statistical details, including the statistical test, the exact value of *n*, precision measure, and statistical significance, are reported in the figures and figure legends.

3. Results and discussions

3.1. Preparation of optimal anDC depending on DC: substrate ratio changes

anDCs were generated by the co-assembly of the DC NVs, which was extracted from matured DC, and nanoparticles in a fluidic system (Fig. 1a). We assumed that the DC NVs could be coated on the substrate (NPs) when the DC vesicles and substrates passed through the filter membrane together. To confirm the fluid structure and rate of envelopment of NPs in the pores, we performed computational fluid dynamics modeling by means of the COMSOL program with 2-dimensional axis asymmetric model, as shown in Figures S1 and S2 (a detailed modeling design is summarized in the supplementary information). Figure S1 indicates the modeling design and parameters to observe fluid flow when the 60-nm size NP passes through the 100-nm pore membrane. For simplification, the NP and membrane pore are assigned as a wall, and the fluid information was set as similar to the cell suspension solution. As shown in Figure S2, the simulation result, the cross-sectional fluid dynamics results revealed that the fluid velocity reaches maximum through the choked section (membrane pore) and decreases to 0 at the NP surface by the Venturi effect, as also described Bernoulli's Equation in Fig. 1a. In a narrow pore membrane, the maximum velocity near the NP surface occurs when the NP passes across the pore, allowing the NP to be smoothly coated with a DC membrane. Therefore, according to the simulation results, the process could be conducted during the first enveloping process in the following order: (1) the DC vesicle and substrate are moved to the membrane pore via fluid flow, and the DC vesicle approaches and attaches to the NP surface by electrostatic interaction in the area where the velocity is 0. (2) DC vesicles spread and fuse with NPs as the fluid flow and (3) envelope both ends with a rapid fluid rate ($v=\max$) (Enlarged fluid illustration in Fig. 1a). At this enveloping process, phospholipid and glycoprotein deposition on the outer leaflet and inner leaflet of membrane bilayers is asymmetric, the relatively positively charged inner leaflet tends to be attached to the negatively charged substrate, resulting in a right-side-out orientation. Therefore, an additional repeated envelopment process could increase the coverage of the anDCs. The finally produced anDC has a different coverage rate depending on the surface area fraction of particles to DC vesicles (X_{DM}) (Fig. 1a). The DC membrane surface area fraction (X_{DM}), which represents the area ratio of the DC membrane to the substrate, can be one of the main factors responsible for surface coverage and efficacy (Figure S3). To obtain high-quality anDCs with high coverage and thermodynamic stability, we prepared anDCs with different X_{DM} values. Both DCs and particles are considered spherical in shape; we calculated and estimated the DC membrane area fraction relative to that of the substrate. Prior to our demonstration, we believed that an anDC having thermodynamically stable and DC-like functions can be obtained when X_{DM} is approximately 1. The morphology of the final products, which depends on different X_{DM} values (0–2), was measured by scanning electron microscopy (SEM), revealing that DC vesicles were likely to be overcoated at $X_{DM} > 1$ (see the yellow area for $X_{DM}=2$) (Figure S3). The surface coverage tended to increase with a high X_{DM} . In contrast, the surface potential values decreased because more DC membranes with low zeta potential (-28.95 mV) were enveloped (Fig. 1b). Photoluminescence (PL) measurements also demonstrated DC vesicle coverage on the substrate after treatment with Nile red, which can exhibit fluorescence when combined with hydrophobic materials such as the cell membrane. The inset graph shows a linear increase in the fluorescence intensity depending on X_{DM} in the 650–700 nm range (Figure S4).

To measure the thermal stability of anDCs at different X_{DM} values, we confirmed the results using thermogravimetry (TG) analysis. Fig. 1c shows derivative thermogravimetry (DTG) curves in the range of 40 °C to 120 °C for the pure PS and cell membrane-enveloped PS nanocomposites under investigation. In this temperature range, TG (shown in

Figure S5a) and DTG curves of all samples except pure PS ($X_{DM}=0$) indicated that the degradation proceeded via two steps—degradation of the (1) cell membrane (occurred around 80 °C) and (2) pure PS, respectively. An enlargement of the DTG spectra for the second degradation step is depicted as an inset in Fig. 1c to clearly show the peak positions and their shifts. Differential scanning calorimetry (DSC) thermograms verified that the endothermic peak changed with coverage (Figure S5b). This indicates that membranes have been coated on the surface of PS, and the variation in the peak is associated with the degree of coating. It is well known that the endothermic peak of PS varies depending on the degree of surface coating [42]. Table S1 is a summary of transition temperatures for all different X_{DM} and glass transition temperature (T_g) of the membrane-coated PS with different X_{DM} values is highest at 1 except pure PS. These results indicate that heat energy absorbed by the particles manufactured at $X_{DM}=1$ increased, whereas particles with higher or lower X_{DM} values absorbed only a small amount

of heat energy to reach the glass transition temperature. However, the lipid bilayer phase transition temperature (T_m ; see Figs. 1c, 5b and Table S1) and the remaining weight % after degradation (see Figure S5c) were the lowest when $X_{DM}=1$ because the DC membrane was fully enveloped on the PS particles. To maintain the curvature of the cell membrane, phosphatidylethanolamine (PE) should be the major ingredient in the inner leaflet of the lipid bilayer. PE has *cis* double bonds, resulting in a further decrease in T_m [43]. Furthermore, the residual weight % decreased when the particle was thermally stable. Fig. 1d shows the maximum decomposition temperature (T_{dm}), and Figure S5d indicates that the thermal stability of the cell membrane-enveloped PS is higher than that of the pure PS sample (See Table S1). This is consistent with the thermal stability characteristics of PS reported previously, indicating that maximizing the coverage of DC membranes can enhance thermal stability [42]. Thus, at moderate X_{DM} values and an area fraction of one, the cell membrane is fully coated on the substrate NP

