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In vivo targeting of adoptively transferred T-cells with antibody- and cytokine-conjugated liposomes ☆

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ABSTRACT

In adoptive cell therapy (ACT), autologous tumor-specific T-cells isolated from cancer patients are activated and expanded *ex vivo*, then infused back into the individual to eliminate metastatic tumors. A major limitation of this promising approach is the rapid loss of ACT T-cell effector function *in vivo* due to the highly immunosuppressive environment in tumors. Protection of T-cells from immunosuppressive signals can be achieved by systemic administration of supporting adjuvant drugs such as interleukins, chemotherapy, and other immunomodulators, but these adjuvant treatments are often accompanied by serious toxicities and may still fail to optimally stimulate lymphocytes in all tumor and lymphoid compartments. Here we propose a novel strategy to repeatedly stimulate or track ACT T-cells, using cytokines or ACT-cell-specific antibodies as ligands to target PEGylated liposomes to transferred T-cells *in vivo*. Using $F(ab')_2$ fragments against a unique cell surface antigen on ACT cells (Thy1.1) or an engineered interleukin-2 (IL-2) molecule on an Fc framework as targeting ligands, we demonstrate that >95% of ACT cells can be conjugated with liposomes following a single injection *in vivo*. Further, we show that IL-2-conjugated liposomes both target ACT cells and are capable of inducing repeated waves of ACT T-cell proliferation in tumor-bearing mice. These results demonstrate the feasibility of repeated functional targeting of T-cells *in vivo*, which will enable delivery of imaging contrast agents, immunomodulators, or chemotherapy agents in adoptive cell therapy regimens.

1. Introduction

Immunotherapy treatments stimulating a patient's own immune system to attack tumors are beginning to show signs of clinical efficacy, demonstrating that the immune system can be harnessed for cancer therapy even in patients with advanced disease [1–3]. Among many immunotherapy strategies in development, adoptive cell therapy (ACT) with autologous tumor-specific T-cells has shown particularly striking results in recent phase I clinical trials [3,4]. In this approach, autologous T-cells isolated from tumor biopsies or peripheral blood are treated with cytokine/stimulatory cocktails to promote expansion of large numbers of tumor-reactive cells that can be reinfused into the patient, following which the transferred cells can home to disseminated tumor sites and destroy metastatic tumors. ACT therapy using completely autologous patient-derived tumor-infiltrating lymphocytes [4,5] or patient T-cells transduced with genetically engineered T-cell receptors [3,6] (TCRs, either exogenous TCR chains or chimeric antigen receptors composed of synthetic antigen-binding Ig domains fused with TCR signaling components) have been demonstrated to elicit objective response rates in up to 70% of patients with advanced metastatic melanoma [4–7] and dramatic cures in chronic lymphoblastic leukemia [3].

Despite these promising early clinical findings, efforts to increase the fraction of patients showing complete responses (complete and durable elimination of all detectable tumors) are needed. To this end, a variety of strategies are being explored, including manipulation of lymphocyte differentiation state during *ex vivo* expansion [8–10], combination treatments where patients receive adjuvant drug therapies

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designed to support ACT T-cells [4,8,11], and genetic modification of T-cells to express supporting costimulatory or cytokine genes [12–14]. We recently demonstrated that the efficacy of ACT can be dramatically enhanced by conjugation of cytokine- or drug-loaded nanoparticles (NPs) to the surfaces of T-cells *ex vivo* prior to transfer into tumor-bearing recipients [15,16], creating T-cell "pharmacytes". T-cell-bound particles provided pseudo-autocrine drug delivery to the transferred cells that greatly increased the effective potency of adjuvant drugs while simultaneously minimizing systemic exposure to these potent supporting signals. This approach allowed autocrine delivery of interleukin cytokines that dramatically enhanced the efficacy of ACT T-cells in a metastatic melanoma model [15] and the delivery of immunosuppression-blocking drugs that enhanced expansion of T-cells within large established tumors in a prostate cancer model [16].

A limitation of the pharmacyte approach is the one-time nature of the intervention: ACT T-cells can only be loaded once with a cargo of adjuvant drug prior to transfer, and the duration of stimulation is inherently limited by expansion of the cell population *in vivo*, since cell-bound particles are diluted with each cell division. We hypothesized that a strategy to target supporting drugs to T-cells with nanoparticle drug carriers directly *in vivo* would enable transferred lymphocytes to be repeatedly stimulated with supporting adjuvant drugs, and thereby provide continuous supporting signals over the prolonged durations that might be necessary for elimination of large tumor burdens. Such "re-arming" of T-cells with supporting drugs could be achieved by repeated administration of targeted particles, allowing adoptively-transferred T-cells to be restimulated multiple times directly *in vivo*, while the use of internalizing targeting ligands would minimize the likelihood of immune responses against the nanoparticle carrier. To our knowledge, only two prior studies have attempted to target nanoparticles to T-cells *in vivo* [17,18]. In both of these studies, particles were targeted to T-cells *via* peptide-MHC ligands that bind to specific T-cell receptors. However, peptide-MHC-functionalized nanoparticles have recently been shown to deliver an anergizing/tolerizing signal to T-cells [18,19] — which is ideal for treating graft rejection or autoimmunity, but runs counter to the goals of cancer immunotherapy.

Here we report on initial results illustrating the feasibility of specifically targeting ACT T-cells *in vivo* using stimulatory or non-stimulatory immunoliposomes. We synthesized and characterized PEGylated liposomes conjugated with 2 types of targeting molecules: (1) antibodies against unique cell surface antigens expressed only by the ACT T-cells (here, we employ the congenic marker Thy1.1), mimicking unique surface markers introduced clinically in genetically-engineered ACT T-cells [20,21]; and (2) recombinant interleukin-2 (IL-2), a cytokine that binds the trimeric IL-2 receptor (IL-2R) expressed by activated T lymphocytes [22]. These two ligands provide contrasting targeting strategies; anti-Thy1.1 provides highly specific targeting without overt stimulation of target cells, while IL-2 provides potentially less specific targeting (IL-2R can be expressed by some endogenous T-cells) but also delivers a direct stimulatory signal to T-cells. We characterized the efficacy of targeting particles to anti-tumor T-cells *in vitro* and internalization of liposomes triggered by these ligands, and then analyzed targeting of ACT T-cells *in vivo* in healthy animals and in a model of metastatic melanoma. Targeted liposomes labeled T-cells in multiple systemic compartments *in vivo*, with anti-Thy1.1 liposomes binding to >90% of transferred cells following a single injection. Further, we demonstrate the proof of concept that targeted stimulatory IL-2-liposomes can drive repeated expansion of ACT T-cells *in vivo*, following multiple periodic injections of targeted vesicles. We believe this strategy could provide a safe means to amplify the efficacy of ACT while avoiding systemic toxicity associated with many adjuvant drug treatments, and would be translatable to other immunotherapy settings, such as enhancement of cancer vaccines and therapeutic interventions in infectious diseases such as HIV.

2. Material and methods

2.1. Materials

All lipids and polycarbonate membranes (0.2 μm) for size extrusion were from Avanti Polar Lipids (Alabaster, AL) and used as received. DiD, ACK lysis buffer, Calcium Phosphate Transfection Kit, HEK293 Free Style Cells, Max Efficiency® DH5α™ Competent cells and Phoenix Eco viral packaging cells were obtained from Invitrogen Life Technologies (Grand Island, NY). Anti-thy1.1 (clone 19E12) and mouse IgG_{2a} isotype control antibodies were purchased from BioXCell (West Lebanon, NH). Dithiothreitol (DTT), Fluorescein isothiocyanate (FITC) isomer I, Concanavalin A Type VI (ConA), and Triton X-100 were from Sigma-Aldrich (St. Louis, MO) and used as received. Recombinant IL-2 and IL-7 were purchased from PeproTech (Rocky Hill, NJ). Anti-mouse CD16/32, anti-CD25, anti-CD25-Alexa 488, anti-CD8-PE, anti-Thy1.1, anti-Thy1.1-Percp-Cy5.5 and anti-Thy1.1-FITC were from eBioscience (San Diego, CA). Anti-mouse vβ13 T-cell Receptor-FITC was purchased from Becton Dickinson (Franklin Lakes, NJ). Protein A agarose column and Amicon Ultra-15 30 kDa MWCO Centrifugal Filter Units were from Millipore (Billerica, MA). Polyethylenimine (PEI) was from Polysciences (Warrington, PA). $F(ab')_2$ Preparation Kits, BCA Protein Assay Kits, and Zeba desalting columns were from Pierce Thermo Scientific (Rockford, IL). IL-2 ELISA Kits were obtained from R&D Systems (Minneapolis, MN). Ficoll-Pague Plus was from GE Health Care (Waukesha, WI). EasySep™ Mouse $CD8^+$ T Cell Enrichment Kit was from Stemcell (Vancouver, BC, Canada). Collagenase II and Hank's Balanced Salt Solution were purchased from (Gibco-Invitrogen, Carlsbad, CA). EndoFree Plasmid Maxi Kit was from Qiagen (Valencia, CA). Retronectin Recombinant Human Fibronectin Fragment was from Clontech (Mountain View, CA). D-luciferin was from Caliper Life Sciences (Hopkinton, MA). B16F10 melanoma cells were from American Type Culture Collection (Manassas, VA). IL-2-Fc was a generous gift from Dane Wittrup's lab at MIT.

2.2. Preparation of IL-2-Fc and anti-Thy1.1 $F(ab')_2$

IL-2-Fc is a bivalent fusion protein of the C-terminus of murine wild type IL-2 linked to a mouse IgG_{2a} backbone [23]. A D265A mutation was introduced in the IgG_{2a} Fc region to minimize interaction of IL-2-Fc with Fc receptors [24]. IL-2-Fc gene was transformed into DH5α cells *via* heat shock and extracted after clone expansion using an EndoFree Plasmid Maxi Kit following the manufacturer's instructions. HEK293 Freestyle cells were transfected with IL-2-Fc gene/polyethylenimine (PEI) complexes and grown in roller bottles at 37 °C for a week before harvest. Cells were spun down and secreted IL-2-Fc in the supernatant was purified by gravity flow/elution through Protein A agarose columns and concentrated by using centrifugal filter units (Amicon Ultra-15 30 kDa MWCO).

Monoclonal antibodies (Abs) against Thy1.1 were digested with pepsin to generate the $F(ab')_2$ using a $F(ab')_2$ Preparation Kit following the manufacturer's instructions. IL-2-Fc and anti-Thy1.1 $F(ab')_2$ concentrations were determined by the BCA Protein Assay Kit. IL-2-Fc bioactivity concentration relative to wild type murine IL-2 was quantified by an IL-2 ELISA Kit.

2.3. Synthesis of IL-2-Fc-liposomes and anti-Thy1.1-liposomes

Vacuum dried lipid films composed of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000 (maleimide-PEG_{2000}-DSPE)/cholesterol/hydrogenated Soy L-α-phosphatidylcholine (HSPC) in a molar ratio of 2.5/27.5/69 together with 1% of a fluorescent lipophilic tracer dye 1,1′-dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD) were rehydrated in 250 μl of 50 mM HEPES/150 mM NaCl-buffer

(pH6.5). Lipids were vortexed every 10 min for 1 h at 62 °C to form vesicles and size extruded through a polycarbonate membrane (0.2 μm). After washing in excess phosphate buffered saline (PBS) pH7.4 and spinning down by ultracentrifugation at 110,000 ×*g* for 4 h, liposomes were re-suspended in 100 μl PBS/1.4 mg of lipids.

IL-2-Fc and anti-Thy1.1 F(ab′)$_2$ were coupled to liposomes as previously described [23]. Briefly, Ab or cytokine (4–8 mg/ml) was treated with 1.8 mM DTT in the presence of 10 mM EDTA at 25 °C for 20 min to expose hinge region free thiols. DTT was subsequently removed by using Zeba desalting columns before mixing with maleimide-bearing liposomes (1 mg protein/1 mg lipid) in PBS pH 7.4. After incubation for 18 h at 25 °C on a rotator, excess protein was removed by ultracentrifugation in excess PBS (if aggregation occurs, liposomes were size extruded through a 0.2 μm polycarbonate membrane at 37 °C before ultracentrifugation). Liposome sizes were characterized before/after coupling by dynamic light scattering (90Plus Particle Size Analyzer, Brookhaven, Holtsville, NY).

2.4. Quantification of targeting ligands coupled to liposomes

Anti-Thy1.1-FITC was concentrated to 4–8 mg/ml using Ultra-15 Centrifugal Filters before coupling to liposomes as previously described. After liposomes were solubilized in 2% Triton X-100 at 37 °C for 5 min with gentle vortexing, FITC fluorescence was measured at ex/em wavelengths of 490/520 nm using a fluorescence plate reader (Tecan Systems, San Jose, CA) and converted to protein concentrations using standard curves prepared from serial dilutions of neat anti-Thy1.1-FITC stock solutions. IL-2-Fc-Lips were solubilized in the same manner and the amount of IL-2 coupled was determined using an IL-2 ELISA Kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

2.5. Activation of pmel-1 Thy1.1$^+$CD8$^+$ T-cells

Animals were cared for in the USDA-inspected MIT Animal Facility under federal, state, local and NIH guidelines for animal care. Spleens from pmel-1 Thy1.1$^+$ mice were ground through a 70 μm cell strainer and red blood cells were removed by incubating with ACK lysis buffer (2 ml/spleen) for 5 min at 25 °C. After 1 wash in PBS, the remaining cells were cultured at 37 °C in RPMI 1640 medium containing 10% fetal calf serum (FCS). ConA at a final concentration of 2 μg/ml and IL-7 at 1 ng/ml were added to activate and expand splenocytes. After two days, dead cells were removed by Ficoll-Pague Plus gradient separation and CD8$^+$ T-cells were isolated *via* magnetic negative selection using an EasySep™ Mouse CD8$^+$ T Cell Enrichment Kit. Purified CD8$^+$ T-cells were re-suspended at 1.5×10^6/ml RPMI containing 10 ng/ml recombinant murine IL-2. After 24 h, cells were washed 3 times in PBS and re-suspended in 100×10^6/ml for adoptive transfer.