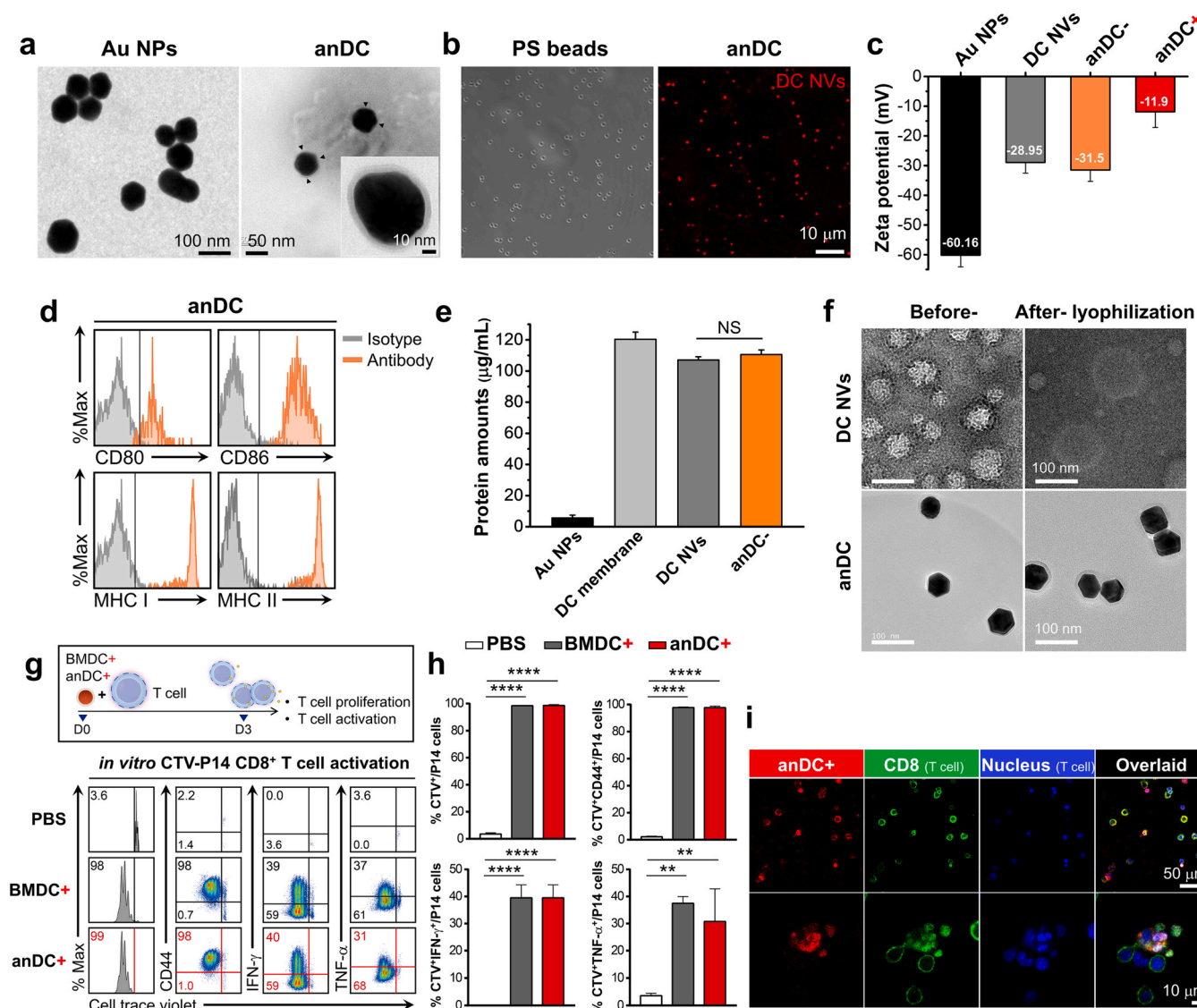


Fig. 2. Characterization of optimized anDC. (a) TEM images of bare Au NPs and anDC. (b) Confocal microscopic images of anDC prepared by 1 mm microbeads. Red fluorescence is DiD-tagged DC membrane. (c) Surface zeta potential of Au NPs, DC membrane, anDCs and antigen-pulsed anDCs. (d) Flow cytometry analysis of the expression of DC-specific surface antigens (CD80, CD86, MHC class I, and MHC class II) on aggregated anDCs. (e) Protein amount in each material measured by a BCA assay. (f) TEM images before and after lyophilization of bare DC NVs and anDCs. (g) The experimental scheme of the co-culture assay in vitro. Flow cytometry data showing the proliferation of P14 CD8⁺ T cells assessed by a CTV dilution assay. The frequencies of CTV⁺, CTV⁺CD44⁺, CTV⁺IFN- γ ⁺, and CTV⁺TNF- α ⁺ in P14 CD8⁺ T cells were analyzed by flow cytometry. The numbers in the plots indicate the percentages of CTV⁺, CTV⁺CD44⁺ or CTV⁺CD44⁺, CTV⁺IFN- γ ⁺ or CTV⁺IFN- γ ⁺, CTV⁺TNF- α ⁺ or CTV⁺TNF- α ⁺ in the P14 CD8⁺ T cell populations. (h) The frequencies of CTV⁺, CTV⁺CD44⁺, CTV⁺IFN- γ ⁺, and CTV⁺TNF- α ⁺ in P14 CD8⁺ T cells are summarized in the bar graph. (i) Fluorescence images of colocalization of anDCs (DiD; red) around T cells (CD8⁺ and DAPI; green and blue) after 72 h of co-culture.

surface, similar to the membrane structure of live DCs, and the highest thermal stability can be inferred.

Through the physicochemical data, it was confirmed that anDC was formed most stably when $X_{DM}=1$. Therefore, we tried to confirm the degree of T cell activation according to the anDC ratio (Fig. 1e). The therapeutic efficacy of anDCs at different X_{DM} values was also validated by *in vitro* T cell activation. The P14 CD8⁺ T cells (specific to the LCMV GP₃₃₋₄₁ epitope) labeled with Cell Trace Violet (CTV), which can figure out the proliferation index of cell, were co-cultured with anDCs for 72 h. The strongest T cell activation was observed at $X_{DM}=1$, which showed the highest T cell proliferation index in terms of CTV (Fig. 1f) coupled with activation marker (CD44) expression, and production of effector cytokines (IFN- γ , TNF- α , and IL-2) as shown in the heatmap (Fig. 1g) and flow cytometry raw data (Figure S6). This is because a DC membrane with high coverage can stabilize anDCs with strong interactions between lipids under optimal conditions, which can offer thermodynamic stability and strong priming activity for antigen-specific T cells. If the membranes are not fully coated overall ($X_{DM}<1$), they may detach in the media environment and fail to function properly. Similarly, if the membranes are over-coated ($X_{DM}>1$), the outermost membranes are more likely to be isolated easily, which could affect the membranes attached to the particle surface. Therefore, it can be anticipated that optimal and continuous antigen presentation could be achieved when $X_{DM}=1$, and the results indeed corroborated this expectation.

3.2. Characterization of anDCs at $X_{DM}=1$

By applying an optimal DC membrane coating ratio, bare Au NPs and anDCs were prepared and measured by transmission electron microscopy (TEM) (Fig. 2a), confirming that the 60–80 nm-sized Au NPs were successfully covered with a DC membrane with a thickness of 5–7 nm. When the fluorescent dye DiD was used to stain the DC NVs followed by co-assembly with PS particles, the fluorescence image of the anDCs confirmed that the DC vesicles were located on the surface of the particles (Fig. 2b). The redshift in the UV–vis absorbance spectrum of anDC compared with that of bare Au NPs similarly demonstrated that DC vesicles were successfully assembled onto the NPs (Figure S7). In addition, the surface net charge of anDC dramatically decreased to -31.5 mV, similar to the value of the DC membrane (Fig. 2c). After pulsing anDC with the GP₃₃₋₄₁ peptide, which is composed of 9 amino acids, the surface charge potential decreased due to the net positive charge of the antigen.

Since DC vesicles are possibly coated in the right-side-out direction [29], we expected that the surface proteins and conjugated antibodies would be deposited in an outward fashion on the anDCs. Indeed, the staining of anDCs with fluorochrome-labeled mAbs against CD80, CD86, MHC class I, and MHC class II, which are major surface molecules of DCs, showed that DC surface molecules existed stably outside the anDCs (Fig. 2d). The SDS-PAGE (Figure S8) and BCA assays (Fig. 2e) revealed that the number and molecular weight distribution of proteins were maintained even after the preparation of anDCs, suggesting that protein loss and denaturation occur less frequently during the anDC preparation. After lyophilization of anDC, the morphological changes were negligible, compared to those of the DC NVs that underwent size changes (Fig. 2f). Furthermore, DC membrane-coated PS particles were prepared to confirm whether the cell membrane maintains its vesicle formation even after selective etching of the core particles (Figure S9a). As a result of treating DC-coated PS particles with tetrahydrofuran (THF), only the PS cores were selectively extracted from them (Figure S9b). Accordingly, even if the core particles are removed or extracted, anDCs can maintain the antigen presentation function as DC NVs. This reveals that the core NPs in anDCs could play a role in support of the DC NVs to rigidly exist on the core surface.

To validate the effect of anDCs on the activation of antigen-specific T cells *in vitro*, P14 CD8⁺ T cells were co-cultured with GP₃₃₋₄₁ peptide-pulsed anDCs (anDC+) or GP₃₃₋₄₁ peptide-pulsed bone-marrow-

derived dendritic cell (BMDC+) for 72 h. Then, we compared the levels of proliferation and activation of P14 CD8⁺ T cells by tracking the CTV dilution and analyzing the expression of surface CD44 and intracellular effector cytokines (Fig. 2g). Co-cultured P14 CD8⁺ T cells exhibited a significant proliferation index only when anDCs or BMDC were pulsed with the GP₃₃₋₄₁ peptide (Fig. 2h). Additionally, proliferating P14 CD8⁺ T cells displayed rapid expression of the T cell activation marker CD44 and robust production of the effector T cell cytokines IFN- γ and TNF- α . Notably, the ability of anDCs to stimulate T cell proliferation and activation was comparable to that of BMDCs. This observation suggests that anDCs derived from BMDCs maintain the original function of DCs in terms of antigen-specific CD8⁺ T cell activation. Furthermore, immunohistochemical analysis confirmed that anDCs pulsed with the GP₃₃₋₄₁ peptide (anDC+) were in close physical contact with P14 CD8⁺ T cells (Fig. 2i). Owing to their close physical contact, anDCs might efficiently augment the proliferation and function of antigen-specific CD8⁺ T cells *in vitro*.