For bioluminescence imaging experiments, Click Beetle Red luciferase (CBR-luc) [16] was introduced into pmel-1 T-cells (post Ficoll purification and magnetic selection) by retroviral transduction. Phoenix Eco viral packaging cells were seeded at 4×10^6 cells/10 cm tissue culture dish in 10 ml DMEM medium containing 10% FCS. After incubation overnight at 37 °C, phoenix cells were exchanged with 10 ml fresh DMEM with 10% FCS, transfected with CBR-luc plasmid and Phoenix Eco plasmid using a Calcium Phosphate Transfection Kit and cultured at 32 °C for 24 h. DMEM was then replaced with 6 ml RPMI containing 10% FCS and transfected Phoenix Eco cells were incubated for another 24 h. Supernatant containing the retrovirus-packaged CBR-luc gene was collected and replaced with fresh RPMI for another 24 h incubation. Supernatant was collected again and combined with that collected 24 h earlier, and sterile filtered (0.45 μm). Six-well non-tissue culture plates (BD Falcon) were coated with 1 ml RetroNectin (15 μg/ml) 18 h at 4 °C, then excess RetroNectin was aspirated. Pmel-1 T-cells post ficoll purification and magnetic selection were suspended in filtered viral sups (RPMI collected previously) with 10 ng/ml IL-2 at 1.8×10^6/ml, 3 ml was added to each RetroNection-coated well, and spinoculation was conducted by centrifuging at 2000 ×*g* for 1 h at 25 °C. Transduced T-cells were then incubated at 37 °C. Six hours later, 1 ml of fresh RPMI was added with 10 ng/ml IL-2. Transduced, activated pmel-1 T-cells were used 1 day later for adoptive transfer studies.

2.6. In vitro liposome binding to T-cells

DiD-labeled protein-conjugated liposomes (0.7 mg lipids in 100 μl) were incubated with 15×10^6 activated pmel-1 Thy1.1$^+$ T-cells in 1 ml complete RPMI supplemented with 10% FCS for 30 min at 37 °C with gentle agitation every 10 min. In competitive conjugation assays, 100-fold molar excess soluble IL-2-Fc or anti-Thy1.1 free antibody (compared to the amount coupled to liposomes) was added 30 min before targeting liposomes to saturate IL-2 or Thy1.1 receptors on the cells, respectively. For IL-2-Fc-Liposome (IL-2-Fc-Lip) competition assays, 2.5×10^6 activated pmel-1 CD8$^+$ T-cells were mixed with 2.5×10^6 naïve C57Bl/6 splenocytes in 100 μl complete RPMI with 10% FCS. The cell mixture was incubated with or without 0.24 mg/ml soluble IL-2-Fc, followed by incubation with 0.07 mg/ml IL-2-Fc-Lip for 30 min at 37 °C with total volume topped up to 300 μl. For competition assays with anti-thy1.1.-liposome (anti-Thy1.1-Lip), 0.15 mg/ml liposomes (Lip) were incubated with a mixture of 2.5×10^6 activated pmel-1 T-cells and 2.5×10^6 naïve C57Bl/6 splenocytes (with or without pre-blocking by 1.34 mg/ml anti-Thy1.1). Cells without any liposomes added served as a control for cellular autofluorescence and cells conjugated with 0.15 mg/ml IgG_{2a}-liposomes (IgG_{2a}-Lip) were used to test non-specific binding of non-targeting liposomes. For all *in vitro* conjugation experiments, cells were stained with anti-CD8 and anti-Thy1.1 after two washes in ice cold PBS to remove unbound liposomes, and analyzed by flow cytometry on a BD FACS Canto except competition assays which were done on a BD LSR II.

2.7. Titration of liposome concentration for in vitro conjugation

Varying amounts of DiD-labeled anti-Thy1.1-Lip were added to 5×10^6 activated pmel-1 Thy1.1$^+$ T-cells in 100 μl complete RPMI with 10% FCS. The total volume for all groups was topped up with RPMI with 10% FCS to 300 μl and incubated at 37 °C for 30 min. After two washes in ice cold PBS to remove unbound liposomes, cells were resuspended in FACS buffer, surface stained with fluorescently labeled anti-CD8 and anti-Thy1.1, and analyzed by flow cytometry on a BD LSR II.

2.8. Internalization of liposomes

Anti-Thy1.1-Liposomes were labeled with 1% (mol) carboxyfluorescein-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine lipid (CF-DOPE) instead of DiD during the synthesis stage and incubated with 6×10^6 activated pmel-1 Thy1.1$^+$ T-cells/0.7 mg of lipids for 60 min at 4 °C with gentle agitation every 15 min. After two washes in ice cold PBS pH7.4 to remove unbound liposomes, T-cells were resuspended in RPMI and aliquotted into four equal portions for 0, 2, 4 and 6 h time points, respectively. After each incubation interval at 37 °C, T-cells were washed 2× in ice cold PBS and re-suspended in flow cytometry buffer (2% FCS in PBS). Cells were placed on ice to minimize further internalization and analyzed by flow cytometry on a BD LSR II. Cells were also imaged directly without fixation on a Zeiss LSM 510 laser scanning confocal microscope.

2.9. Adoptive transfer and in vivo liposome targeting

Albino C57BL/6 female mice 6–8 weeks of age were from the Jackson Laboratory (Bar Harbor, ME). One day before adoptive transfer, mice were sublethally lymphodepleted with 5 Gy total body irradiation. 15×10^6 activated pmel-1 $CD8^+$ T-cells in 150 μl PBS were injected intravenously (i.v.) into each recipient animal. DiD-labeled immunoliposomes (1.4 mg lipids) were re-suspended in 100 μl PBS and injected i.v. immediately or three days after adoptive transfer. Twenty-four hours after administration of liposomes, mice were euthanized and blood, lymph node, and spleen cells were analyzed by flow cytometry on a BD FACS Canto to assess liposome binding to T-cells.

2.10. Adoptive transfer of CBR-Luc T-cells and bioluminescence imaging

B16F10 melanoma cells were suspended at 1×10^6 cells/200 μl in Hank's Balanced Salt Solution and injected i.v. to induce lung metastases in albino C57Bl/6 mice (day −8). Animals were then sublethally lymphodepleted by total body irradiation (5 Gy) 7 days post tumor inoculation (day −1). Pmel-1 $CD8^+$ T-cells transduced with CBR-Luc (12×10^6) were resuspended in 150 μl PBS and administered i.v. one day after lymphodepletion (day 0). IL-2-Fc-Lip (1 mg of liposomes) or PBS was injected i.v. immediately after ACT and on day 6. D-Luciferin, a substrate for CBR-luc, was suspended in PBS (15 mg/ml) and 150 mg luciferin/kg body weight was injected Intraperitoneally (i.p.) in anesthetized animals 10 min before bioluminescence imaging acquisitions (5 min, 3 min, 2 min and 1 min) on a Xenogen IVIS Spectrum Imaging System (Caliper Life Sciences). Images were collected every two days starting from day 0 (2 h after ACT) to day 14. Living Image software version 3.0 (Caliper Life Sciences) was used to acquire and quantitate the bioluminescence imaging data sets. To compare the stimulatory effects of soluble IL-2 and IL-2-Fc-Lip, a similar experiment was repeated with 1st/2nd dose as 0.5 mg/1 mg IL-2-Fc-Lip or 10 μg/20 μg soluble recombinant wild type mouse IL-2 (PeproTech, Rocky Hill, NJ), equivalent to the amount of IL-2 coupled on IL-2-Fc-Lip). On day 12 mice were sacrificed and T-cells from inguinal lymph nodes were collected and surface stained for CD8 and vβ13 before analyzing by flow cytometry on a BD FACS Canto to assess the percentage of tumor-specific T-cells in each group.