3.3. Storage stability of optimized anDCs

Although the BMDCs are effective for T cell activation, there are major barriers to limiting the efficacy of DC-based therapy. First, the DC lifetime is short *in vivo*, which cannot induce sufficient CD8⁺ T cell activation to control the tumor. Furthermore, live BMDCs are inconvenient for storage, and their viability and functions could be affected by long-term storage in low temperatures [11]. On the other hand, anDCs can be employed as an immunotherapy agent even after storage, as long as surface proteins on the DC vesicles remain intact. To demonstrate the stability of anDCs after long-term storage, we prepared anDCs and treated them with lyophilization (L), suspended them in $1 \times$ PBS (S), and centrifuged them to produce pellets (P). Then, each anDC was stored at 25°C (L) and -20°C , 4°C (S and P) for 10, 20, or 30 days (Fig. 3a). On the specified days, stored anDCs and freshly prepared anDCs or BMDCs were co-cultured with P14 CD8⁺ T cells to determine whether their activation potential was retained, respectively. Figs. 3b and 3c show the percentages of proliferating and activated T cells and population of CD44⁺ in T cells, respectively. In comparing fresh anDCs and BMDCs, L- and S-state anDCs maintained their T cell proliferating potential even after 30 days of storage (Fig. 3b). However, the T cell proliferation capacity of anDCs in the P state gradually decreased with storage time. The cytokine release trend showed as a heatmap for stored and fresh anDCs, with a reddish heatmap color indicating more expression and release level for their targets (Figs. 3d–3g). Interestingly, long-term stored L- and S-anDC+ revealed better T cell activation potential compared to the BMDC+, which means no physical and biological changes on anDC throughout the long-term storage. The fresh, L-, and S-anDCs induced T cells to release IFN- γ and TNF- α , whereas P-anDCs stimulated the release of IL-2 rather than IFN- γ . These trends may be attributed to changes in cytokine concentration over time after T cell activation [44], IL-2 releases relatively earlier time point in inflammation than others. Consequently, faster T cell activation appeared to occur immediately after T cell co-culture for fresh and L- and S-anDCs than for P-anDCs. This result implies that differences in the storage method can determine the stability of anDCs. Gravity and centrifugation forces may induce lipid-lipid interactions between anDCs, which can delay and mitigate antigen presentation to T cells and their differentiation. However, lyophilized samples, similar to fresh samples, could be stored in a stable state, resulting in an instant T cell activation response even after 30 days of storage. Therefore, different from the inconvenient storage process of BMDCs, which requires ultra-low temperatures under a static environment, the anDCs enable quality control with simple long-term storage.

3.4. *in vivo* ability of anDCs to activate antigen-specific T cells and inhibit tumor growth

Next, we investigated the ability of anDCs to induce antigen-specific

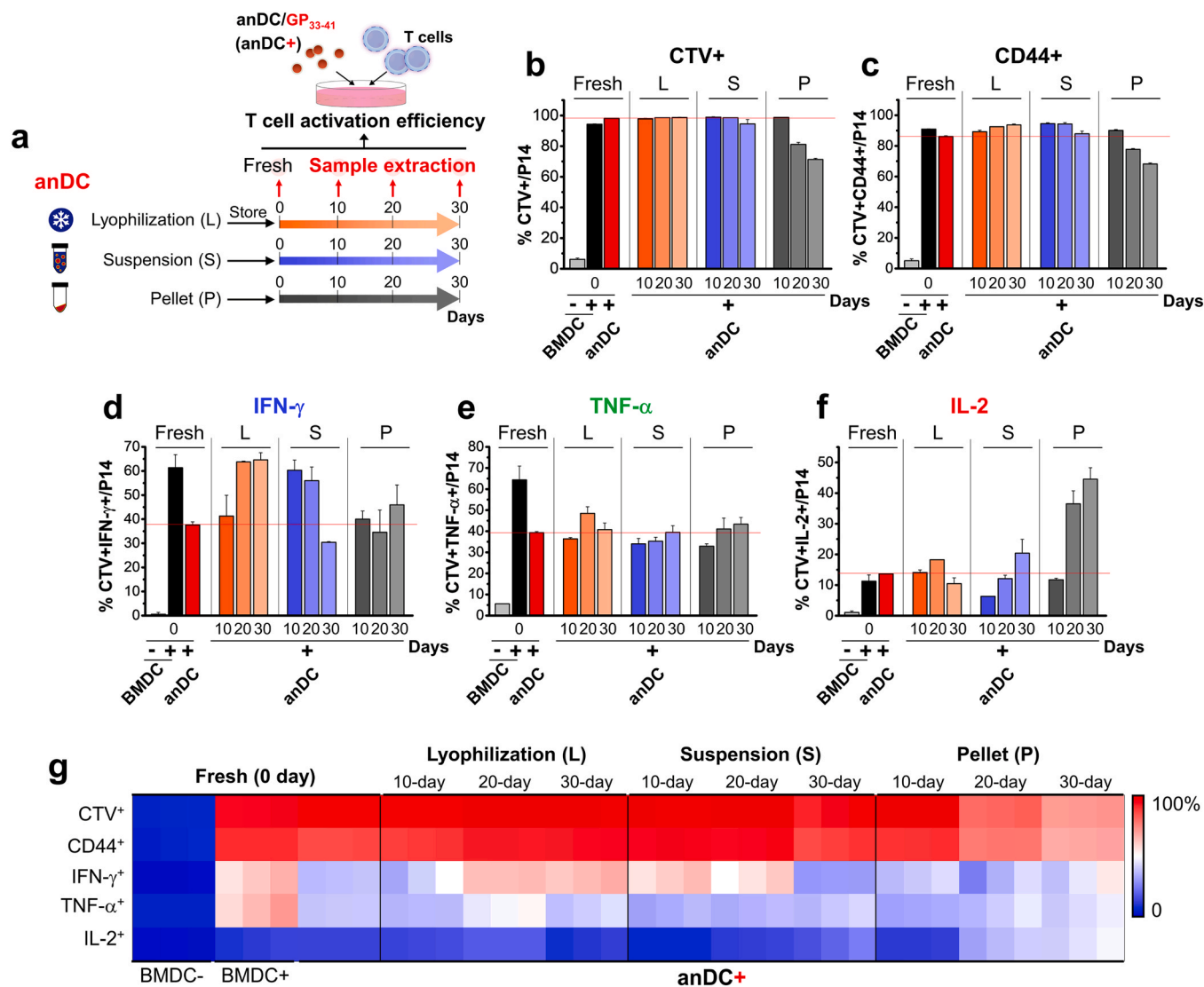


Fig. 3. Long-term storage stability of anDC. (a) Experimental illustration of the storage stability assay for anDCs. Three different storage processes (lyophilization (L), suspension (S), and pellet (P)) were assessed for the T cell activation potential. (b–f) Graphs showing the proliferation and activation of P14 CD8⁺ T cells according to the flow cytometry results. (g) overall T cell activation results from fresh anDCs or stored anDCs illustrated by heatmap.

T cell response *in vivo*. We isolated CD8⁺ T cells from P14 Thy1.1 mice and labeled them with CTV. CTV-labeled P14 Thy1.1⁺CD8⁺ T cells were adoptively transferred to Thy1.2⁺ naïve mice. Twenty-four hours after adoptive T cell transfer, GP₃₃₋₄₁ pulsed BMDC⁺ or anDC⁺ was transferred to Thy1.2⁺ naïve mice, and the transferred P14 Thy1.1⁺CD8⁺ T cell in the spleen were analyzed 48 h post-DC transfer. This procedure allowed us to track the proliferation and function of exogenously transferred donor T cells (P14 Thy1.1⁺CD8⁺ T cells) separately from the endogenous T cells (Thy1.2⁺CD8⁺ T cells) in recipient mice (Fig. 4a and Figure S10). Consistent with *in vitro* data (Fig. 2g), when the mice were injected with BMDC⁺ or anDC⁺, both P14 CD8⁺ T cells exhibited substantial levels of *in vivo* proliferation; however, anDCs induced higher levels of T cell proliferation than BMDCs (~74%) in Fig. 4b and S11. Interestingly, the frequencies of cytokine-producing P14 CD8⁺ T cells among CTV⁺ cells were higher in the BMDC co-culture than in the anDC co-culture. This result indicates that anDCs, compared to BMDCs, can induce sufficient T cell proliferation *in vivo* but cannot further stimulate T cell differentiation, resulting in relatively low levels of T cell effector cytokines.

Before validation of effect to antitumor response of anDC, we monitored whether anDC can reach and persist well in tumor

microenvironment. First, we subcutaneously inoculated syngeneic CT26 tumor cells, murine colorectal carcinoma, to mouse. At 5 days after tumor inoculation, we subcutaneously (s.c.) or intravenously (i.v.) injected near-infrared fluorescent cyanin dye (DiR)-labeled anDC pulsed with AH-1 peptide (anDC⁺), a H-2 L^d-restricted CD8⁺ T cell epitope expressed from CT26 tumor (Fig. 4c). Then, we analyzed distribution of DiR-labeled anDC⁺ in multiple organs of tumor-inoculated mice over time. The anDC was well detected in multiple organs including tumor in both groups for 72 h (Fig. 4d). Interestingly, when we injected anDC subcutaneously, we found it remained better on tumor than when we intravenously delivered anDC *in vivo*. Otherwise, anDC was more accumulated in spleen, liver and lymph node rather than tumor when mice were intravenously injected (Fig. 4d). Therefore, it is suggested that the subcutaneous delivery of anDC might be more effective in delivering anDC to tumors without any side-effects induced by accumulation of anDC in multiple organs, not tumor burden. Also, through the intravenous injection of anDC, the accumulated anDC to lymph node can have a more possibility to present antigens to T cells; the administration routes of anDCs could be changed by the tumor types and target organs.

Next, we examined whether anDCs could affect tumor growth. Mice were inoculated with syngeneic MC38 adenocarcinoma cells, and 5 or 10

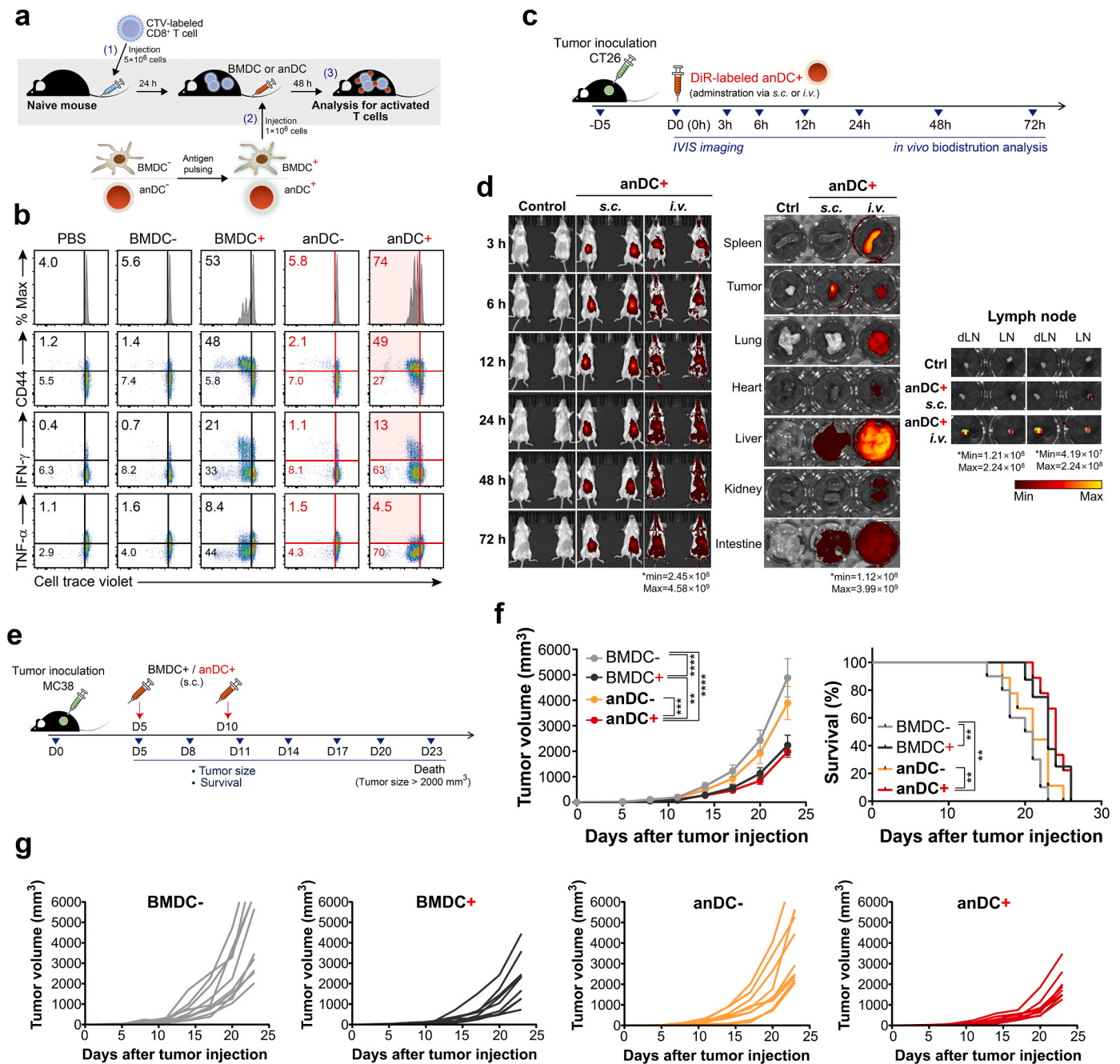


Fig. 4. *in vivo* functionality of anDCs to induce antigen-specific T cell responses and inhibit tumor growth. (a) The experimental scheme of the *in vivo* antigen-specific T cell proliferation assay. (b) Flow cytometry data showing the proliferation of P14 CD8⁺ T cells assessed by a CTV dilution assay. The frequencies of CTV⁺, CTV⁺CD44⁺, CTV⁺IFN- γ ⁺, and CTV⁺TNF- α ⁺ in P14 CD8⁺ T cells were analyzed by flow cytometry. The numbers in the plots indicate the percentage of CTV⁺, CTV⁺CD44⁺ or CTV⁺CD44⁺, CTV⁺IFN- γ ⁺ or CTV⁺IFN- γ ⁺, CTV⁺TNF- α ⁺ or CTV⁺TNF- α ⁺ in the P14 CD8⁺ T cell populations. (c) Experimental scheme of *in vivo* anDCs biodistribution. After tumor inoculation, DiR-labeled anDC⁺ was administered via subcutaneously (s.c.) or intravenously (i.v.). (d) IVIS images of real-time anDC biodistribution over time and *ex vivo* biodistribution of anDC in the main organs after 72 h. (e) The experimental scheme of therapeutic treatment of anDCs in an MC38 tumor model *in vivo*. (f) The overall tumor growth and (g) individual tumor growth of mice are represented. (h) The survival of tumor-bearing mice has been summarized. Data were analyzed by two-way ANOVA with Tukey's multiple comparison test or Mantel-Cox log-rank test. The graphs show the mean $\pm$ SEM. Data represent at least two independent experiments (n=1–10/group). N.S., not significant. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

days later, they subcutaneously received BMDCs or anDCs, which were unpulsed or pulsed with p15E peptide, a H-2 K^b-restricted CD8⁺ T cell epitope expressed from MC38 tumor (Fig. 4e). Subcutaneous transfer of BMDC⁺ or anDC⁺ exhibited substantial delay in tumor growth compared to that of unpulsed BMDC- or anDC- (Fig. 4 f and 4g). In line with tumor growth inhibition, mice that received peptide-pulsed BMDCs or anDCs lived longer (Fig. 4 h). Collectively, our data demonstrated that anDCs pulsed with MHC class I-restricted tumor antigens were able

to inhibit tumor growth as effectively as BMDCs.