2.11. Sample preparation for flow cytometry

Inguinal lymph nodes and spleens were ground through a 70 μm cell strainer and washed once with ice cold PBS. Splenocytes were then lysed with ACK lysis buffer (2 ml/spleen) for 5 min at 25 °C to remove red blood cells before washing in ice cold PBS. Blood samples were lysed 2× with 1 ml ACK lysis buffer for 5 min at 25 °C and then washed 1× with ice cold PBS. All cells were washed in FACS buffer (PBS with 2% Fetal Calf Serum) once before surface-staining with Ab. After staining, cells were washed 2× in FACS buffer and analyzed on a BD FACS Canto Flow Cytometer. All data were processed using FlowJo software.

2.12. Statistical analysis

Statistical analysis was done using GraphPad Prism software and two-tailed unpaired t-tests were conducted between groups of experimental data. Graphs show the mean ± SEM of sample groups.

3. Results and discussion

3.1. Synthesis and characterization of IL-2-Fc-Lip and anti-Thy1.1-Lip

To generate cytokine- or antibody (Ab)-conjugated liposomes (IL-2-Fc-Lip or anti-Thy1.1-Lip) for T-cell targeting, PEGylated

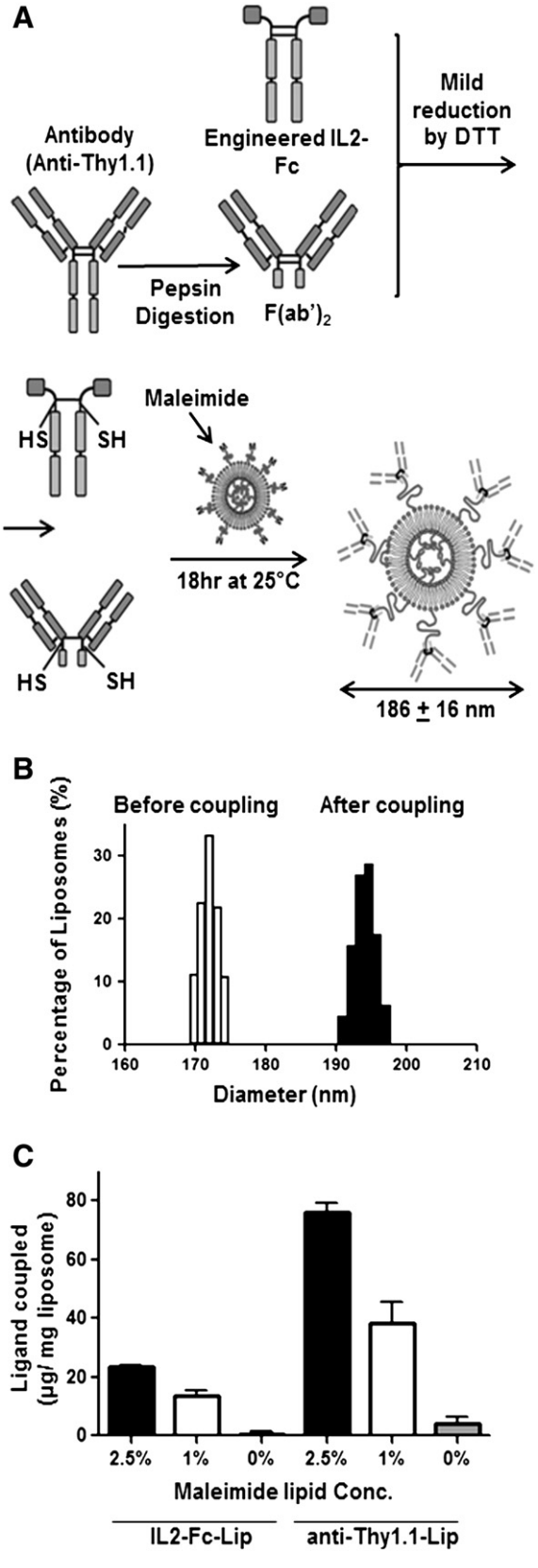


Fig. 1. T-cell-targeted liposome synthesis and characterization. (A) Schematic of immunoliposome preparation. (B) Typical particle size distributions for liposomes before antibody conjugation (open bars) and after conjugation (black filled bars) determined by dynamic light scattering. (C) Quantification of ligand (IL-2 cytokine equivalent or anti-Thy1.1) coupled to liposomes incorporating different mole fractions of maleimide-PEG lipid: 2.5% (black filled bar), 1% (open bar) or 0% (striped bar), assessed by IL-2 ELISA and measuring FITC-labeled anti-Thy1.1 incorporation respectively.

liposomes incorporating maleimide-headgroup PEG-lipids (Mal-PEG-DSPE) were prepared from high-T_m lipids and sized by membrane filtration to a mean diameter of 173 ± 13 nm (Fig. 1A, B). Murine IL-2 fused to the C-terminus of mouse IgG_{2a} Fc or anti-Thy1.1 F(ab′)$_2$ were coupled to the maleimide termini of PEG chains to serve as targeting ligands of the immunoliposomes. To minimize interaction of liposomes with phagocyte Fc receptors, a D265A mutation was introduced in the Fc portion of IL-2-Fc [24] and F(ab′)$_2$ fragments of anti-Thy1.1 monoclonal antibodies were generated by pepsin digestion. Prior to F(ab′)$_2$/cytokine coupling, IL-2-Fc and anti-Thy1.1 F(ab′)$_2$ were mildly reduced by DTT to expose hinge region free thiols for reaction with the liposome maleimide functional head groups. We tested liposomes containing two different mole fractions of maleimide-PEG lipid (1 or 2.5 mol% of total lipids), and found greater targeting ligand conjugation with higher fractions of maleimide-lipid, as expected (Fig. 1C). Negligible IL-2 or F(ab′)$_2$ binding to liposomes was observed in the absence of the maleimide reactive groups. Liposomes with 2.5 mol% mal-PEG-DSPE gave 23 ± 2 μg of IL-2 (cytokine equivalent, or 1.4 nmol IL-2) or 76 ± 7 μg of anti-Thy1.1 (0.5 nmol F(ab′)$_2$)/mg lipid after overnight coupling at 25 °C. As shown in Fig. 1B, targeting ligand conjugation caused a slight increase in the mean size of the vesicles from 173 ± 13 nm to 186 ± 16 nm.

3.2. IL-2-Fc-Lip and anti-Thy1.1-Lip binding to T-cells in vitro

To generate a target population of T-cells to be used in adoptive transfer studies, CD8$^+$ T-cells from pmel-1 Thy1.1$^+$ mice (which express a transgenic T-cell receptor specific for the gp100 antigen of melanoma [25]) were isolated by magnetic negative selection from activated splenocytes, and expanded by culturing with IL-2 for 1 day to obtain an elevated expression of CD25 (the α-chain of the trimeric IL-2 receptor) compared to naive pmel-1 or naive polyclonal C57Bl/6 CD8$^+$ T-cells (Fig. 2A and data not shown). Fluorescently labeled PEGylated vesicles showed very low background binding to activated pmel-1 T-cells following a 30 min incubation at 37° *in vitro*, but IL-2-Fc-Lip or anti-Thy1.1-Lip containing 1% or 2.5% maleimide functional groups efficiently bound to activated pmel-1 T-cells (Fig. 2B). The Mean Fluorescence Intensities (MFI) of cells after conjugation with different types of liposomes was quantified; the high expression levels of Thy1.1 on pmel-1 T-cells led to much greater per-cell binding of anti-Thy1.1-Lip *vs.* IL-2-Fc-Lip (Fig. 2C). For both targeting ligands, liposomes containing 2.5 mol% mal-PEG-DSPE (and therefore with higher ligand densities) achieved much greater binding to T-cells than vesicles with 1 mol% of the maleimide lipid, with MFIs of bound liposomes increased by 6-fold and 4-fold for anti-Thy1.1-Lip and IL-2-Fc-Lip, respectively (Fig. 2B, C).