3.5. Preparation of α CTLA-4-conjugated anDCs for enhanced cancer regression

As previously observed, anDCs effectively delayed the tumor growth, but the *in vivo* therapeutic response was comparable to that of BMDCs (Fig. 4). Thus, to boost the immune response, we additionally

engineered monoclonal antibody against CTLA-4 (α CTLA-4) onto anDCs and tested their ability to activate antigen-specific T cells *in vitro* and *in vivo* (Fig. 5a). α CTLA-4, a type of ICI, can further reinforce the costimulatory activity of DCs by facilitating the binding of surface B7.1/2 on anDCs to CD28 on T cells, especially in draining lymph nodes, where

the transferred anDCs preferentially reside and prime antigen-specific T cells [45]. Furthermore, this enhanced costimulatory activity of DCs can increase the frequency/number of CD8⁺ T cells with enhanced effector function [45]. In addition, CTLA-4 is a well-known target for regulatory T cells (Treg), which are one of the CD4⁺ T cell subsets, having

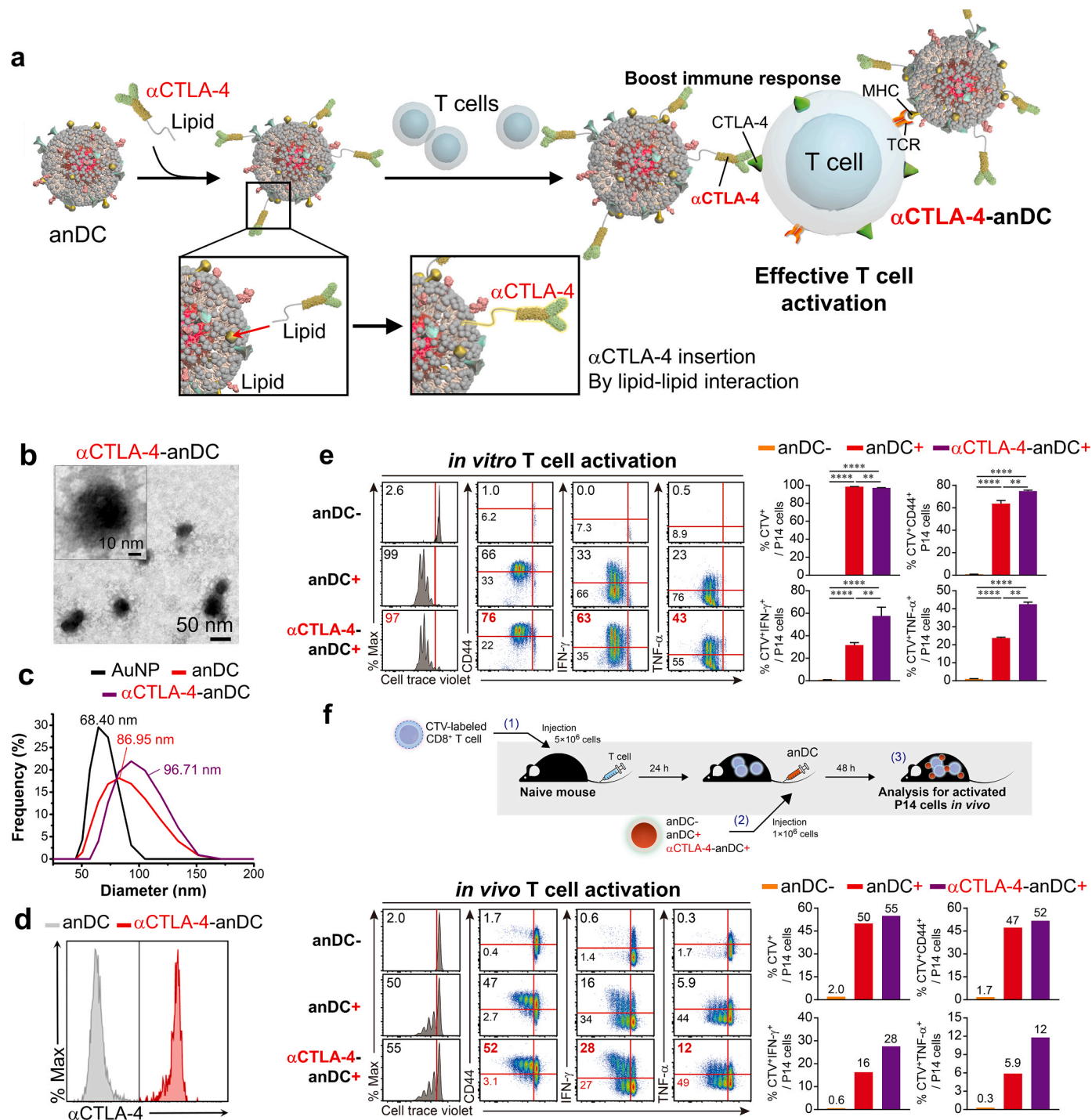


Fig. 5. Characterization of anDC and α CTLA-4-conjugated anDCs. (a) Illustration of the conjugation of α CTLA-4 onto anDCs by lipid-lipid interactions. As a result, α CTLA-4-conjugated anDCs can boost the immune response. (b) TEM images of anDCs and α CTLA-4-anDCs. (c) Size distribution of bare substrate, anDC and α CTLA-4-anDC. (d) Fluorescence intensity of anDCs and α CTLA-4-conjugated anDCs by insertion of fluorescence-labeled α CTLA-4. (e) Flow cytometry data showing the proliferation of P14 CD8⁺ T cells by anDC-, anDC+ and α CTLA-4-anDC+ assessed by a CTV dilution assay *in vitro*. The frequencies of CTV⁺, CTV⁺CD44⁺, CTV⁺IFN- γ ⁺, and CTV⁺TNF- α ⁺ in P14 CD8⁺ T cells were analyzed by flow cytometry. The numbers in the plots indicate the percentage of CTV⁺, CTV⁺CD44⁺ or CTV⁺IFN- γ ⁺ or CTV⁺TNF- α ⁺ in the P14 CD8⁺ T cell populations. The frequencies of CTV⁺, CTV⁺CD44⁺, CTV⁺IFN- γ ⁺, and CTV⁺TNF- α ⁺ in P14 CD8⁺ T cells are summarized in the bar graph. (f) Flow cytometry data showing the proliferation of P14 CD8⁺ T cells assessed by a CTV dilution assay *in vivo*.

immunosuppressive function [46]. Therefore, by conjugation α CTLA-4 to anDCs, we can expect the increased number of CD8⁺ T cells along with enhanced effector function and decreased number and function of Treg cells *in vivo*.

Since the phospholipid 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) can facilitate insertion into the DC membrane by lipid-lipid interactions, we prepared DSPE-polyethylene glycol (PEG) (2000)-carboxylic acid and covalently bound it to α CTLA-4 by an EDC/NHS reaction (Figure S12). After conjugation, the number of newly generated amide bonds was confirmed using IR and Raman spectroscopy. The Raman spectrum results in Figure S13 reveal a dramatically decreased peak intensity of the C-H bond to the N-H bond for DSPE-PEG- α CTLA-4 (final product) compared to that of DSPE-PEG-succinimide (intermediate product). This was due to the conjugation of α CTLA-4, a protein with a sufficient amide bond. We measured the molecular weight of each molecule using the MALDI-TOF/TOF. As shown in Figure S14, the expected molecular weight of DSPE-PEG (2000)-COOH is 2780.380 g/mol, the same as the molecular weight provided by the manufacturer. Significantly, the broad peak ranges from 1000 to 3500 are distributed, which indicates that various molecular weights were synthesized. After the EDC/NHS treatment of DSPE-PEG-COOH, an intermediate succinimide-grafted molecule was obtained. The molecular weight peak was positively shifted, correlated to the molecular weight of succinimide (99.09 g/mol). For the final product (DSPE-PEG- α CTLA-4), the estimated molecular weight was approximately 152,235 g/mol after conjugation with 150 kDa α CTLA-4. Therefore, we conclude that α CTLA-4 was successfully covalently conjugated to DSPE-PEG-COOH.

After the synthesis, we prepared α CTLA-4-anDCs and measured their morphology using TEM and atomic force microscopy (AFM). After the generation of α CTLA-4-conjugated anDCs, a rough structure around the anDCs was created, probably because α CTLA-4 was linked to the anDCs to form a globular structure (Fig. 5b) [47]. This was also observed in the AFM images and line profiling indicated by the red arrow around the anDC (Figure S15). The estimated molecular weight of DSPE-PEG (2000)- α CTLA-4 was approximately 150 kDa, which can be converted into a size of 3.51 nm [48]; therefore, the diameter of the α CTLA-4-conjugated anDCs increased to 96.71 nm (Fig. 5c). In addition, staining α CTLA-4-conjugated anDCs with fluorochrome-labeled anti-CTLA-4 mAb revealed that α CTLA-4 was successfully fused into the anDCs (Fig. 5d).

Next, the α CTLA-4-anDCs were tested their ability to activate antigen-specific T cells *in vitro* (Fig. 5e and S16) and *in vivo* (Fig. 5f). As shown in Fig. 5e, when anDC+ were conjugated with α CTLA-4, they induced P14 CD8⁺ T cell proliferation with an activity similar to that of non-conjugated anDC+ *in vitro* (~98%). In contrast, α CTLA-4-anDC+ showed a significantly improved ability to promote the differentiation of P14 CD8⁺ T cells, thereby 2-fold increasing the frequency of effector cytokine-producing P14 CD8⁺ T cells for IFN- γ and TNF- α . These tendencies were also found to be the same in *in vivo* T cell proliferation experiment (Fig. 5f). Similarly, when α CTLA-4 were conjugated with DC NV+, there was little difference in the proliferation level of OT-1 CD8⁺ T cells compared to α CTLA-4-anDC+, but the amount of IFN- γ or TNF- α produced from OT-1 CD8⁺ T cells was significantly reduced (Figure S16). This means that the presence of substrates facilitates the lipid-lipid interaction and makes it more robust. Given the similar ability of anDCs to induce T cell differentiation compared to BMDCs, these results indicate that α CTLA-4 conjugation is an optimized way to enhance the ability of anDCs to stimulate T cell activation *in vitro* and *in vivo*. In addition, the anDC could be decorated with other ICIs or immunologic adjuvants for effective T cell activation and differentiation on demand, implying that the anDC can be applicable for further personalized cancer immunotherapy.

3.6. Therapeutic efficacy of α CTLA-4-conjugated anDCs in 3 different tumor models

We finally examined whether α CTLA-4 conjugated anDCs were more effective for tumor regression than non-conjugated anDCs in MC38 and CT26 tumors (Fig. 6a). In MC38 tumor-bearing C57BL/6 mice, anDC+ showed dramatic tumor suppression effect compared to control (anDC-). Furthermore, when the α CTLA-4-anDC+ was administered, almost of tumor volume was diminished and three of the nine mice that received α CTLA-4-anDC+ were completely free of tumor burden (Figs. 6b and 6c; CR=3/9). Consistent with tumor growth, in terms of survival, most of the mice in the control groups, which received anDC- or anDC+, had already died at twenty-six days post tumor inoculation. However, all the mice that received α CTLA-4-anDC+ were still alive. In addition, we figured out the efficacy of α CTLA-4-anDC+ by using another murine tumor model, which BALB/c mice were subcutaneously inoculated with CT26 tumor cells (Figs. 6d and 6e). In line with previous data of MC38 model, α CTLA-4-anDC+ also significantly delayed CT26 tumor growth, whereas anDC- or anDC+ did not (Figs. 6d and 6e). At twenty-six days post tumor inoculation, half of the mice that received α CTLA-4-anDC+ survived, whereas all the mice that received anDC- or anDC+ had already died (Fig. 6d).

Next, to investigate whether these results could be replicated in a highly aggressive and therapy-resistant murine tumor model, we assessed the function of α CTLA-4-conjugated anDCs in TC-1-luciferase lung cancer model [49]. Six days post tumor inoculation, anDCs were intravenously injected, followed by one additional consecutive administration on day 11 (Fig. 6f). Every 4 days, IVIS image data were obtained to measure tumor growth. Among three groups, only α CTLA-4-anDC+ repressed tumor growth compared to anDC- or anDC+. Consistent with MC38 tumor data, in TC-1 luciferase model, we also cannot detect any tumor burden *in vivo* at day 18 (Fig. 6g). This result was validated by the signal intensity and total flux [p/s] (Fig. 6h), and a smaller number of nodules in the lung was found (Fig. 6i). Taken together, our results demonstrated that α CTLA-4-anDCs could successfully elicit anti-tumor immunity in multiple tumor models by providing a transformation of “cold tumor” into “hot tumor” to increase antitumor immune response [3].

3.7. Augmented anti-tumor immune response through alteration of tumor-infiltrating immune cells by therapeutic treatment of α CTLA-4-conjugated anDCs

To elucidate the α CTLA-4-conjugated anDC-mediated alterations in tumor-infiltrating immune cells that cause tumor regression, we sacrificed MC38-bearing mice 48 h after the second administration of anDCs and examined the phenotype and function of tumor-infiltrating lymphocytes (TILs) (Fig. 7a). Comparing α CTLA-4-anDC+ treated mice with anDC- or anDC+ treated mice, the frequency and absolute number of CD8⁺ T cells were significantly increased in α CTLA-4-anDCs treated mice. In contrast, Treg cells were significantly decreased, resulting in a higher CD8⁺ T/Treg cell ratio in α CTLA-4-anDC+ treated group (Figs. 7b and 7c). When we assessed correlation between tumor volume and TILs, significant positive correlations were exhibited between tumor volume and the frequency of CD8⁺ T cells or ration of CD8⁺ T cells/Treg cells. In addition, the frequency of Treg cells showed obvious negative correlations when compared with inoculated tumor volume (Fig. 7d). Furthermore, we measured the expression of several molecules in PD-1 and Tim-3, the molecules expressed on the cell surface of activated CD8⁺ T cells during persistent antigen exposure, cytotoxic serum protease protein Granzyme B (GzmB), and proliferation marker Ki-67. When mice were received with α CTLA-4-anDC+, CD8⁺ T cells in tumor microenvironment exhibited more activated and functional phenotype in terms of increased PD-1 and GzmB expression coupled with augmented proliferation capacity of higher Ki-67 expression (Figs. 7e and 7f). Then, we measured the expression of these molecules in immunosuppressive