To evaluate the specificity of anti-Thy1.1-Lip and IL-2-Fc-Lip binding, we assessed T-cell labeling in the presence of competing free IL-2-Fc or anti-Thy1.1 Abs added to a 1:1 mixture of naïve C57Bl/6 lymphocytes and pmel-1 T-cells 30 min before the targeted vesicles. IL-2-Fc-Lip bound to activated pmel-1 T-cells, but not naïve C57Bl/6 CD8$^+$ T cells that lack IL-2 receptors (Fig. 2D middle left). Pre-blocking pmel-1 T-cells with soluble IL-2-Fc blocked 90% of binding to pmel-1 T-cells (Fig. 2D middle right, E). Similarly, anti-Thy1.1-Lip selectively targeted pmel-1 CD8$^+$ T cells but not naive C57Bl/6 (Thy1.2$^+$) CD8$^+$ T cells (Fig. 2D bottom left). Pre-incubation of pmel-1 T-cells with anti-Thy1.1 lowered anti-Thy1.1-Lip binding by 99% (Fig. 2D bottom right, E). Autofluorescence and non-specific binding of non-targeted control IgG_{2a}-Lip were negligible (Fig. 2D top left and right, E). As expected from the pM affinity of IL-2 for its receptor [26,27] and the typical nM affinity of commercial antibodies, liposomes at concentration of 0.4 mg/ml (equivalent to 2 nM of liposomes) labeled 100% of activated pmel-1 T-cells *in vitro*, and liposome binding reached a plateau at concentrations higher than 0.4 mg/ml (equivalent to 2 nM liposomes) (Fig. 2F). Thus, both IL-2- and anti-Thy1.1-targeted stealth liposomes achieve specific and avid binding to primed pmel-1 CD8$^+$ T-cells. Even when the concentration was titrated down to 0.1 mg/ml, nearly 100% of cells were still labeled with liposomes, albeit with fewer liposomes bound per cell.

3.3. Internalization of Anti-Thy1.1-conjugated liposomes

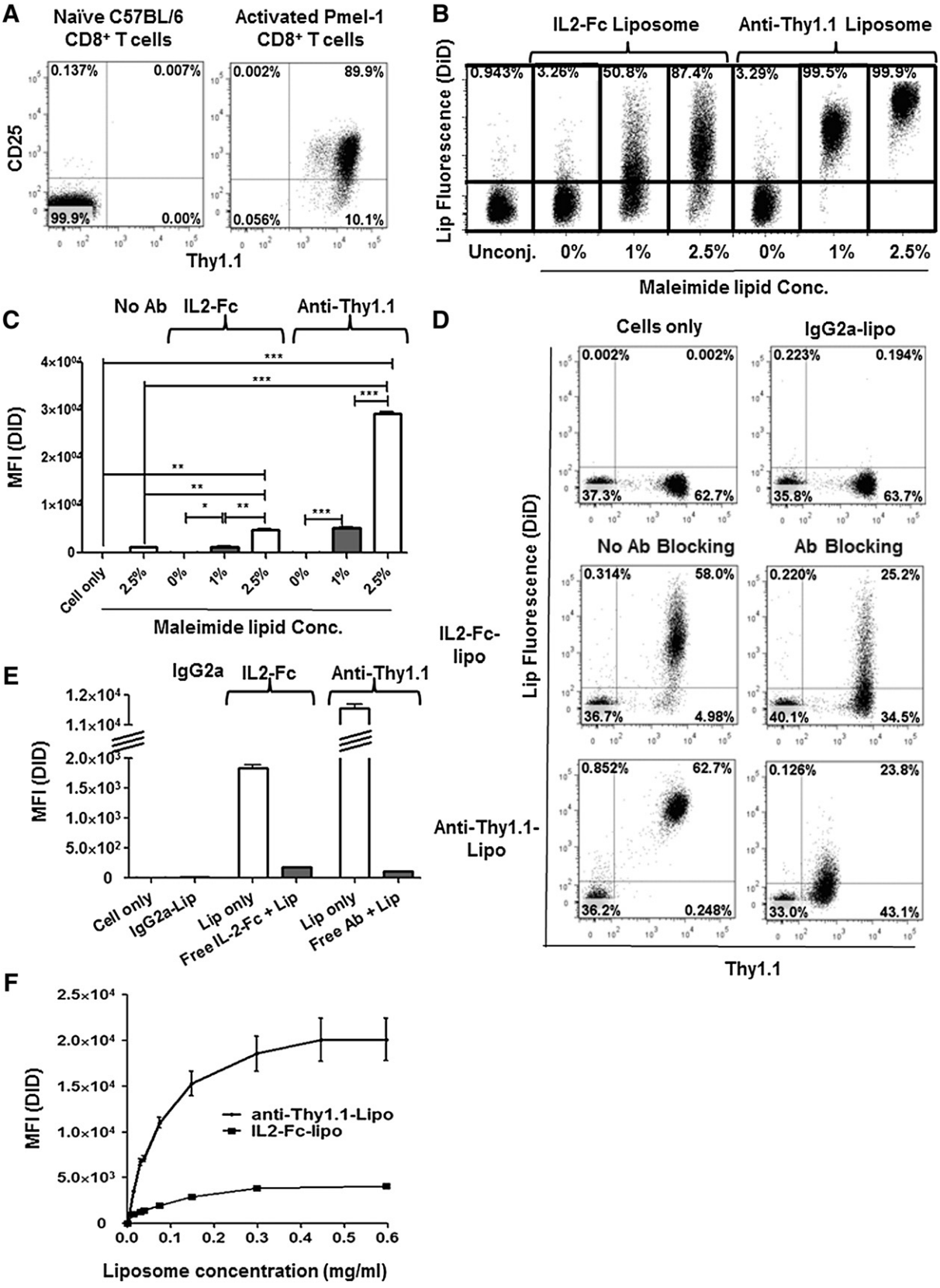
We previously reported that IL-2-Fc-conjugated liposomes are rapidly internalized by activated T-cells *in vitro* [28]. To determine whether anti-Thy1.1-Lip would also trigger liposome endocytosis, we added anti-Thy1.1-Lip incorporating a carboxyfluorescein (CF)-headgroup lipid to pmel-1 T-cells at 4 °C to allow binding without internalization, then warmed the cells to 37 °C and assessed cell-associated fluorescence over time. Fluorescein has highly pH-sensitive fluorescence that is strongly quenched at acidic pHs [29]. The high avidity of liposome binding to cells led to no measurable release of free liposomes into the supernatant over 6 h at 37 °C (not shown); we thus attributed loss of the CF tracer signal to endocytic uptake by labeled cells. Over a time course of 6 h, the MFI of liposome-labeled T-cells steadily dropped, corresponding to roughly 90% internalization over this time course (Fig. 3A). Confocal imaging also showed that anti-Thy1.1-Lip fluorescence initially localized to the plasma membrane of labeled cells was largely lost by 6 h (Fig. 3B).