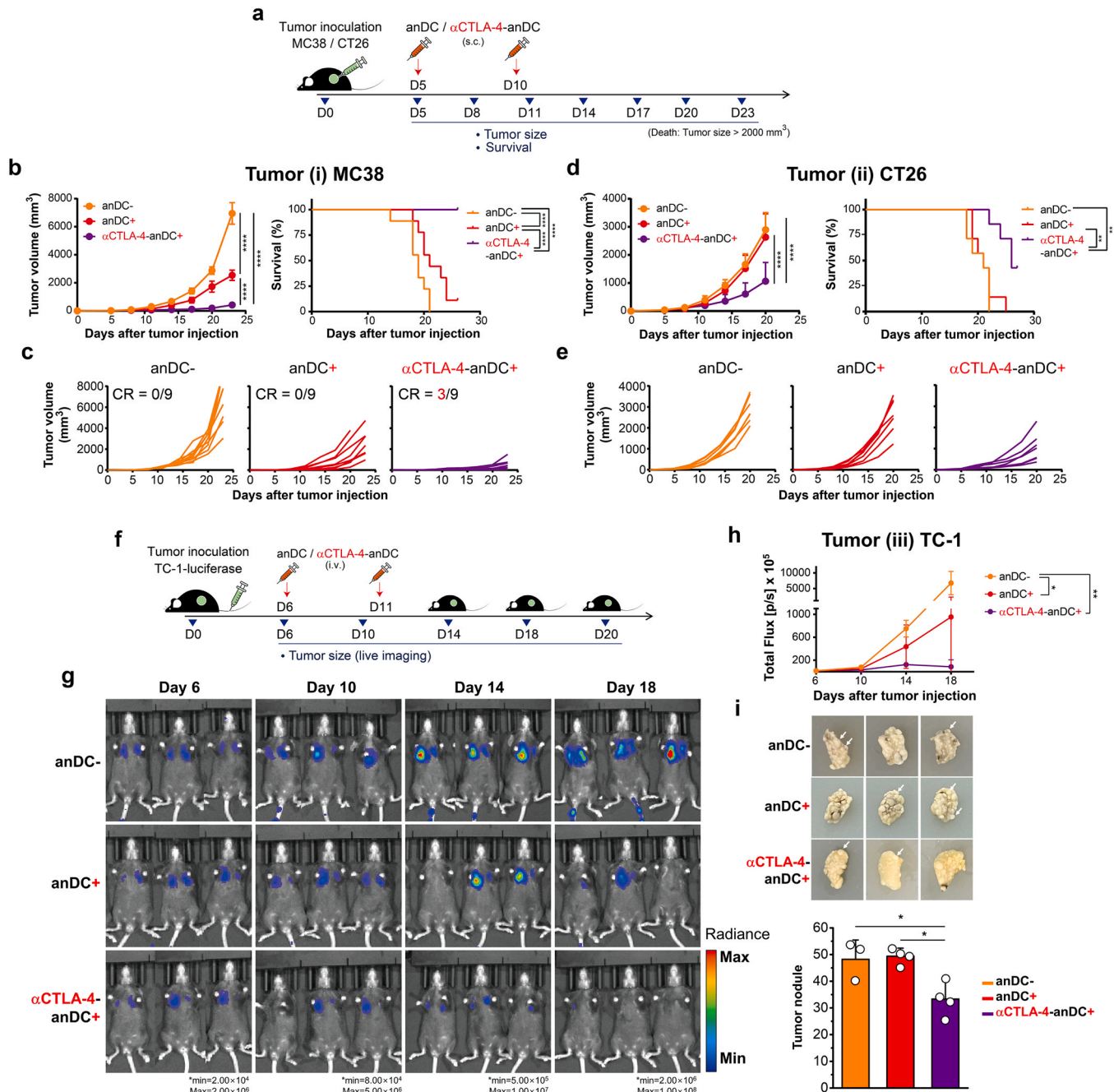


Fig. 6. Dramatically restrained tumor growth by α CTLA-4-conjugated anDCs in multiple tumor models. (a) The experimental scheme of therapeutic treatment of anDCs in the MC38 and CT26 tumor model. (b) The individual tumor growth and survival rate (c) and overall tumor growth of MC38 are represented. (d) The individual tumor growth and survival rate (e) and overall tumor growth of CT26 are represented. (f) The experimental scheme of therapeutic treatment of anDCs in the TC-1-luciferase tumor model. (g) Bioluminescence kinetics are shown for the TC-1-luciferase tumor-bearing mice and expressed in photons per second per square centimeter per steradian (p/s/cm²/sr). (h) and overall total flux of bioluminescence from TC-1-luciferase tumor are represented. (i) (left) Photograph of TC-1 tumor after sacrifice. White arrows represent tumor nodule in lung. (right) Graph for tumor nodule numbers in lung. Data were analyzed by two-way ANOVA with Tukey's multiple comparison test or Mantel-Cox log-rank test. The graphs show the mean $\pm$ SEM. Data are representative of a single experiment (n=3–9/group). N.S., not significant. * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001.

Tregs. In contrast to CD8⁺ T cells, both PD-1 and Tim-3 are known as functional molecules in Treg, which means higher expression of PD-1 and Tim-3 on Tregs can better suppress other immune cells. When we assessed functional markers of tumor infiltrated Treg cells, consistent with decreased tumor volume, Treg cells in α CTLA-4-anDC+ injected mice expressed lower levels of PD-1 and Tim-3, which suggested that they had less immunosuppressive function (Figs. 7g and 7h).

Next, we evaluated effector function of tumor specific CD8⁺ T cells by restimulating them with p15E peptide. To validate this, we isolated

CD8⁺ T cells from tumor after anDC treatment and then restimulated them with p15E peptide *ex vivo*. Tumor-specific CD8⁺ T cells from the α CTLA-4-conjugated anDCs treated group exhibited a significantly enhanced capacity to produce the effector cytokines IFN- γ , TNF- α , and IL-2 compared to those from the anDC- or anDC+ treated tumor (Figs. 7i and 7j). In addition, when CD4⁺ and CD8⁺ T cells were restimulated *ex vivo* with phorbol myristate acetate (PMA)/ionomycin to evaluate general T cell effector function, the CD8⁺ T cells from α CTLA-4-conjugated anDCs group also exhibited enhanced production of effector cytokines