3.4. In vivo targeting of IL-2-Fc-Lip and anti-Thy1.1-Lip in healthy animals

Next, we tested the capacity of anti-Thy1.1-Lip and IL-2-Fc-Lip to target pmel-1 T-cells *in vivo* in healthy mice. PEGylated liposomes conjugated with isotype control murine IgG_{2a} were prepared to serve as a control non-T-cell-targeting liposome. To model clinical adoptive T-cell therapy, recipient Thy1.2$^+$ C57Bl/6 mice were lymphodepleted by sub-lethal irradiation, followed by i.v. injection of 15×10^6 activated pmel-1 Thy1.1$^+$CD8$^+$ T-cells one day later. Lymphodepletion removes cytokine sinks and regulatory T-cells to allow more efficient expansion and effector function of transferred T-cells [30,31]. To assess T-cell targeting, IgG_{2a}-Lip, IL-2-Fc-Lip, or anti-Thy1.1-Lip fluorescently labeled with the non-pH-sensitive tracer DiD were injected i.v. either immediately after adoptive transfer or 3 days after T-cell injection. Twenty-four hours after liposome injection, cells from the blood, lymph nodes (LNs), and spleens were analyzed by flow cytometry to assess binding of fluorescent liposomes (Fig. 4A). Thy1.1 expression allowed liposome binding to transferred pmel-1 T-cells to be distinguished from endogenous T-cells (Fig. 4B). Sample flow cytometry

Fig. 2. *In vitro* binding of IL-2-Fc-Lip and anti-thy1.1 F(ab′)$_2$-Lip to primary T-cells. (A) Flow cytometry analysis of cell surface expression of CD25 and Thy1.1 on naïve C57BL6/J (Thy1.1$^-$) splenocytes *vs.* activated pmel-1 Thy1.1$^+$CD8$^+$ T-cells. (B, C) Pmel-1 CD8$^+$ Thy1.1$^+$ T-cells were incubated with 0.7 mg/ml (per 15×10^6 cells) DiD-labeled liposomes (IL-2-Fc or anti-Thy1.1 F(ab′)$_2$ conjugated) for 30 min at 37 °C in complete RPMI, then analyzed by flow cytometry for liposome binding. Shown are representative flow cytometry scatter plots (B) and quantification of Mean Fluorescence Intensity (MFI) of pmel-1 T-cells as a function of mol% of mal-PEG-DSPE included in the vesicles (C). (D, E) Activated pmel-1 CD8$^+$ T-cells were mixed with naive C57BL/6 splenocytes in a 1:1 ratio and incubated with 0.07 mg/ml IL2-Fc-Lip or 0.15 mg/ml anti-Thy1.1-Lip for 30 min at 37 °C, then analyzed by flow cytometry. (D) Shown are scatter plots representing liposome fluorescence on naive C57BL/6 CD8$^+$ T cells (Thy1.1$^-$) and activated pmel-1 CD8$^+$ T-cells (Thy1.1$^+$) with/without 0.24 mg/ml soluble IL2-Fc or 1.34 mg/ml anti-Thy1.1 antibody added for 30 min prior to addition of liposomes. Cells incubated with 0.15 mg/ml IgG2a-lipo are also shown. (E) Quantification of the MFI of Pmel-1 CD8$^+$ T cells when bound with respective liposomes or pre-blocked by free Ab/IL-2. (F) Titrated concentrations of fluorescent liposomes were added to 5×10^6 activated pmel-1 T-cells and incubated at 37 °C for 30 min, then analyzed by flow cytometry for MFI of T-cell-associated liposomes. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

histograms are shown in Fig. 4C, illustrating conjugation efficiencies of IgG_{2a}-Lip, IL-2-Fc-Lip, and anti-Thy1.1-Lip obtained when liposomes were injected immediately after ACT T-cells. The percentage endogenous or ACT $CD8^+$ T-cells labeled by each type of liposome in the blood (Fig. 4D), lymph nodes (Fig. 4E) and spleens (Fig. 4F) were assessed; this analysis revealed that IgG_{2a}-Lip exhibited low binding to both T-cell populations. In contrast, anti-Thy1.1-Lip labeled nearly 100% of the transferred T-cells in the blood and spleen whether



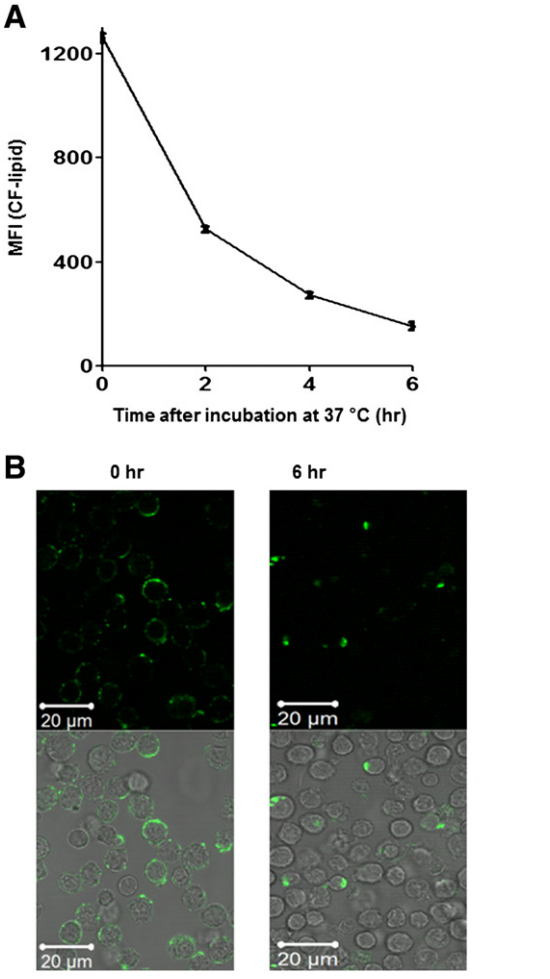


Fig. 3. Internalization of Thy1.1-targeted liposomes. Carboxy-fluorescein (CF)-labeled anti-Thy1.1-Lip (1.4 mg/ml) were incubated with 12×10^6 activated pmel-1 $CD8^+$ T-cells in 500 μl RPMI containing 10% FCS for 1 h at 4 °C, washed, then incubated in RPMI at 37 °C until analysis by flow cytometry 2 h, 4 h or 6 h later. (A) MFI of T-cell-associated CF fluorescence. (B) Confocal images of cells at time zero or after 6 h at 37 °C. Scale bar = 20 μm.

injected on day 0 or day 3. The slightly greater background binding of isotype control IgG2a-Lip to ACT *vs.* endogenous T-cells in spleens was found to be an artifact of the liposome dose; injection of lower liposome doses of 0.18 mg (approximately ~0.1 mg/ml liposomes in the blood) led to a similar efficiency of specific T-cell binding but eliminated the low differential background binding to ACT *vs.* endogenous T-cells (data not shown). A lower fraction of T-cells in lymph nodes were labeled by anti-Thy1.1-Lip following a day 3 injection, which may reflect a combination of poor entry of targeted liposomes into LN and/or incomplete recirculation of T-cells from LN back into the blood in the 24 h time window between liposome injection and our analysis. Anti-Thy1.1-Lip also showed low levels of background binding to endogenous ($Thy1.1^-$) T-cells. IL-2-Fc-Lip labeled the majority of pmel-1 T-cells in the LNs, spleen and blood when injected just after T-cells, and also showed relatively low binding to endogenous T-cells. However, injection of IL-2Fc-Lip on day 3 led to relatively poor T-cell labeling in the blood and LNs, while still labeling a majority of ACT T-cells in the spleen. Poor labeling by IL-2-Fc-Lip on day 3 reflected rapid downregulation of the IL-2R *in vivo* following T-cell transfer in the absence of antigen (data not shown). Thus, both IL-2-Fc and anti-Thy1.1 $F(ab')_2$ can be effective for specifically targeting adoptively transferred T-cells *in vivo*, though modulation of IL-2R levels by T-cells over time is an important consideration for achieving effective targeting.