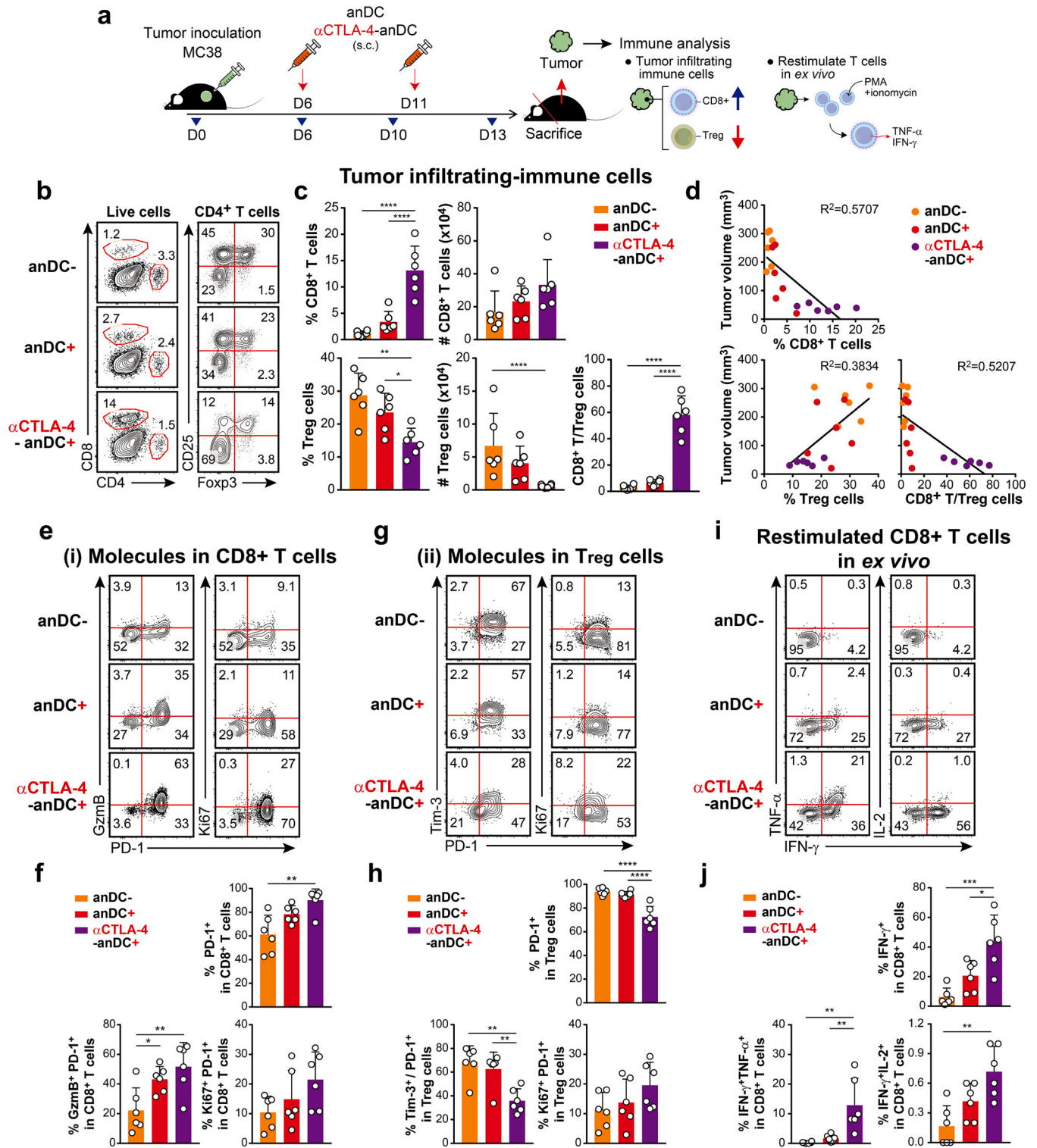


Fig. 7. Phenotypal and functional characters of tumor-specific T cell response induced by αCTLA-4-conjugated anDCs. (a) Schematic illustration of analysis of tumor-infiltrating immune cells and restimulating T cells ex vivo isolated from MC38-bearing mice with anDC, anDC/p15E, or αCTLA-4-conjugated anDC/p15E treatments twice on 6 and 11 days post tumor inoculation. (b) Representative flow cytometry plots of tumor-infiltrating immune cells. (c) Frequency and number of CD8⁺ T cells or Treg cells and the ratio of CD8⁺ T/Treg cells to Tregs. (d) Individual correlations between tumor volume and CD8⁺ T cells, Treg cells, and the ratio of CD8⁺ T/Treg cells to Tregs (e, f) Representative flow cytometry plots (e) and frequencies (f) of molecules indicated by CD8⁺ T cells. (g, h) Representative flow cytometry plots (g) and frequencies (h) of the molecules indicated by Treg cells. (i, j) CD8⁺ T cells were stimulated ex vivo with MC38 epitope peptide (p15E, KSPWFTTL). Representative FACS plots (i) and frequencies (j) of IFN-γ⁺, IFN-γ⁺TNF-α⁺, and IFN-γ⁺IL-2⁺ in CD8⁺ T cells. (k) Individual correlation between the frequency of IFN-γ⁺ or IFN-γ⁺TNF-α⁺ in CD8⁺ T cells and the frequency of PD-1⁺, GzmB⁺PD-1⁺, and Ki-67⁺PD-1⁺ in CD8⁺ T cells. Data were analyzed by (c, f, h, j) one-way ANOVA with Tukey's multiple comparison test or (d, k) line indicating the linear regression best fit. Data are represented as the mean ± SEM, with each dot indicating one mouse. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

(Figure S17). In addition, positive correlations between cytokine functionality and the expression of PD-1, Gzm B, and Ki-67 in CD8⁺ T cells indicated that the tendency of phenotype and function was consistent (Fig. 7k).

Collectively, these data demonstrated that α CTLA-4-anDCs modulated composition of tumor infiltrating immune cells towards increased CD8⁺ T/Treg cell ratio along with hyper-proliferative CD8⁺ T cells having augmented effector functions and less immunosuppressive Tregs as shown in Fig. 8. Blocking CTLA-4 pathway by conjugated antibody on anDCs might decrease CTLA-4-associated inhibitory signaling pathway on CD8⁺ T cell, resulting in increased frequency and effector cytokine function. In line with these effect on CD8⁺ T cells, in Treg cells, inhibition of CTLA-4 pathway might induce their decreased suppressive function. Therefore, in tumor microenvironment, alterations of immune system induced by α CTLA-4-anDC injection resulted in successful tumor regression.

4. Conclusion

In this study, we successfully prepared an artificial nano-platform with optimal effects was created by reinterpreting the membrane of antigen-presenting cells, the DCs, as a material and producing immortal nanoDC (anDC). We first optimized anDCs to amplify the efficacy of T cell activation by variation of DC membrane and substrate ratio. Through the encapsulation of Au NPs with DC membrane compactly, anDCs were designed to activate T cells as effectively as endogenous DCs but with longer stability than BMDCs and further optimized for production and storage for 30 days. Furthermore, the Au NP core in anDC can provide long-term T cell activation activity and retention *in vivo* compared to a naive DC membrane. This study also proposed a new modular concept that can maximize function and efficacy by engineering lipid-conjugated α CTLA-4 around the anDCs. The α CTLA-4-

functionalized anDCs showed significant advances *in vitro* and *in vivo* T cell activation potential. From a therapeutic's perspective, α CTLA-4-conjugated anDCs showed dramatically enhanced tumor regression effects in multiple murine tumor models, improving the tumor suppression capacity of tumor-infiltrating immune cells *in vivo*.

Thus, we developed an anti-tumor drug that is practically usable, can be stored for a long time, and has excellent efficacy over current approaches. We anticipate that functionalized anDCs when combined with patient tumor-derived neoantigen epitopes for T cells can be a game-changing therapeutic tool in precision medicine to treat cancer patients.

CRediT authorship contribution statement

Moonhyun Choi: Visualization. **Chae-Won Moon:** Conceptualization, Investigation, Methodology, Writing – original draft. **Taihyun Kim:** Conceptualization, Investigation, Methodology, Writing – original draft. **Tae Gun Kang:** Conceptualization, Investigation, Methodology, Writing – original draft. **Sang-Jun Ha:** Project administration, Supervision, Writing – review & editing. **Daheui Choi:** Conceptualization, Investigation, Methodology, Writing – original draft. **Sangmin Lee:** Supervision. **Jinke Hong:** Project administration, Supervision, Writing – review & editing. **Yoojin Lee:** Visualization. **Sungwon Jung:** Visualization. **Yoogyong Oh:** Visualization. **Taeho Kim:** Visualization. **Da-Hae Kim:** Investigation, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

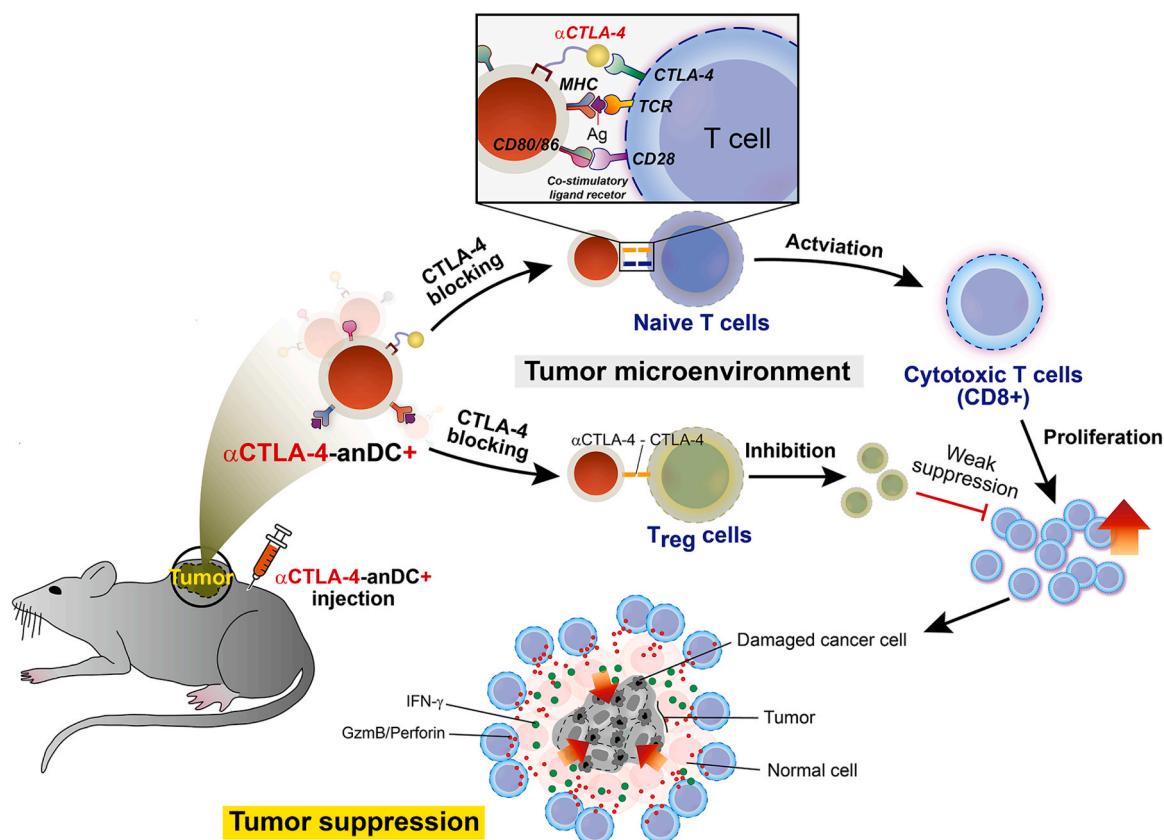


Fig. 8. Illustration on the overall tumor suppression effect by α CTLA-4-anDC according to the immune analysis in tumors. The α CTLA-4-anDC/Ag showed substantial tumor regression effect by enhanced CD8⁺ T cell activation and inhibition of Treg cells.

Data Availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.nantod.2024.102237](https://doi.org/10.1016/j.nantod.2024.102237).

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