3.5. IL-2-Fc-Lip permit repeated boosting of ACT T-cells in a murine lung metastasis model

To test the potential functional impact of stimulatory T-cell targeted liposomes, we assessed the response of pmel-1 melanoma-specific T-cells *in vivo* during ACT treatment of B16F10 tumors in a metastatic lung tumor model. B16F10 melanoma cells were injected *via* the tail vein to allow lung metastases to establish for 10 days, then animals were lymphodepleted and received adoptive transfer of luciferase-expressing pmel-1 melanoma-specific $CD8^+$ T-cells (Fig. 5A). In one group of animals, T-cell expansion was followed over time by bioluminescence imaging without further treatment, while in other two groups of mice, the adoptively-transferred cells were boosted on days 0 and 6 by injection of IL-2-Fc-Lip. Adoptively transferred cells without further adjuvant support showed a low level persistence in the tumor-bearing recipients that gradually declined over 14 days, as expected in the absence of additional stimulation or protection from tumor immunosuppression [25] (Fig. 5B, C). In contrast, following injection of the first dose of IL-2-Fc-Lip, pmel-1 T-cells expanded 3-fold more than the control T-cell therapy group. These boosted T-cells began to contract again between day 4 and day 6, but following a second dose of IL-2-Fc-Lip, re-expanded to an even greater level, reaching a peak by day 10 with 6-fold greater T-cell numbers relative to the T-cell-only treatment group (Fig. 5B, C). To assess the relative potency of stimulation achieved by IL-2-Fc-Lip compared to traditional systemic IL-2 therapy, we repeated this ACT experiment and compared the expansion of T-cells following injection of IL-2-Fc-Lip or soluble IL-2 (at an equivalent total amount of cytokine to that bound to the liposomes) on day 0 and day 6. Flow cytometry analysis of T-cells pooled from the inguinal lymph nodes 12 days after adoptive transfer confirmed that the frequency of tumor-specific $CD8^+$ T-cells (pmel-1 T-cells express the Vβ13 TCR β chain) was nearly 3 times greater in animals that received IL-2-Fc-Lip injections compared to T-cells alone (Fig. 5D–E). Further, soluble IL-2 at these doses showed no enhancement in T-cell expansion. The difference between the potency of IL-2-Fc-Lip and soluble IL-2 may reflect the very short half-life of IL-2 *in vivo* [32], which the PEGylated liposomes may partly overcome. Notably, this enhanced potency was not accompanied by overt toxicity as assessed by changes in animal weights during the therapy (data not shown). Thus, IL-2-targeted liposomes allow multiple boosts of ACT T-cells *in vivo*, leading to repeated waves of T-cells expansion in tumor-bearing animals, which exceed the response elicited by systemic free IL-2.

4. Conclusions

Here we synthesized and characterized antibody- and cytokine-decorated immunoliposomes targeting unique cell surface antigens or activation markers on T-cells, respectively. Targeting liposomes bound to ACT T-cells specifically *in vitro*, and further, anti-Thy1.1-Lip also labeled nearly 100% of transferred T-cells in systemic compartments and most of transferred T-cells in LN *in vivo* following a single injection of targeted vesicles. Despite its lower targeting specificity compared to anti-Thy1.1-Lip, IL-2-Fc-Lip was able to repeatedly boost transferred T-cells *in vivo* in tumor-bearing animals and provide direct stimulation to ACT T-cells. We focused here on an initial characterization of *in vitro* and *in vivo* T-cell targeting, and future work will examine the therapeutic efficacy of ACT cell targeting with drug-loaded liposomes. However, these results demonstrate the concept of repeated targeting of ACT T-cells for adjuvant stimulation *in vivo*. This data sets the stage for functional targeting of supporting adjuvant drugs or imaging contrast agents to T-cells, in order to enhance the efficacy of ACT and/or permit longitudinal tracking of ACT T-cells *in vivo*.

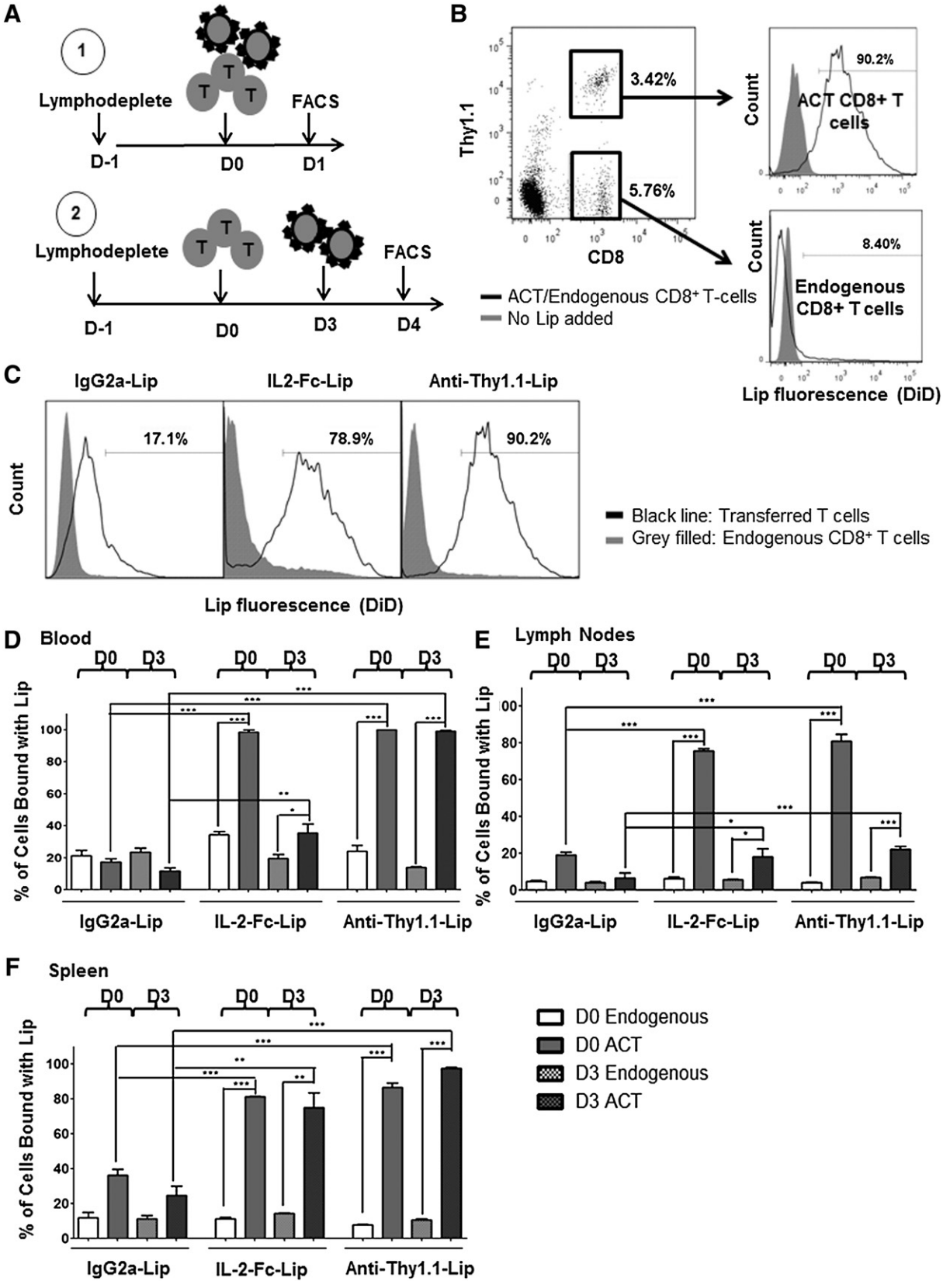


Fig. 4. IL-2-Fc- and anti-Thy1.1-Liposomes target transferred T-cells *in vivo*. C57Bl/6 mice received i.v. adoptive transfer of 15×10^6 pmel-1 $CD8^+Thy1.1^+$ T-cells, followed by i.v. injection of 1.4 mg IL-2-Fc-Lip, anti-Thy1.1-Lip, or isotype control IgG_{2a}-Lip either immediately after the T-cells or 3 days after the T-cells. Liposome binding to cells recovered from lymphoid organs and blood was analyzed 24 h after liposome injections by flow cytometry. (A) Timeline of injections and analysis. (B) Representative flow cytometry plots illustrating gating strategy for analysis of liposome binding to transferred pmel-1 T-cells or endogenous $CD8^+$ T-cells. (C) Representative histograms of pmel-1 T-cell or endogenous $CD8^+$ T-cell labeling following day 0 liposome injections. (D–F) Quantification of percentages of endogenous or transferred T-cells labeled by day 0 or day 3 liposome injections in the blood (D), lymph nodes (E), and spleen (F). $n = 5$ animals/group for IgG_{2a}-Lip and anti-Thy1.1-Lip and $n = 3$ for IL-2-Fc-Lip. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

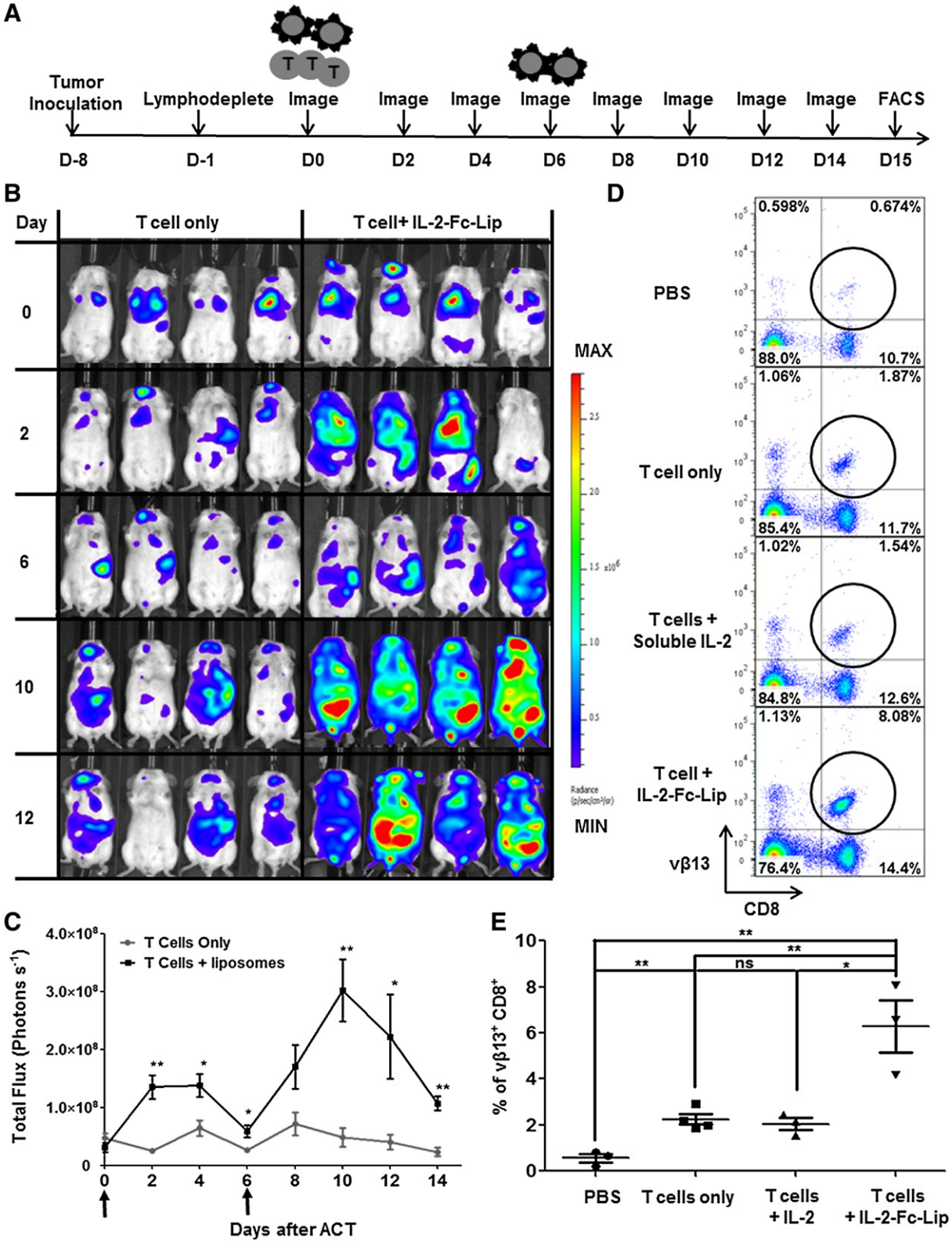


Fig. 5. IL-2-Fc-liposomes allow repeated expansion of target ACT T-cells *in vivo* in tumor-bearing animals. (A–C) B16F10 tumor cells (1×10^6) were injected i.v. into albino C57Bl/6 mice and allowed to establish lung metastases for 7 days. Animals were then sublethally lymphodepleted by irradiation and received i.v. adoptive transfer of 12×10^6 luciferase-expressing pmel-1 $CD8^+$ T-cells the next day. One group of mice additionally received injections of IL-2-Fc-Lip (1 mg, carrying 60 μg IL-2-Fc or 20 μg IL-2 cytokine equivalent) i.v. immediately after T-cell transfer and again on day 6. (A) Timelines of cell/liposome injections and bioluminescence imaging of T-cells. (B) Representative bioluminescent images of ACT T-cells over time. (C) Quantification of average whole-body T-cell bioluminescence over time. (D–E) Groups of C57B/6 mice with established lung metastases were left untreated or were treated with T-cells as in A, then received either IL-2-Fc-Lip or equivalent total doses of systemic free IL-2 (10 μg day 0, 20 μg day 6) injected i.v. on day 0 and day 6. (D) Sample flow cytometry analyses showing percentages of tumor-specific (vβ13 TCR^+) $CD8^+$ T-cells among T-cells in inguinal lymph nodes on day 12 after adoptive transfer. (E) Quantification of average frequency of tumor-specific (vβ13 TCR^+) $CD8^+$ T-cells in inguinal lymph nodes 12 days after adoptive transfer. $n = 3$–4 animals/group. *, $p < 0.05$; **, $p < 0.01$.

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